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Physiological response and miRNA-mRNA interaction analysis in the head kidney of rainbow trout exposed to acute heat stress

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ABSTRACT

The rainbow trout is a cold-water fish cultured in China. Heat stress has a serious impact on the summer survival and the yield of rainbow trout. A better understanding of the regulatory response of rainbow trout to heat stress will help in determining the relationship between heat stress signaling pathways and adaption mechanisms and help contribute to breeding new high-temperature tolerant strains of rainbow trout. In this study, the 48-h median lethal temperature (48h-LT_{50}) of rainbow trout was determined as 22.5°C. We developed control (16°C) and heat-treated (22.5°C) groups and extracted RNA from the head kidney tissues for high-throughput sequencing to study the microRNA (miRNA) expression profiles. Twelve up-regulated and five down-regulated miRNAs were identified between the control and heat-treated groups. A total of 22 target genes were predicted for 6 of the differentially expressed miRNAs, including 31 negative miRNA–mRNA interactions. Important regulatory pathways under heat stress are related to the metabolism and immune responses of the rainbow trout. Our findings provide preliminary data for investigating the high-temperature molecular mechanism of the rainbow trout and can help producers to reduce the economic losses caused by high temperature weather.

1. Introduction

The rainbow trout (*Oncorhynchus mykiss*) is a freshwater, cold-water fish native to the Pacific coast of North America. It was not until the 1950s that the rainbow trout was introduced to China for breeding and research. In China, because of the high nutritional and economic value of the rainbow trout, it is called as “water ginseng.” It has a beautiful body shape and color and is regarded as an ornamental fish. It is also a model animal in fish research (Li et al., 2017). The rainbow trout has become a primary aquaculture product bred in northeast and northwest China, and thus plays an important role in developing cold water resource utilization and promoting rural tourism culture. The rainbow trout has high requirements of water quality and environment and prefers to live in clean, low-temperature, flowing water with high dissolved oxygen. The normal temperature range for rainbow trout growth is 12-18°C. Researchers have found that when the water temperature is higher than 18°C, the rainbow trout stops eating, and when the water temperature is more than 25°C, it begins to die (Matthews et al., 2010). However, with global warming, continuous hot weather in summer has seriously affected the survival and production of the rainbow trout in northwestern China. According to our monitoring data in 2016, the Yellow River (Liu Jiaxia section) in Gansu showed a temperature rise from June to September, and the number of days with a water temperature of 18°C reached more than 112 days. This has become one of the biggest problems for the large-scale production of rainbow trout. However, there are few studies on the heat resistance regulation and reaction mechanism in rainbow trout.

The head kidney is the main hematopoietic and immune organ of fish (Geven et al., 2017), which is similar to mammalian bone marrow and is involved in the production and storage of red blood cells (RBCs). As an immune organ, the head kidney is similar to the mammalian adrenal gland and functions in non-specific immunity and removing foreign bodies and necrotic cells. Therefore, it can monitor changes in the external environment and play an important role in maintaining homeostasis of the internal environment along with coordinating and regulating various systems (Tort et al., 2011b; Rebl et al., 2017). To investigate the intolerance of rainbow trout to high temperature, biochemical methods were used to detect superoxide dismutase (SOD) activity, lysozyme activity, and malondialdehyde content in the head kidney tissues, so as to evaluate the injury to tissues and organs during heat stress. These results will be helpful for further studies on the molecular mechanisms of high-temperature tolerance in rainbow trout.

MicroRNAs (miRNAs) are short, 18-25 nucleotides sequences, with diverse biogenesis pathways and regulatory mechanisms. They are

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widely involved in nearly all known biological processes such as regulation of growth, immune systems, endocrine system, and responses to environmental stimuli (Bizuayehu et al., 2014). Li et al. (2017) found 128 differentially expressed (DE) miRNAs in the liver of rainbow trout under heat stress, which were mainly involved in protein metabolism, energy metabolism, and the immune system. The present study is the first report on miRNA-mRNA interactions in rainbow trout under heat stress. We analyzed the physiological effects of heat stress on rainbow trout to explore the damage and mechanism of heat stress, which indicate that further breeding of heat-resistant rainbow trout species is warranted.

2. Materials and methods

2.1. Experimental fish

All experimental fish (425-day-old, average weight 118 ± 5 g) were obtained from the same family. They were selected from Gansu Fisheries Research Institute (Linxia, China). Before the experiment, all the fish were adapted for 2 weeks to the indoor water-cycling system, with the water temperature maintained at 16°C, using the three sets of electric heating rods (3000 w) and automatic temperature controllers. The pH of the water was 7.4 ± 0.2 with dissolved oxygen > 7 mg L^{-1}.

2.2. Experimental management

This experiment consisted of two parts. In the first part, we determined the 48-h median lethal temperature (48h-LT_{50}) of the rainbow trout under high temperature stress. The 48h-LT_{50} of the rainbow trout was determined by setting five gradient temperatures (20, 21, 22, 23, and 24°C). The water temperature was rapidly raised to each of the experimental temperatures within 2 h. Three biological replicates were performed for each temperature treatment (10 fish per tank). The cumulative mortality of each group within 48 h was calculated and the 48h-LT_{50} of fish was obtained by linear interpolation. The 48h-LT_{50} was used as the next experimental water temperature.

In the second part, we investigated the behavior and physiological changes in the rainbow trout at 48h-LT_{50}. The fish were randomly placed in six tanks, including three 48h-LT_{50} tanks (LT group) and three 16°C tanks (control (CO) group). The cumulative mortality in each group was calculated at 0, 2, 4, 8, 12, 24, 36, and 48 h after heat stress.

2.3. Ethics statement

All animal experiments were performed in accordance with the protocol approved by the Animal Ethics Committee of Gansu Province, China. The head kidney and caudal blood were collected following the requirements for administration of experimental animals in Gansu, China.

2.4. Experimental sampling and processing

We randomly captured three fish from each group (LT group and CO group) at 0, 2, 4, 8, 12, 24, 36, and 48 h after heat stress. The fish were quickly anesthetized using 200 mg·L^{-1} tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA). Blood samples were collected from the caudal vein using a vacuum blood collection tube of containing ethylenediamine tetraacetic acid (EDTA) anticoagulant. The head kidneys were collected and divided into two parts and immediately frozen in liquid nitrogen, then stored at −80°C. One part was used to determine superoxide dismutase (SOD), lysozyme activity, and malondialdehyde content; another part was used for miRNA expression analysis.

2.5. Sample analysis

2.5.1. Blood biochemical analysis

Three fish were randomly selected from each group at 8 time points (0, 2, 4, 8, 12, 24, 36, and 48 h) after heat stress. The numbers of white blood cells (WBCs) and red blood cells (RBCs) were measured (p;/32q54546s) using an automatic blood cell analyzer (BC-5120, Shenzhen Mindray Bio Medical Co., Ltd., Shenzhen, China). SOD, lysozyme activity, and malondialdehyde content were measured from the head kidney tissues according to the manufacturer's instructions (Beauchamp et al., 1971; Dominguez et al., 2015; Zhang et al., 2008). All test kits were purchased from the Nanjing Jiancheng Biological Engineering Institute (Nanjing, China).

2.5.2. Small RNA library construction and sequencing

The small RNA (sRNA) was extracted from the head kidney using a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The quality and quantity of the extracted sRNA were assessed using an Agilent 2100 (Agilent, CA, USA) Bioanalyzer. sRNA with 28S/18S ≥ 1.5 and RNA integrity number > 8.0 was selected for further analysis. Six sRNA libraries were built including CO-1, CO-2, CO-3, LT-1, LT-2, and LT-3. Six libraries were sequenced, and the reads were assembled according to standard procedures (Qiang et al., 2017a).

2.5.3. Differential expression analysis, prediction of target genes and bioinformatic analysis

Differentially expressed genes (DEGs) were determined based on the MA-plot (Yang, 2002). To improve the accuracy of the results for DEGs, miRNAs with |fold changes| ≥ 2 and Q-value ≤ 0.001 were considered significantly DE. For more accurate prediction of target genes by DE miRNAs, two kinds of software (RNAhybrid and miRanda 3.3a) were used to identify the miRNA binding sites. The results of the two algorithms were combined and the overlaps were calculated. The major biological functions of the target genes corresponding to the DE miRNAs could be determined by the gene ontology (GO: http://www.geneontology.org) function. The Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG: http://www.genome.jp/kegg/pathway.html) was used to determine the most important biochemical metabolic pathways and signal transduction pathways including the DE miRNAs target genes.

2.6. Interaction analysis of miRNA-mRNA

In order to predict all the possible positively and negatively correlated miRNA-mRNA pairs, we employed ACGT101-CORR 1.1 to construct the miRNA-mRNA regulatory network. Based on all the sample-matched miRNA and mRNA sequencing data, we selected the negatively correlated DE miRNA-mRNA pairs for screening. The interaction network of screening pairs was constructed using Cytoscape software (http://www.cytoscape.org/).

2.7. Data analysis

The Pheatmap function in R software was used for hierarchical clustering analysis based on significantly different miRNA results. DE miRNAs based on normalized deep-sequencing counts were identified using Student's *t*-tests according to the experimental design. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to conduct one-way analysis of variance for the experimental data on early heat stress response. A threshold of $P < 0.05$ was considered statistically significant.

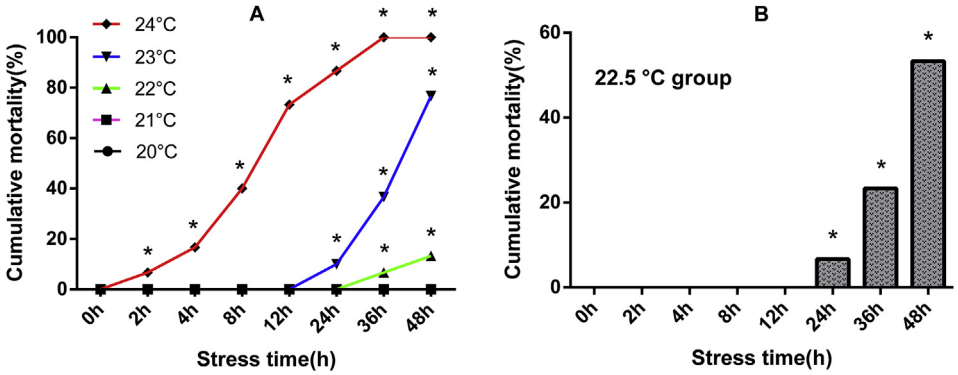

Fig. 1. Cumulative mortality in the rainbow trout under heat stress at different sampling points (A). Cumulative mortality of the rainbow trout under the 48-h median lethal temperature (48h-LT_{50}) stress (B). * indicates significant differences between CO and heat stress groups ($P < 0.05$).

3. Results

3.1. Assessment of the 48-h median lethal temperature (48h-LT_{50}) heat stress in the rainbow trout

Fig.1A shows a significant difference in the cumulative mortality rate of rainbow trout within 48 h under different stress temperatures. In certain temperature ranges, the number of deaths and the death rate were increased obviously with the increase in water temperature. At 20°C and 21°C, there were no rainbow trout deaths within 48 h. At 22°C, the fish began to die in 36 h and cumulative mortality was 13.33%. At 23°C, the cumulative mortality began to sharply rise in 36 h and 48 h (36.67% and 76.67%), respectively. At 24°C, the fish began to die in 2 h and the cumulative mortality reached 100% within 36 h. By linear interpolation, we determined that the 48h-LT_{50} was 22.5°C [y = 21.9047 + 0.0143x].

Fig. 1B shows the cumulative mortality of rainbow trout under 48h-LT_{50} stress. At 16°C (CO group), the rainbow trout always swam in an orderly, uniform manner, almost in the same direction. However, in the 48 h post-22.5°C stress (LT group), the rainbow trout began to show some behavioral changes. At 6 h, the rainbow trout showed restlessness, increased alertness, significantly increased speed of swimming, and outward jumping from time to time. At 12 h, some fish gradually stopped at the bottom of the tank and swimming was reduced. At 24 h, some fish lost their balance and began to die. At 48 h, the mortality rose sharply and reached 53.3%.

3.2. Effect of heat stress on blood cell counts

In the 48 h post-22.5°C stress, compared with the CO group, the number of red blood cells (RBCs) and white blood cells (WBCs) in the LT group was first increased and then decreased (Fig. 2). In the LT group, the number of RBCs increased significantly from 0.28×10^{12} at 12 h to a maximum of $0.34 \times 10^{12}\ L^{-1}$ at 24 h and then decreased to $0.25 \times 10^{12}\ L^{-1}$ at 48 h. Similarly, compared with the CO group, the number of WBCs increased significantly from $194.2 \times 10^{9}\ L^{-1}$ at 8 h to $217.7 \times 10^{9}\ L^{-1}$ at 24 h, and then decreased significantly to $163.2 \times 10^{9}\ L^{-1}$ at 48 h.

3.3. Effect of heat stress on biochemical indices in the head kidney

In the 48-h period post-22.5°C stress, compared with the CO group, there were significant differences in superoxide dismutase (SOD) activity (A), lysozyme activity (B), and malondialdehyde content (C) in the head kidney (Fig. 3). The SOD activity in the LT group increased significantly from 82.3 U·(mg prot)$^{-1}$ at 2 h to a maximum of 113.7 U·(mg prot)$^{-1}$ at 12 h and then gradually decreased to 47.3 U·(mg prot)$^{-1}$ at 48 h.

Lysozyme activity in the LT group increased significantly from 0.7 μg·(mg prot)$^{-1}$ at 0 h to a maximum of 2.2 μg·(mg prot)$^{-1}$ at 36 h and then decreased slightly to 2.1 μg·(mg prot)$^{-1}$ at 48 h. These values were higher in the LT group than in the CO group at every time point.

The malondialdehyde content in the LT group increased significantly from 5.2 nmol·(mg prot)$^{-1}$ at 2 h to a maximum of 11.3 nmol·(mg prot)$^{-1}$ at 48 h. These values were higher in the LT group than in the CO group at every time point.

An increase in water temperature (from 20°C to 24°C) significantly increased the mortality of the rainbow trout after 48 h under heat stress (Fig. 1). By linear interpolation, we determined that the 48-h median lethal temperature (48h-LT_{50}) was 22.5°C. At 24 h post-22.5°C stress, some fish began to show an imbalance and soon died. RBC and WBC counts in the heat-stressed rainbow trout increased gradually from 0 h to 24 h and reached peak values at 24 h, then gradually decreased from 36 h to 48 h (Fig. 2). Based on the above results, the thermal stress for

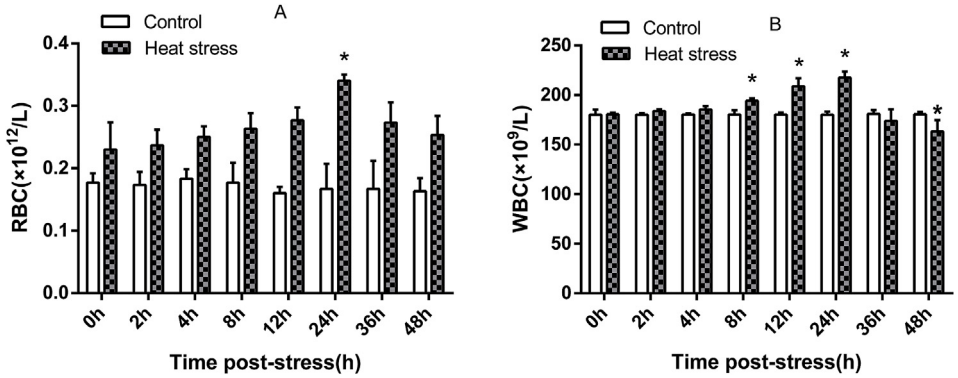

Fig. 2. Counts of red blood cells (RBCs) and white blood cells (WBCs) in the rainbow trout between CO and heat stress groups over 48 h. Note: Data are expressed as the mean ± SE. * indicates significant differences between CO and heat stress groups ($P < 0.05$).

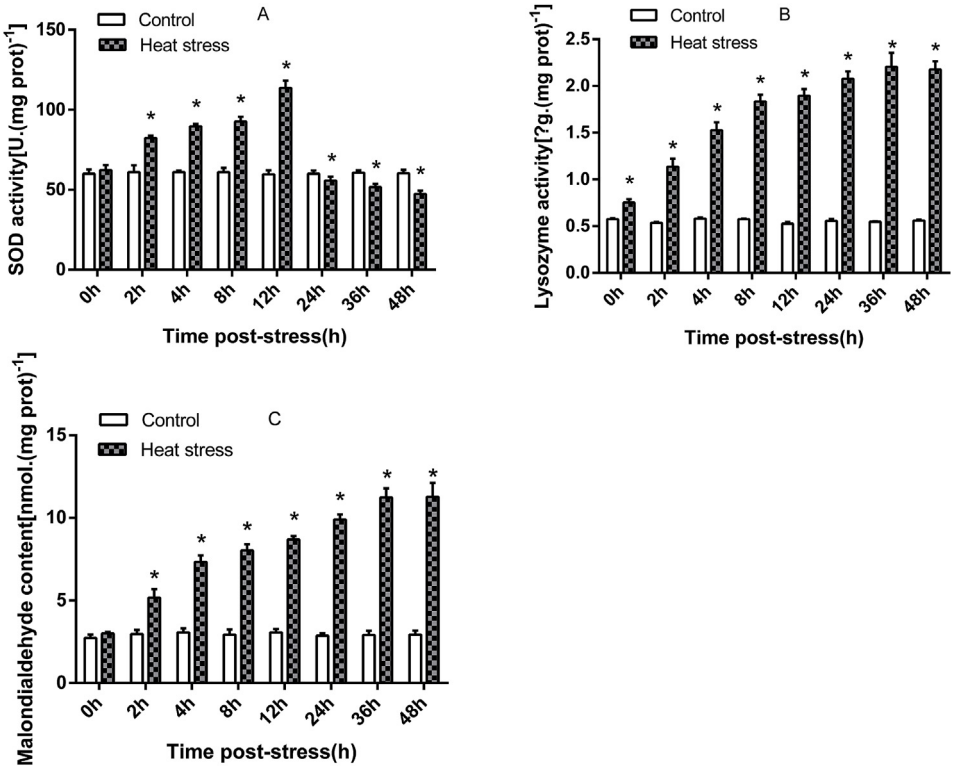

Fig. 3. Superoxide dismutase (SOD) activity (A), lysozyme activity (B), and malondialdehyde content (C) in the head kidney of the rainbow trout under 48h-LT_{50} stress. Note: Data are expressed as the mean ± SE. * indicates significant differences between CO and heat stress groups ($P < 0.05$).

24 h post-22.5°C was an important threshold for the rainbow trout. Therefore, head kidney tissues of the rainbow trout after 24 h post-22.5°C of heat stress were used to study the molecular regulation mechanism of heat stress.

3.4. Expression profiling of miRNAs in head kidney under 48h-LT_{50} stress.

Six miRNA libraries were constructed, including three CO groups (CO-1, CO-2, and CO-3) and three treatment groups (LT-1, LT-2, and LT-3). The raw reads of six libraries were obtained, which were 27741138, 29102968, 28885811, 28889075, 28353346, and 29215090, respectively. After deleting the adaptor sequences and low-quality readers, clean reads were obtained from the six libraries, which were 24874106, 25097034, 25062254, 26412743, 25899891, and 26690731, respectively. In the clean reads, 300 predicted novel miRNAs and 804 conserved miRNAs were detected.

Expression profiling of miRNAs: 534 miRNAs were significantly down-regulated, and 483 miRNAs were significantly up-regulated at 24 h post-22.5°C stress. According to transcripts with at least 100 reads in each of the libraries, 17 DE miRNAs were identified, including 12 up-regulated and 5 down-regulated (Table 1). Cluster analysis of 17 DE miRNAs showed that these miRNAs were divided into five biological processes (Fig. 4).

3.5. Prediction of target genes and associated regulated pathways

The RNAhybrid and miRanda 3.3a toolbox were used to predict the target genes of DE miRNAs and determine the possible functions of DE miRNAs related to heat stress. In order to understand the functional distribution of different genes, the gene ontology (GO) database was used to conduct functional classification statistics of target genes (Qiang et al., 2017b). A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted to further understand the biological functions of target genes. Six biological pathways were found at 24 h under 48h-LT_{50} stress, indicating that most of the known DE miRNAs were predicted to be regulated by one or more genes involved in the heat-stress response, mainly including lipid metabolism, energy metabolism, amino acid metabolism, cellular processes, and human diseases (Fig. 5).

Based on the *Oncorhynchus mykiss* transcriptome sequence data, the GO and KEGG annotations, and the miRNA and mRNA expression patterns (Qiang et al., 2017c), we screened potential target genes of miR-10c-5p, miR-133-3p, miR-133a-3p, miR-16c-5p, and miR-27e, which were related to heat stress. The negative expression levels were detected between the following miRNA-mRNA pairs (Fig. 6): miR-133-3p/133a-3p-mediator complex subunit 16 (MED16), miR-133-3p/133a-3p- 2,4-dienoyl-CoA reductase 2 (DECR2), miR-10c-5p-mitogen-activated protein kinase kinase kinase 7 (MAP3K7), miR-16c-5p-acetyl-CoA carboxylase alpha (ACACA), and miR-27e- ATP/GTP binding protein 1 (AGTPBP1).

4. Discussion

In fish, stress indicates a state in which the homeostasis of the organism is destroyed and is stimulated by an internal or external environment (Chrousos, 1992). Upon environmental changes, such as high temperature (Li et al., 2017; Qiang et al., 2017c), the body adjusts through compensation or adaptation mechanisms to overcome stress factors. However, if the animal experiences acute and intense stress, the anti-stress mechanism may lose its value and lead to destruction of homeostasis, and even occurrence of growth inhibition, reproductive disorders, and reduced disease resistance (Wendelaar, 1997). From previous studies (Wendelaar, 1997; Thomas et al., 1994), we have learned that fish counter some stress factors by stimulating oxygen intake, mobilizing energy, and redistributing the energy used for growth and reproduction, leading to a stress response that inhibits the fish immune function and normal growth. Temperature stress is a parameter included in the study on fish stress and is more important than in other

Table 1
Differentially expressed (DE) miRNAs in the rainbow trout head kidney between the control (CO) and 22.5 °C-stressed (LT) groups.

miRNA	CO expression	LT expression	Fold change (log_2LT/CO)	Regulation (LT vs CO)	P-value
miR-16c-5p	17.280	740.390	5.678	up	< 0.01
miR-128	2.3167	47.253	4.594	up	< 0.01
miR-27e_1	16.900	279.167	4.312	up	< 0.01
miR-17a-5p	22.377	252.693	3.741	up	< 0.01
miR-489	1.717	18.550	3.681	up	< 0.01
miR-24-3p	39.727	427.577	3.667	up	< 0.01
miR-10c-5p	213.537	2215.550	3.623	up	< 0.01
miR-133-3p	2.140	21.837	3.582	up	< 0.01
miR-206	13.433	120.613	3.393	up	< 0.01
miR-1a-3p	2.433	20.737	3.319	up	< 0.01
miR-133a-3p	11.753	94.933	3.243	up	< 0.01
miR-133a-3p_1	2.930	21.910	3.125	up	< 0.01
miR-139-5p	680.573	11.407	-5.660	down	< 0.01
miR-202_1	484.057	11.543	-5.145	down	< 0.01
miR-462_1	5060.667	164.053	-4.699	down	< 0.01
miR-202-3p_1	33.397	2.230	-3.661	down	< 0.01
miR-202-5p_2	203.473	15.723	-3.453	down	< 0.01

These 17 miRNAs had transcripts with at least 100 reads in each of the libraries.

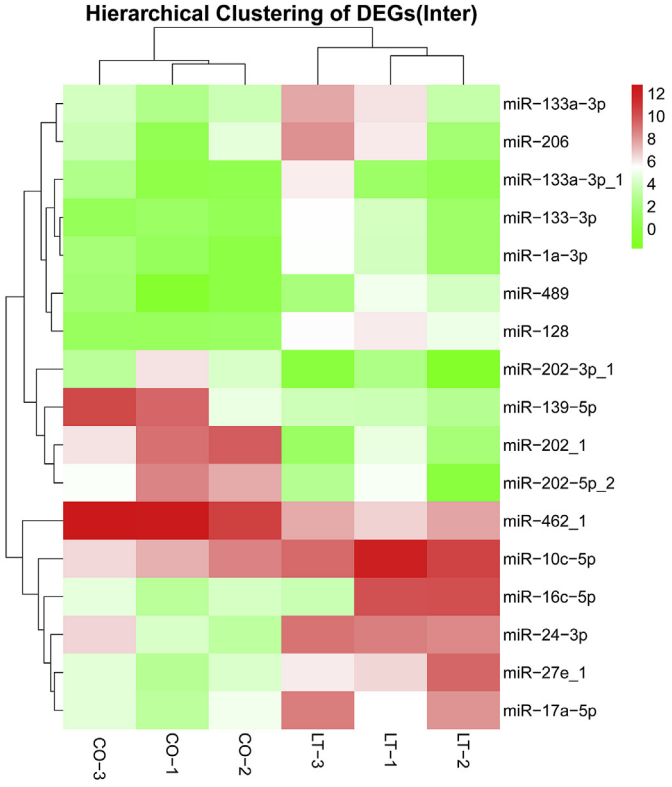

Fig. 4. Hierarchical clustering of differentially expressed (DE) miRNAs between the control (CO) and 22.5°C-stressed (LT) groups.

vertebrates. This is mainly owing to the higher dependence of fish on the water environment. High temperature can directly damage the fish internal environment and thus inhibit its immune function and growth, but the underlying molecular mechanism is still unknown (Qiang et al., 2017c). In our study, five stress temperatures (20, 21, 22, 23, and 24°C) were set up to evaluate the 48-h cumulative mortality of rainbow trout. Results showed that the 48-h cumulative mortality rate was lower at temperatures less than 22°C, indicating that the rainbow trout could overcome the high temperature environment through its own adaptation mechanisms. However, when the water temperature reached 23°C for 24 h, the rainbow trout began to swim erratically and tried to jump out of the water to escape the unsuitable living environment, and showed a significant increase in cumulative mortality rate. This suggests that 23°C may be the maximum temperature at which the rainbow trout can survive. Then, according to the linear interpolation method, 22.5°C was determined as 48h-LT_{50} of the rainbow trout.

Blood plays an important role in maintaining the stability of the internal environment of the body. It has many important physiological functions and its components are relatively stable. In order to adapt to major changes in the environment, the body may cause blood cell counts to change, which can partly reflect the health of fish (Qiang et al., 2017c; Bao et al., 2018). RBCs or erythrocytes are the most abundant cells in blood, accounting for 40-50% of the total blood volume. The main component of mature RBCs is hemoglobin. The concentration of hemoglobin is positively correlated with the number of RBCs, which play an important role in the transport of oxygen and carbon dioxide (Farrell, 2011). In this study, at 48 h post-22.5°C stress, a marked increase in the number of RBCs was observed at 24 h followed by a decrease. This suggests that as the metabolic rate of respiration increases with heat stress, the body needs more oxygen and energy to maintain homeostasis. Therefore, within a certain range, the number of RBCs increased significantly. Previous studies have also reported that heat stress increases the production of RBCs (Dewilde et al., 1967). However, after 24 h of heat stress, as the RBC counts decrease, the ability of fish to resist stress may begin to diminish. In addition, compared to mammals, the number of WBCs or leukocytes in fish is very high. WBCs are involved in the immune defense system of fish (Farrell, 2011). They are phagocytic and produce antibodies and other immune active substances (Ellis, 2006). In this study, at 48 h post-22.5°C stress, a marked increase in the number of WBCs was observed at 24 h, followed by a decrease. This could be due to acute heat stress exacerbating the inflammatory response in rainbow trout, where excess bacteria or toxins are eliminated by large amounts of WBCs, which also explains the significant increase in the number of WBCs in the early stages of stress and a sharp decline after 24 h. Previous studies (Qiang et al., 2017c) have reported a significant decline in the number of WBCs in tilapia after acute heat stress.

The head kidney is an important hematopoietic and immune organ of fish, and its integrity may help maintain homeostasis. It functions in nonspecific immunity and can monitor and regulate the changing environmental conditions (Qiang et al., 2017d). Although highly adapted to the high-temperature environment and well balanced in the antioxidant process, the head kidney is highly sensitive to oxidative stress and thus produces a stress response, leading to a higher reactive oxygen species (ROS) formation rate and damage to the antioxidant system (Abele et al., 2004). The primary function of the antioxidant system is to remove excessive free radicals and ROS to prevent cell damage (Zhang et al., 2004). Previous studies have shown that excess free

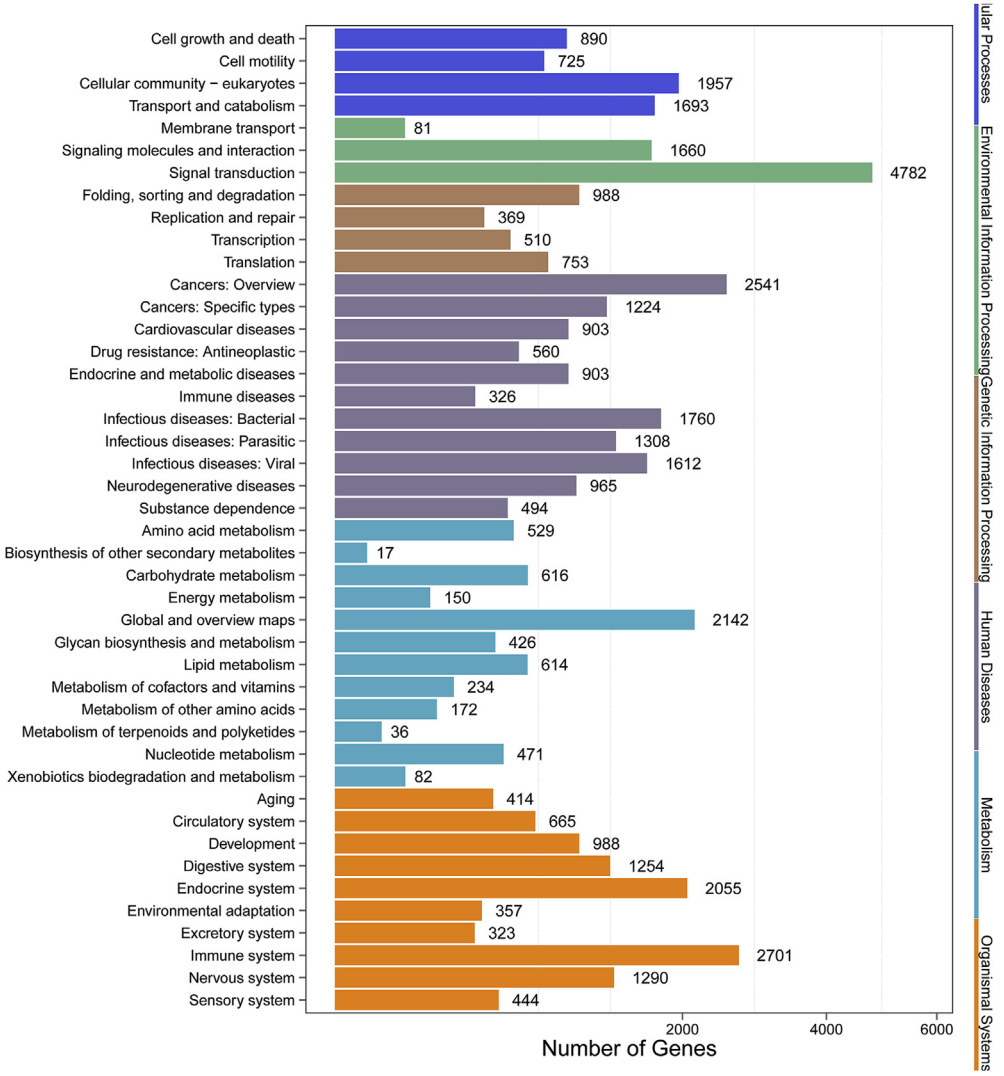

Fig. 5. Classification of the enriched KEGG pathways for the miRNA of the rainbow trout exposed to heat stress.

radicals under heat stress can cause oxidative damage by increasing lipid peroxidation, which can be monitored by malondialdehyde content (He et al., 2015). Malondialdehyde, a cytotoxic end-product of lipid peroxidation, and its content indicate metabolic disorders and the extent of tissue oxidative damage (Qiang et al., 2018a). In this study, the malondialdehyde content in rainbow trout head kidney gradually increased up to 48 h post-22.5°C stress, which indicates that during heat stress, the lipid metabolism of the body is disturbed, resulting in oxidative damage. Acute cold stress has been also reported to increase malondialdehyde content in the head kidney of *Oreochromis niloticus* (Qiang et al., 2018b).

Lysozyme is an important humoral immune factor that plays an important role in resisting microbial invasion. Lysozyme is also an opsonin that promotes phagocytosis and can directly activate complement and macrophages. The non-specific immune level of fish can be reflected by changes in lysozyme activity (Qiang et al., 2018a, b). Studies have shown that lysozyme activity increases significantly when the fish are exposed to acute cold stress (Qiang et al., 2018b). Dominguez et al. (2015) found that the activity of plasma lysozyme in Nile tilapia increased at 28°C heat stress for 2 and 4 weeks, but decreased significantly at 33°C for 4 weeks. In the early stage of heat stress, the increase of lysozyme activity indicates that the body plays an important role in fighting infection. In this study, at 48 h post-22.5°C stress, significantly increased lysozyme activity was observed in the head kidney of the rainbow trout. It can be inferred that the body can resist the infection of some pathogenic microorganisms in the early stage of heat stress. However, this result was different from other studies, which may be because the stress time used in this study was too short to observe a subsequent decline in lysozyme activity. Previous studies have found that cold stress inhibits the activity of lysozyme in the serum of different fish (Ndong et al., 2007; Tort et al., 1998).

SOD is an important antioxidant enzyme in the biological defense system and plays an important role in maintaining normal physiological balance (Qiang et al., 2018a). SOD can remove excessive ROS and prevent excessive ROS from damaging cell membranes and causing lipid peroxidation. In the early stage of heat stress, the increase in SOD activity indicated that the organism improved the mechanism of resistance to stress. With the increase in stress, a large amount of ROS accumulation leads to the formation of oxidative stress. Studies have shown that temperature stress can significantly increase the activity of SOD in different fish, including yellow catfish (*Pelteobagrus fulvidraco*) (Qiang et al., 2018a) and tilapia (*Oreochromis niloticus*) (Qiang et al., 2018b). In this study, at 48 h post-22.5°C stress, a significantly increased SOD activity was observed at 12 h, which was then decreased in the head kidney of the rainbow trout. It can thus be inferred that ROS in the head kidney cannot be completely cleared after 12 h at post-22.5°C stress. Qiang et al. (2018b) found that cold stress may lead to reduced SOD activity, increased malondialdehyde content, and may adversely

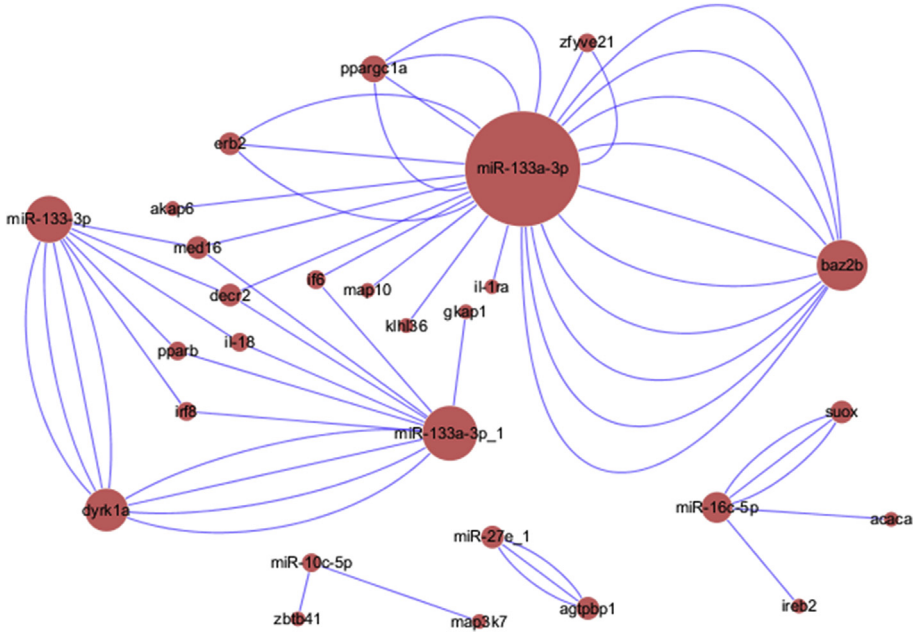

Fig. 6. Analysis of the miRNA-mRNA negative correlation network. The network contains 31 negatively correlated miRNA-mRNA interactions (22 DE target mRNAs for 6 DE miRNAs) and was constructed using Cytoscape software.

affect the physiological adaptability of tilapia, eventually leading to death.

We studied the early response in the head kidney of rainbow trout exposed to heat stress by high throughput sequencing of CO and 22.5°C-treated (LT) libraries. A total of 97.76%, 96.79%, 97.41%, 96.82%, 97.98%, and 94.51% of valid reads in the CO-1, CO-2, CO-3, LT-1, LT-2, and LT-3 libraries, respectively, were mapped to the reference rainbow trout genome. The sequencing data show that the six libraries have good overall consistency. miRNAs are involved in a plethora of important biological processes in maintaining homeostasis in fish. The miR-10 family, miR-27, miR-133 and miR-133a are involved in a variety of important biological processes, including gene regulation, tumor development, cell proliferation and differentiation. Five potential target genes were identified and their functional characteristics showed that they were mainly involved in metabolism and organismal systems pathways, including lipid metabolism, carbohydrate metabolism, endocrine regulation, and immune regulation.

In this study, the expression levels of miR-10c-5p in the LT library were notably higher than those in the CO library. Previous studies have shown that the miR-10 family, comprising short non-coding RNAs, is highly conserved and involved in the expression and regulation of the Hox gene (Tehler et al., 2011). The miR-10 family members are also reportedly involved in regulating the development of some tumors. For example, the expression level of miR-10b-5p in Huntington chorea brain tissue is significantly increased, and is closely related to the disease stage and rate of progression (Hoss et al., 2015). In pituitary adenomas, the expression level of miR-10b was closely related to the tumor diameter and invasion degree (Luo et al., 2013). Bioinformatics software was used to predict the potential target gene for miR-10c-5p, and it was found that up-regulation of miR-10c-5p may inhibit the expression of MAP3K7 mRNA. MAP3K7 is also called transformed growth factor activated kinase-1. It plays a role in cell proliferation by regulating a variety of cellular pathways. For example, down-regulation of MAP3K7 promotes apoptosis in breast cancer cells (Zhou et al., 2017). miR-133 and miR-133a are essential in mediating cell proliferation, differentiation, and anti-apoptosis. In this study, the expression levels of both miR-133 and miR-133a in the LT library were notably higher than those in the CO library. Xu et al. (2007) showed that miR-133 regulates the differentiation and proliferation of cardiac and skeletal muscles, and is preferentially expressed in these cells. miR-133 plays an anti-apoptotic role in the regulation of cardiomyocyte apoptosis induced by oxidative stress in H9c2 rat ventricular cells (Xu et al., 2007). miR-133a was considered as a tumor suppressor, with a significant inhibitory effect on the proliferation and apoptosis of bladder cancer cell lines by silencing the target gene TAGLN2 (Yoshino et al., 2011). In this study, up-regulation of miR-133-3p and miR-133a-3p may inhibit the expression of MED16. MED16 plays a key role in preventing oxidative damage to cells (Sekine et al., 2015). In addition, the up-regulation of miR-133-3p and miR-133a-3p may inhibit the expression of DECR2. DECR2 is an enzyme that plays an important role in the degradation of unsaturated fatty acids (Dommes et al., 1981). The miR-27 family plays an important role in the regulation of adipogenic differentiation. Lin et al. (2010) believed that the miR-27 family is down-regulated during adipogenic differentiation, and that overexpression of miR-27 inhibits adipocyte formation, without affecting myogenic differentiation. Previous studies have shown that miR-27 is an anti-fat generation miRNA that damages mitochondrial function (Kang et al., 2013). In this study, up-regulation of miR-27e may inhibit the expression of AGTPBP1. AGTPBP1 plays a role in regulating lymphoid development, and studies have shown that the absence of AGTPBP1 can inhibit the development of T cells (Christoph et al., 2015). In mammals, miR-16 is an important biomarker for cancer development. For instance, miR-16-5p is down-regulated in early gastric cancer and may serve as a biomarker for early screening and progress evaluation of the disease (Zhang et al., 2015). However, reports on the role of the miR-16c-5p in fish are scarce. In this study, up-regulation of miR-16c-5p may inhibit the expression of ACACA. ACACA is a key regulatory enzyme of fatty acid synthesis, and plays a pivotal role in the regulation of cellular fatty acid fluxes (Travers et al., 2005).

5. Conclusions

In conclusion, the 48h-LT_{50} acute heat stress may cause free radical metabolic disorders as a result of oxidative stress, increased malondialdehyde content, and lysozyme activity in the head kidney of the rainbow trout, with SOD activity increased at first followed by a decrease. These results show that homeostasis may be damaged at 24 h post-22.5°C stress, which probably contributed to cell injury in the

rainbow trout, eventually leading to death. The expression profiles of miRNAs in the head kidney at 24 h post-22.5°C stress were then studied. Seventeen DE miRNAs were found between the CO and LT libraries, including twelve up-regulated and five down-regulated miRNAs. Five potential target genes were identified, and their functional characteristics showed that they were mainly involved in metabolic processes and immune regulation pathways. These findings provide preliminary data for investigating the high-temperature molecular mechanism of rainbow trout, and can help producers reduce their economic losses caused by high-temperature weather conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.05.014.

Author's contributions

Jian-Lin Wang and Chang-Qing Zhou conceived and designed the experiments. Chang-Qing Zhou, Peng Zhou, Yan-Li Ren, and Li-Hui Cao performed the experiments. Chang-Qing Zhou performed the data analysis and wrote the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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