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Transcriptomic responses to heat stress in gill and liver of endangered *Brachymystax lenok tsinlingensis*

Ping Li [a,b,c], Qigen Liu [a,b,c], Jiale Li [b,c], Feng Wang [d], Sien Wen [d], Ning Li [e,*]

[a] Center for Research on Environmental Ecology and Fish Nutrition of the Ministry of Agriculture and Rural Affairs, Shanghai Ocean University, Shanghai 201306, China
[b] Key Laboratory of Freshwater Aquatic Genetic Resources of the Ministry of Agriculture and Rural Affairs, Shanghai Ocean University, Shanghai 201306, China
[c] National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai 201306, China
[d] Yellow River Fisheries Research Institute, Chinese Academy of Fishery Science, Xi'an 710086, China
[e] Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA

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ABSTRACT

Global warming significantly affects fish, particularly cold-water fish, because increased temperature adversely impacts their abilities to grow or reproduce, and eventually influences their fitness or even causes death. To survive, fish may alter their distribution or behavior to avoid the stress, and perhaps acclimate or evolve resistance to the elevated temperature. *Brachymystax lenok tsinlingensis* is an endangered cold-water species in China, and it has been found to alter the altitudinal distribution, decrease swimming efficiency and develop resistance under heat exposure, which badly impact the continuing conservation work. To better protect them, it is essential to understand how they respond to thermal stress behaviorally and physiologically. Therefore, the fish were exposed to 24.5 °C and based on the time taken for them to lose equilibrium, they were separately sampled as sensitive and tolerant groups. Both gill and liver tissues were collected from both groups for transcriptome sequencing. Sequencing results demonstrated that control and tolerant groups were similar in transcriptomic patterns and sensitive groups differentially expressed more genes than tolerant ones, suggesting the gene expression of tolerant groups may return to base levels as exposure time increased. Tissue differences were the major factor affecting gene expression, and they also displayed different physiological responses to heat stress. Consistent with other studies, heat shock response, immune response, metabolic adjustment and ion transport were found to be triggered after exposed to elevated temperature. The findings would contribute to a better understanding of responding mechanisms of fish to thermal stress and provide guidance for future conservation programs.

1. Introduction

Global climate change has significant effects on organisms, especially on aquatic ectotherms, for which water temperature is a prominent environmental factor. The shifts in temperature may pose a serious threat to their capabilities to develop, grow or reproduce, and ultimately affect their fitness and even cause death (Huey and Kingsolver, 1989). To persist, organisms may alter their geographic distribution or behavior to avoid the stress, and perhaps they may acclimate through physiological plasticity or evolve resistance over generations through natural selection (Beitinger and Fitzpatrick, 1979; Huey and Kingsolver, 1989; Angilletta Jr and Angilletta, 2009; Poloczanska et al., 2013). Particularly under the globally increased water temperature suggested by the Intergovernmental Panel on Climate Change (IPCC), to understand the biological and physiological mechanisms of organisms' responses to elevated temperature is of vital importance to the conservation of wild animals and aquaculture of farmed animals.

Due to the decreasing cost and increasing throughput of transcriptome sequencing technique, it has been widely used as a useful approach to identify specific genes and pathways that are responsive to a given stressor. In fish species, the mechanisms of their responses to heat stress have been well studied by RNA-seq despite of diverse thermal exposure regimes (e.g. magnitude and duration; fluctuating versus stable temperature) (Liu et al., 2013; Smith et al., 2013; Bilyk and Cheng, 2014; Logan and Buckley, 2015; Narum and Campbell, 2015; Barat et al., 2016; Guo et al., 2016; Qian and Xue, 2016; Oomen and

* Corresponding author.
E-mail address: ningli@usc.edu (N. Li).

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Hutchings, 2017; Y. Li et al., 2017; Huang et al., 2018; Lyu et al., 2018; Li et al., 2019). Consistently, heat shock response is the hallmark, during which the heat shock proteins (*HSPs*) cope with stress-induced denaturation of other proteins to prevent their cyto-toxic aggregation (Feder and Hofmann, 1999; Logan and Buckley, 2015). Additionally, immune functions have been primarily induced since high temperature could influence their immune defense and increase the virulence and transmission of certain pathogens (Watson et al., 1998; Decostere et al., 1999; Cairns et al., 2005). It has also been demonstrated that acute stress caused expression changes in a series of evolutionarily conserved stress-responsive genes and pathways involved in oxidation reduction, energy metabolism, apoptosis and homeostasis (Liu et al., 2013; Smith et al., 2013; Guo et al., 2016; Huang et al., 2018; Lyu et al., 2018; Li et al., 2019), revealing the power of RNA-seq to assist in elucidation of the fundamental mechanisms of the responses to thermal stress.

Brachymystax lenok is a cold-water salmonid species present across eastern Siberia and portions of northern Mongolia, China and Korea (Froufe et al., 2004). This species usually inhabits upland streams where year-round temperature is below 20 °C and dissolved oxygen level is above 10 mg/L (Yoon et al., 2015). In China, *B. lenok tsinlingensis* is considered as a post-glaciation invader and then was stagnated in streams of Qinling mountain, which makes it one of the southernmost salmonid species (Li, 1984; Xing et al., 2015). However, the wild stocks have experienced dramatic declines in the past few decades due to overfishing and deteriorated habitats caused by mining and dam construction (Ren and Liang, 2004; Zhao and Zhang, 2008). Now it has been listed in the China Red Data Book of Endangered Animals and classified as a second-class state protected wild animal in China since 1998 (Yue and Chen, 1998; Zhao and Zhang, 2008). Similarly in South Korea, it was designated as a threatened species in 2012 because of over-exploitation, pollution, devastated natural habitats and climate change (Yoon et al., 2015). Although this species has been effectively protected through establishment of aquatic wildlife natural reserves and artificial breeding in China (Xing et al., 2015), the number of *B. lenok tsinlingensis* estimated in Qianhe river valley in 2004 has decreased to only 10% of the previous number in 1980s (Ren and Liang, 2004). Remarkably, their lowest inhabiting altitude has elevated from 1000 m to 1200 m (Ren and Liang, 2004), indicating the adverse effects of climate change on this endangered cold-water fish. Notably, our water temperature monitoring near the breeding station from 2017 to 2020 showed that the temperature during the summer periods are close to or above 20 °C for several weeks (Fig. 1), highlighting the increasingly stressful environments for *B. lenok tsinlingensis*.

Thermal tolerance is expected to change across different life stages (embryos, larvae, adults, and spawning adults) due to the variation of oxygen demand and the development of aerobic capacities (Pörtner and Farrell, 2008; Pörtner and Peck, 2010; Dahlke et al., 2020). It was suggested that larvae and non-reproductive adults (which indicates post-metamorphosis stage) are more tolerant to thermal stress than embryos and spawning adults (Dahlke et al., 2020), indicating their less vulnerability to climate warming. The vulnerability to warming is often evaluated based on the difference between the maximum habitat temperature during summer and the upper thermal tolerance limit, which is also called thermal safety margin (TSM) (Dahlke et al., 2020). The TSM of 580 fish species studied from 1981 to 2000 is estimated to be 11.6 ± 0.3 °C (Dahlke et al., 2020), however, the estimated TSM of embryos from 554 species is 4.5 ± 0.4 °C and that of spawners from 543 species is 4.1 ± 0.3 °C, which are significantly narrower than adults (Dahlke et al., 2020). Thus, the estimation of *B. lenok tsinlingensis* TSM will be a useful resource and provide guidance for future life stage-specific conservation programs.

Fish homeostatic mechanisms are highly dependent on their surrounding conditions. Even though the temperature may not reach the lethal limit, the exposure will still lead to their thermal stress responses which are highly likely to cause decreased growth and reproduction, and eventually population failure (Pankhurst and King, 2010; Whitney et al., 2016). Xia et al. (2017) have also observed decreased swimming efficiency of *B. lenok tsinlingensis* under thermal stress. Collectively, it is of great significance to understand their capacities and physiological mechanisms in response to elevated temperature. Gills are continually exposed to external environment, and thus they become a frequent target for stress responses (Harper and Wolf, 2009). Unlike the gills, liver is protected from physical exposure to the environmental factors, however, its responses may also be evident due to its critical role in metabolism and energy reserve (Harper and Wolf, 2009). Therefore, in this study the lethal temperature limit of *B. lenok tsinlingensis* was first assessed, and then they were exposed to the acute heat stress. Both gill and liver tissues from heat-stressed individuals were sampled for RNA-seq. The objective is to identify genes and pathways responsive to heat stress tolerance, which would allow elucidation of responding mechanisms to elevated temperature and identification of biomarkers of heat tolerance in *B. lenok tsinlingensis*.

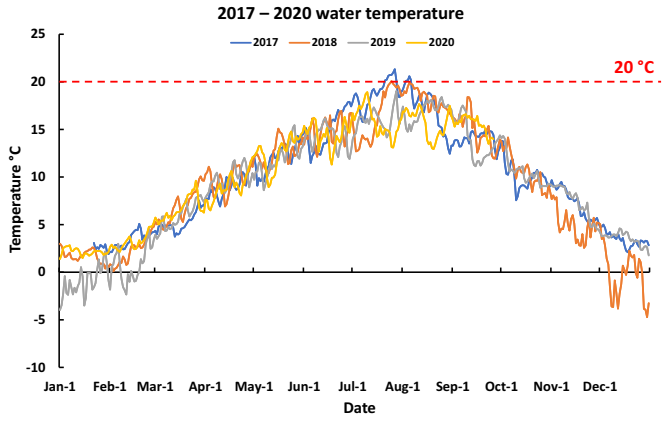

Fig. 1. The daily average water temperature of the stream close to Taibai Aquaculture Station from 2017 to 2020. Blue line represents year 2017, orange line represents year 2018, grey line represents year 2019 and yellow line represents year 2020. The red dot line horizontal to x-axis represents 20 °C line.

2. Materials and methods

2.1. Ethics statement

Artificial breeding and domestication of *B. lenok tsinlingensis* was approved by Taibai Aquaculture Station in Shaanxi Province of China. All procedures involving the handling and treatment of fish were conducted according to the guiding principles of the Chinese Legislation on the Use and Care of Laboratory Animals as well as approved by the Taibai Aquaculture Station.

2.2. Heat stress and sample collection

The fish population was achieved from a conservation-related breeding program which mated 117 female wild fish with 98 male wild fish in Taibai Aquaculture Station (Shaanxi, China) at ambient temperature (~12 °C) in 2017. A total of 200 6-month-old fish (~8 cm body length) were randomly selected from this population for the experiment. The fish were reared in the lab for 2 weeks with continuous aeration (dissolved oxygen > 8.0 mg/L) at ambient temperature. Prior to the heat treatment, a total of 60 fish were randomly selected as the control group and transferred to another tank with the same condition. Several trials have been conducted prior to the experiment to determine the lethal temperature, and to optimize the heating rate to decrease the stress responses as well as to avoid death of fish during the heating period. Finally, 24.5 °C was selected as it killed almost all the fish within two days, which are long enough to distinguish the heat-sensitive and heat-tolerant groups. Heaters were used in the treatment tanks to

increase water temperature by 1 °C/h until it reached 24.5 °C. The dissolved oxygen decreased as the water temperature went up, so aeration was increased to maintain the oxygen level above 6.0 mg/L. During the experimental period, fish were not fed, and water was not changed to avoid confounding factors. The temperature was held constant and loss of equilibrium was suggested to be a sign of stress (Quinn et al., 2011; Liu et al., 2013). No fish showed loss of equilibrium during the heating period. After the temperature reached 24.5 °C, the first and last 18 individuals which showed loss of equilibrium were collected as heat-sensitive and heat-tolerant groups, respectively, and 18 randomly selected fish from the control group were collected as well. Fish were euthanized with MS-222 (200 mg/L) before sample collection. Gill and liver samples were collected due to their high metabolic activities and essential roles in responses to environmental stressors (Harper and Wolf, 2009; Logan and Somero, 2011; Quinn et al., 2011; Liu et al., 2013; Oomen and Hutchings, 2017). Samples were put into liquid nitrogen to avoid RNA degradation and then stored in −80 °C until further RNA extraction.

2.3. RNA extraction, library preparation and sequencing

Total RNA was extracted from both tissues of 54 fish samples using Trizol Reagent (Invitrogen, USA) according to manufacturer's instructions. A NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was utilized to determine RNA concentration and to assess RNA quality. Equal amounts of RNA from six random individuals in each group were pooled for each tissue. A total of 18 sequencing libraries (3 groups * 2 tissues * 3 biological replicates) were constructed using TruSeq RNA Sample Prep Kit (Illumina, USA). Briefly, poly-A isolation was conducted to purify mRNA from the total RNA. After fragmentation, sequences with the length of 200–300 bp were obtained for cDNA synthesis. The synthesized double-stranded DNA was then end-repaired and adenylated at the 3′ ends. Next, sequencing adapters were ligated to the end-repaired fragments, and AMPure XP beads (Beckman Coulter, USA) were used for purification after PCR amplification. The final libraries were quantified by a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA) and quality-assessed by Agilent 2100 Bioanalyzer (Agilent, USA). Eighteen libraries were pooled for sequencing on Illumina HiSeq 2500 platform to obtain paired-end 150 bp reads.

2.4. Differential expression analysis

Low-quality bases (<Q20) and adapter sequences were trimmed by cutadapt v1.2.1 (Martin, 2011). After subsequently removing short reads (<50 bp), the trimmed reads were evaluated by FastQC v0.11.8 (Andrews, 2010), and then mapped to the de novo assembled transcriptome (Wen et al., 2020) for transcript abundance estimation by RSEM v1.3.1 (Li and Dewey, 2011). Genes with half samples having at least 10 read counts were retained for differential expression analysis between tolerant/sensitive groups and control groups within each tissue by DESeq2 v1.22.2 (Love et al., 2014). *P*-values were adjusted to control the false discovery rate by the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Genes with adjusted *P*-value < 0.05 and $|\log2(\text{FoldChange})| > 1$ were considered as differentially expressed genes (DEGs).

2.5. GO and KEGG classification of differentially expressed genes

GO (Gene Ontology) terms of all genes were achieved by Blast2GO v4.0.7 (Conesa et al., 2005), and the KEGG (Kyoto Encyclopedia of Genes and Genomes) Automatic Annotation Server (KAAS) (http://www.genome.jp/tools/kaas/) BBH (Bi-directional Best Hit) method (Moriya et al., 2007) was used to retrieve KEGG pathway annotations. GO terms enrichment analyses of differentially up-regulated and down-regulated genes were performed by a Mann-Whitney *U* test (MWU) in R using the package ape v5.3 (Wright et al., 2015). KOBAS (Wu et al., 2006) was used to identify functionally enriched KEGG pathway in the pairwise comparisons.

3. Results

3.1. Heat treatment

Heating was started from 12 °C at the increasing rate of 1 °C/h. After 3 h (15 °C), the fish started to jump out of water badly and the strong jumping behavior continued until the water temperature reached 20 °C. Fish occasionally jumped out during the increase from 20 °C to 21 °C, but after 21 °C, thick foam started to accumulate on the surface and evident increase in the amount of foam ceased during the rise in temperature from 23 °C to 24 °C.

No fish showed loss of equilibrium during the heating period, but fish started to show signs of stress quickly (one fish every 3–6 min) after the temperature reached 24.5 °C. As shown in Fig. 2, survival of the first and the last 60 fish showing loss of equilibrium were compared to indicate the differences between the heat-sensitive and the heat-tolerant groups. The first 18 heat-sensitive fish were sampled within 86 min. A total of 36 h later, the sampling of heat-tolerant groups was initiated with the first 11 individuals collected within a period of 12 h. The remaining seven fish were then sampled although they were still alive and did not show loss of equilibrium to avoid large variations of exposure time in the heat-tolerant groups.

3.2. Transcriptome sequencing

A total of 18 samples with 3 biological replicates for each tissue within each group were used for transcriptome sequencing. A range from ~42 million to ~53 million clean reads were generated for each sample and aligned to the transcriptome (Table S1). After raw read counts for each sample were filtered by removing genes with less than 10 reads for half of the samples, 27,105 transcripts were retained for principal component analysis (PCA) and clustering analysis to reveal the relationships across samples. As shown in Fig. 3A, two tissues were clearly separated by PC1 (64.2%), and PC2 (11.6%) indicated the differences among groups while control and tolerant ones clustered together within both tissues. Clustering analysis displayed a consistent result with PCA, with quite different expression patterns between tissues and similar patterns between control and tolerant groups within tissues (Fig. 3B). The huge tissue differences were further assessed by comparing two tissues under control condition, and there are 15,274 transcripts (56.4% of the total transcripts analyzed) found to be differentially expressed (Table S2).

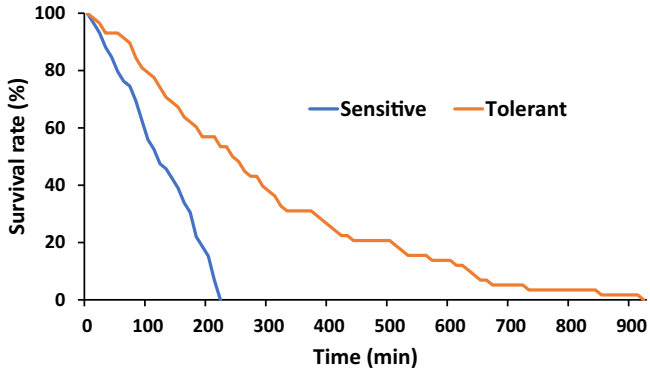

Fig. 2. The survival comparisons between the first (Sensitive group; blue) and the last (Tolerant group; orange) 60 fish showing loss of equilibrium.

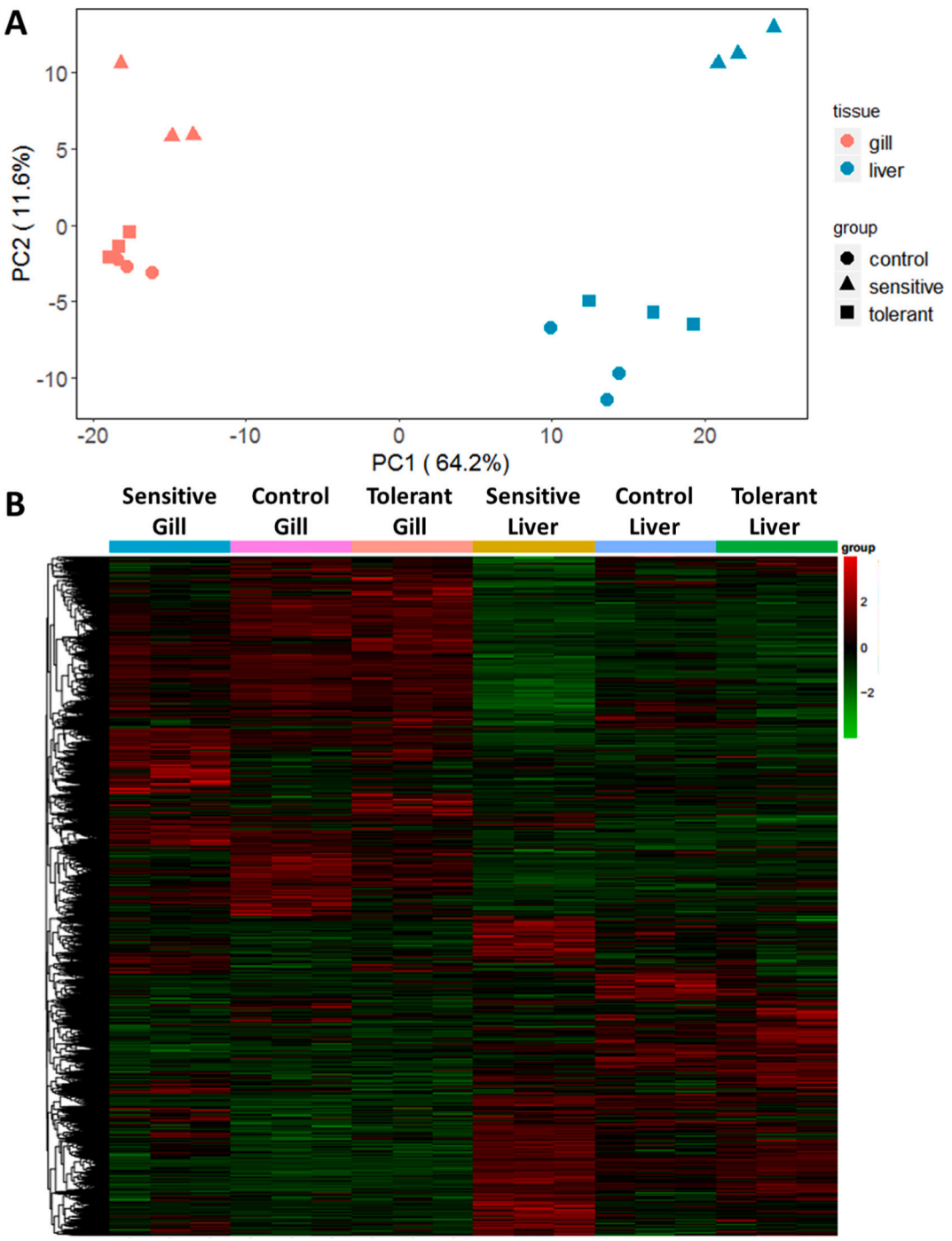

Fig. 3. Expression patterns across groups and tissues. (A) Principal component analysis (PCA) of the top 500 genes in terms of variance across samples. Tissue and group information are indicated beside the plot. The percentage of variance explained by the first two principal components is indicated beside the axes. (B) Heatmap of transformed expression values across samples. Hierarchical clustering analysis showed the relative closeness of samples and genes. The samples from the same group were clustered together and are indicated with the same color on the top of the plot. For each gene, higher expression values are indicated by warmer colors (red), and lower expression values are indicated by colder colors (green).

3.3. Differential expression analysis after exposure to heat stress

To assess how the sensitive and tolerant groups responded to the heat stress, they were compared to the control groups within each tissue for differential expression analyses. As shown in Table 1, the sensitive groups differentially expressed more genes than tolerant groups with up-regulating 3451 and 3736 genes and down-regulating 3337 and 4348 genes while tolerant groups up-regulated 1781 and 1127 genes and down-regulated 1027 and 1356 genes in gill and liver tissues, respectively (Tables S3–S6). Overall, in response to heat stress, gill tissues displayed more up-regulation while liver tissues showed more down-regulation.

Although the comparisons between sensitive and tolerant groups may be biased by the different exposure time, it was still conducted to demonstrate the general pattern of differential expression between the two stressed groups (Tables S7–S8). More up-regulation was consistently observed in the comparisons of tolerant groups with sensitive groups for both tissues, with 3363 up vs. 2821 down in gill and 3176 up vs. 2659

Table 1
The number of differentially expressed genes in each comparison.

	Gill		Liver	
	Sensitive vs. control	Tolerant vs. control	Sensitive vs. control	Tolerant vs. control
Up-regulation	3451	1781	3736	1127
Down-regulation	3337	1027	4348	1356
Total	6788	2808	8084	2483

down in liver. In terms of the number of DEGs, over 20% of the total genes altered gene expression levels between groups.

3.4. Differentially expressed genes shared between groups

As shown in Fig. 4A, although there are not many DEGs shared by two tissues, there is a higher proportion of genes found to be shared between tissues in the sensitive groups (32.3%–38.5%) than the tolerant groups (2.6%–3.0%). In the same tissues, tolerant groups shared a relatively high proportion of genes with sensitive groups in gill (44.1%) and liver (56.3%) while the percentages are much lower within the sensitive groups (17.3%–18.3%; Fig. 4B).

DEGs shared by both sensitive and tolerant fish within both tissues were identified, as we believe the temporally and spatially common DEGs may represent the key responding genes to thermal stress. As shown in Table S9, a total of 29 transcripts were found to be differentially expressed with the same direction (all up- or down- regulated) in all four groups. Heat shock protein 30 (c184836_g2), unc-45 homolog B (c190062_g1) and extracellular calcium sensing receptor (c174424_g1) were highly up-regulated in both tissues of the stressed animals compared with control animals, suggesting their potential as biomarkers or indicators of heat stress.

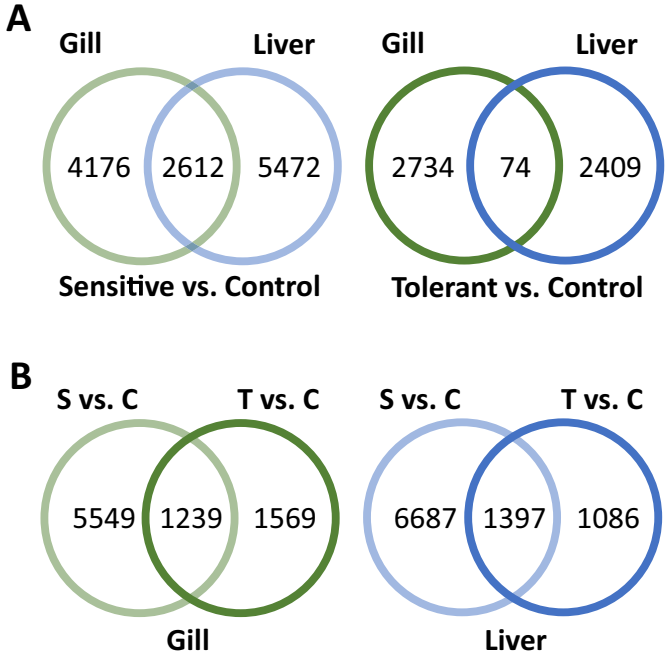

Fig. 4. Venn diagram showing the number of differentially expressed genes (DEGs) shared between tissues (A) and between sensitive and tolerant groups (B). S represents sensitive group, T represents tolerant group and C represents control group. Green and blue circles represent gill and liver tissues, respectively. The lighter color represents sensitive groups while the darker color represents tolerant groups.

3.5. GO and KEGG pathway enrichment of DEGs

GO term "protein folding" was the most significantly up-regulated one in gill of both sensitive and tolerant groups (Table 2). Further in gill, immune response (MHC protein complex) and cytoskeletal reorganization ("intermediate filament" and "keratin filament") related GO terms were up-regulated in the sensitive groups, while tolerant groups up-regulated immune response ("regulation of coagulation" and "immune effector process"), oxidative stress response ("response to oxidative stress" and "oxidoreductase activity"), metabolic process of carbohydrate, amino acid and lipid, hydrolase and endopeptidase (Tables 2 and S10).

As shown in Tables 3 and S10, cytoskeletal reorganization ("intermediate filament" and "keratin filament") was up-regulated and protein synthesis ("ribosome", "ribonucleoprotein complex" and "aminoacyl-tRNA ligase activity") was down-regulated in liver of both sensitive and tolerant groups. Furthermore, GO term "nitrogen compound transport" was up-regulated, while "response to oxidative stress" was down-regulated in the sensitive groups. The tolerant groups up-regulated immune response (MHC protein complex), anion transport and amino acid transport, while down-regulated "protein folding" and "endoplasmic reticulum lumen".

In the KEGG pathway enrichment analysis, "Protein processing in endoplasmic reticulum" was found to be enriched in the gill of sensitive groups while no pathway was enriched in the tolerant groups (Table 4). In the liver of the sensitive groups, aging (longevity regulating pathway), metabolism (phenylalanine metabolism, and ubiquinone and other terpenoid-quinone biosynthesis) and signaling (FoxO signaling pathway and AMPK signaling pathway) related pathways were enriched, while in the tolerant ones, mainly immune system (osteoclast differentiation, Fc gamma R-mediated phagocytosis, NOD-like receptor signaling pathway, Toll-like receptor signaling pathway and hematopoietic cell lineage) and endocrine system (PPAR signaling pathway, thyroid hormone signaling pathway, insulin signaling pathway, regulation of lipolysis in adipocytes and adipocytokine signaling pathway) related pathways were identified to be enriched (Table 4). Additionally,

Table 2
Enriched GO terms in the gill of both sensitive and tolerant groups under the Biological Process category.

GO term	*P*-value	Direction
Sensitive vs. control		
Protein folding	0.0002	Up
Organic anion transport	0.0149	Up
Carboxylic acid biosynthetic process	0.0268	Up
Anion transmembrane transport	0.0268	Up
Carboxylic acid transport	0.0268	Up
Regulation of gene expression	0.0268	Up
Regulation of immune response	0.0163	Down
Negative regulation of response to external stimulus	0.0268	Down
Nucleic acid metabolic process	0.0268	Down
Tolerant vs. control		
Protein folding	0.00004	Up
Regulation of endopeptidase activity	0.0064	Up
Regulation of coagulation	0.0078	Up
Small molecule metabolic process	0.0082	Up
Immune effector process	0.0084	Up
Alpha-amino acid metabolic process	0.0098	Up
Carbohydrate phosphorylation	0.0099	Up
Cellular carbohydrate metabolic process	0.0099	Up
Cellular amino acid metabolic process	0.0205	Up
Response to oxidative stress	0.0303	Up
Negative regulation of cellular protein metabolic process	0.0314	Up
Carbohydrate metabolic process	0.0327	Up
Multicellular organism development	0.0003	Down
Regulation of Ras protein signal transduction	0.0099	Down
Protein phosphorylation	0.0119	Down
Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	0.0193	Down

Table 3
Enriched GO terms in the liver of both sensitive and tolerant groups under the Biological Process category.

GO term	*P*-value	Direction
Sensitive vs. control		
Organic substance transport	0.00006	Up
Nitrogen compound transport	0.0002	Up
Response to oxidative stress	0.0067	Down
Tolerant vs. control		
Developmental process	0.0002	Up
Carboxylic acid transport	0.0075	Up
Organic anion transport	0.0075	Up
Protein phosphorylation	0.0267	Up
Amino acid transport	0.0267	Up
Anion transport	0.0267	Up
Antibiotic metabolic process	0.0267	Up
Organic acid metabolic process	0.0267	Up
Protein targeting	0.0267	Down
Protein folding	0.0267	Down

Table 4
Enriched KEGG pathways in each contrast.

Pathway ID	Pathway description	FDR
Gill sensitive		
ko04141	Protein processing in endoplasmic reticulum	0.0000001
Liver sensitive		
ko04213	Longevity regulating pathway - multiple species	0.0361
ko00360	Phenylalanine metabolism	0.0361
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	0.0361
ko04068	FoxO signaling pathway	0.0361
ko04152	AMPK signaling pathway	0.0467
Liver tolerant		
ko04380	Osteoclast differentiation	0.0010
ko04668	TNF signaling pathway	0.0044
ko03320	PPAR signaling pathway	0.0063
ko00120	Primary bile acid biosynthesis	0.0114
ko04919	Thyroid hormone signaling pathway	0.0114
ko04024	cAMP signaling pathway	0.0119
ko04910	Insulin signaling pathway	0.0119
ko04066	HIF-1 signaling pathway	0.0215
ko04923	Regulation of lipolysis in adipocytes	0.0387
ko04666	Fc gamma R-mediated phagocytosis	0.0387
ko04072	Phospholipase D signaling pathway	0.0387
ko04621	NOD-like receptor signaling pathway	0.0387
ko04920	Adipocytokine signaling pathway	0.0394
ko04620	Toll-like receptor signaling pathway	0.0394
ko04152	AMPK signaling pathway	0.0411
ko04640	Hematopoietic cell lineage	0.0427

lipid metabolism-related pathways were also found to be enriched in the liver of tolerant groups, including primary bile acid biosynthesis, regulation of lipolysis in adipocytes and adipocytokine signaling pathway.

4. Discussion

This study conducted a heat exposure experiment in endangered cold-water fish species *B. lenok tsinlingensis* to assess their behavioral and transcriptomic responses to thermal stress. As temperature increased, they tried to escape the stressful environment by swimming quickly and jumping frequently, and further they started to increase the production of mucus as a response to the increasingly serious heat stress. After they started to loss equilibrium, their gill and liver samples of sensitive and tolerant animals were collected for transcriptome sequencing to investigate their responding mechanisms to heat stress. To our knowledge, this is the first transcriptome study in *B. lenok tsinlingensis* to assess the effects of environmental factors. Considering the overall susceptibility of fish to elevated temperature due to climate change, this study will contribute to a more comprehensive understanding of the influence of global warming on the cold-water fish species and provide insights into the necessity of conservation programs for this endangered species.

4.1. Behavioral responses to heat stress

To mitigate the adverse effects caused by increased water temperature, fish may alter their distribution or behavior to avoid the stressor (Angilletta Jr and Angilletta, 2009). It was also documented that wild stocks of *B. lenok tsinlingensis* in Qianhe river valleys of Qinling Mountains have increased inhabiting lowest-altitude from 1000 m to 1200 m and individuals have been observed to be miniaturized (Ren and Liang, 2004). Thus, for the extreme temperature they rarely encountered during summer, it may be more efficient for them to swim to high altitude, which may isolate them from other populations and then largely restrict the gene flow among populations. The restricted gene flow among fish from various streams may bring much more burden to conservation work of this species due to the large gene differentiation among populations (Zhang et al., 2014; Liu et al., 2015; Jang et al., 2017; P. Li et al., 2017; Liu et al., 2018). Another scenario for fish is to acclimate to the warm water if the temperature is not as lethal as 24.5 °C used in this study. As the cold-water Salmonid fish with the southernmost distribution, *B. lenok tsinlingensis* has displayed increased thermal tolerance and decreased swimming efficiency under elevated acclimation temperature (Xia et al., 2017). Since living in warmer water may result in higher energetic costs, this will definitely lead to a potential trade-off with more energy allocated to homeostasis and less energy available for growth and reproduction (Logan and Buckley, 2015). Therefore, the increased water temperature will pose a serious threat to wild *B. lenok tsinlingensis* populations as well as bring more pressure and challenges to conservation work.

Additionally, foam was found to be accumulated on the surface of tanks due to the increased mucus secretion under thermal stress. Fish epidermal mucus contains innate immune components, and thus provides a non-specific but fast acting protection as the first line of physical defense against pathogens (Dash et al., 2018). Thermal stress can compromise fish defense mechanism and adversely affect their physiological mechanism, which may trigger both non-specific and specific immune responses. In Pacific Sardine (*Sardinops sagax caeruleus*), desquamation and mucus production have been observed under chronic and acute heat stress (Hernández-López et al., 2018). In both common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*), rodlet cells, resembling mucus cells, were undetected unless fish were exposed to stressors such as thermal elevation and heavy metals (Iger and Abraham, 1997). Therefore, the increased foam in the fish tank after the temperature reached 21 °C indicated that they are in a stressful state.

4.2. Transcriptomic responses to heat stress

Thousands of genes were differentially expressed to respond to heat stress in both groups, while heat-sensitive fish differentially expressed almost three times as many genes as the heat-tolerant ones. Although the high number of DEGs may be caused by the usage of de novo assembled transcriptome, the pattern still indicated the diverse transcriptomic responses of both groups to heat stress, which is consistent with the findings of a large number of DEGs in the comparison of tolerant groups with sensitive groups. As shown in Figs. 3 and 4, the relative closeness between tolerant and control groups and the high percentage of shared genes in the tolerant groups with the sensitive groups suggested that most of initial DEGs in the tolerant groups may return to base levels as exposure time increased.

Gill and liver tissues were commonly used for thermal stress experiments as gill is the direct target of environmental stress as well as the primary site of oxygen uptake and osmotic regulation, and liver is the major organ of metabolisms (Harper and Wolf, 2009; Liu et al., 2013). There is an interesting pattern in both sensitive and tolerant fish displaying more up-regulated DEGs in gill while more down-regulated

DEGs in liver. Transcripts differentially expressed in the gill were mainly enriched into: (1) protein processing, (2) ion transport, (3) cytoskeleton, (4) immune response, (5) anti-oxidative stress, and (6) metabolic response, while those in the liver were classified into: (1) metabolic alteration, (2) immune response, (3) endocrine response, (4) cytoskeleton, and (5) ion transport, suggesting the tissue-specific transcriptomic responses to thermal stress.

The up-regulation of *HSP* genes is consistent across fish species responding to heat stress, including rainbow trout (Y. Li et al., 2017; Huang et al., 2018), redband trout (Narum and Campbell, 2015), snow trout (Barat et al., 2016), spotted rainbowfish (Smith et al., 2013), black rockfish (Lyu et al., 2018), blunt snout bream (Li et al., 2019), half-smooth tongue sole (Guo et al., 2016), catfish (Liu et al., 2013), and yellow croaker (Qian and Xue, 2016), which also highlights the importance of transcriptional regulation of *HSP* genes during exposure to elevated temperature. Similarly in the gill, heat stress responses were consistently associated with the up-regulation of GO term "protein folding" and enrichment of KEGG pathway "protein processing in endoplasmic reticulum". It is well known that endoplasmic reticulum function with a number of chaperones and cofactors, including *HSP*s to fold thermally damaged proteins and prevent or reverse cytotoxic aggregation (Fink, 1999).

Thermal stress is commonly able to cause stress-associated inflammatory changes, and thereby induce immune responses. Especially in the tolerant groups, due to the extended exposure time, a series of immune systems were activated, which has been observed in other fish species as well (Smith et al., 2013; Narum and Campbell, 2015; Barat et al., 2016; Guo et al., 2016; Huang et al., 2018; Lyu et al., 2018; Li et al., 2019). At the same time, metabolic changes can result from exposure to elevated temperature due to the increased demand of energy. Liver, as the major organ for metabolic adjustments, revealed down-regulation of protein synthesis-related genes and up-regulation of lipid metabolism and endocrine-related genes, which implicated the dynamic metabolic alterations with acute thermal stress. An NMR-based metabolomics study of plasma and liver in *B. lenok* showed that 24 °C exposure treatment caused similar metabolic adjustments including repression of energy metabolism, shifts in lipid metabolism and alterations in amino acid metabolism (Liu et al., 2019). The findings of dynamic metabolic changes were also consistent with the observations in the liver of another cold-water fish species rainbow trout indicated by transcriptome sequencing (Wiseman et al., 2007).

4.3. Future consideration for conservation

Increased temperature induced behavioral and physiological changes of *B. lenok tsinlingensis*, and eventually caused death of most individuals in this study. In the wild, we hypothesized that behavioral adaptation will influence their altitudinal distribution and thereby bring more challenges to the conservation of this endangered fish species. Although TSM could not be accurately estimated from this study, based on the four-year water temperature monitoring of the stream close to our station from 2017 to 2020, the TSM for adults is estimated to be 3.5 °C (24.5 °C–21 °C), which is far below the value of 11.6 ± 0.3 °C from the estimation of 580 fish species (Dahlke et al., 2020). Given the facts that the estimated TSMs of embryos and spawners are significantly narrower than adults (Dahlke et al., 2020), *B. lenok tsinlingensis* embryos and spawners are suggested to have extremely narrow TSM, probably putting them on the edge of extinction. Consequently, life stage-specific strategies should be considered for future conservation plans, and more attention should be paid to embryos and spawners due to their less vulnerability to elevated water temperature.

Abbreviations

IPCC	Intergovernmental Panel on Climate Change
TSM	thermal safety margin
HSP	heat shock protein
DEG	differentially expressed gene
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
KAAS	KEGG Automatic Annotation Server
BBH	Bi-directional Best Hit
MWU	Mann-Whitney *U* test
PCA	principal component analysis

Data availability statement

Raw sequencing reads have been deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession numbers of SRR8873448 – SRR8873465.

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Declaration of competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2021.100791.

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