Turk. J. Fish.& Aquat. Sci. 19(8), 681-688
http://doi.org/10.4194/1303-2712-v19_8_06

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Effect of Heat Stress on Heat Shock Protein 30 (Hsp30) mRNA Expression in Rainbow Trout (*Oncorhynchus mykiss*)

Xiaoxia Liu[1], Haina Shi[1,2], Zhe Liu[1,*], Yujun Kang[1], Jianfu Wang[1], Jinqiang Huang[1]

[1] Gansu Agricultural University, College of Animal Science and Technology, 1 Yingmencun, Anning District, Lanzhou, 730070, Gansu, China.
[2] Qingyang Academy of Agricultural Sciences, Qingyang, 745000, Gansu, China.

Article History
Received 03 March 2018
Accepted 30 July 2018
First Online 06 August 2018

Corresponding Author
Tel.: +86 139 19085712
E-mail: liuz@gsau.edu.cn

Keywords
Cold-water species
High temperatures
Small heat shock proteins
Tissue-specific gene expression

Abstract

Rainbow trout (*Oncorhynchus mykiss*) is a cold-water species, and often affected by high temperatures. To understand *hsp30* mRNA expression trend and molecular mechanism responding to high temperature stress, real-time qPCR was used to quantify *hsp30* mRNA expression level in different tissues (heart, liver, gill, brain, spleen and head kidney) in control (18°C) and heat-stressed (25°C for 2, 4, 8 and 12 h) groups. Results showed that *hsp30* mRNA in all tissues had already synthesized at 18°C. *hsp30* mRNA expression in all tissues was significantly up-regulated and reached the peak after heat stress at 25°C for 2-4 h ($P<0.05$), then significantly down-regulated at 8 h ($P<0.05$), and finally returned to control level at 12 h and HSR ($P>0.05$). The highest *hsp30* mRNA expression levels were recorded in heart and liver after 2-4 h. However, the most significant changes of *hsp30* mRNA after 2-4 h were observed in gill by 15668.243- and 11452.125-fold compared with 18°C. These results indicated that *hsp30* mRNA expression in rainbow trout was induced by heat stress and responded in a time- and tissue-specific manner. Hsp30 can provide some protection in heat stress, but it is only within a certain range.

Introduction

Small heat shock proteins (sHsps), members of the heat shock protein family, are evolutionarily conserved and widely found in various animals, plants and microorganisms (Brady *et al.*, 1997; Bagnéris *et al.*, 2009; Basha, O'Neill, & Vierling, 2012; Guzzo, 2012; Hanazono *et al.*, 2013; Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013; Prakash, Thomas, Reddy, & Mohandas, 2013). sHsps are molecular chaperones that are induced by different environmental stress factors such as high temperatures, exposure to heavy metals or oxidative stress, and can efficiently bind to non-native proteins and prevent those proteins from irreversible aggregation (Jakob, Gaestel, Engel, & Buchner, 1993; Haslbeck *et al.*, 1999; Rogalla *et al.*, 1999; Li, Liu, He, Gao, & Yuan, 2013). sHsps are involved in the regulation of cell growth and development in multiple organisms (Heikkila, 2003; Marvin *et al.*, 2008; Morrow & Tanguay, 2012; Heikkila, 2017). In addition, sHsps are also involved in the inhibition of apoptosis and the pathogenesis of various diseases in different species (Goplen *et al.*, 2010; Chen *et al.*, 2011; Tóth *et al.*, 2013). However, not all sHsps have similar function. The substrate protection ability of inclusion-body binding protein A (IbpA) and inclusion-body binding protein B (IbpB) in *E. coli* is different. IbpA alone has little protective activity of substrate, but it enhances the activity of molecular chaperone IbpB (Ratajczak *et al.*, 2010). In animals, the activities of different sHsps on different substrates also show great differences.

Hsp30 has the same molecular chaperone function as other sHsps. When cells are stressed or diseased, intracellular proteins usually degenerate in stress or

harsh environments and become abnormal proteins, while Hsp30 can maintain a refolding conformation by binding to these abnormal proteins. When stress disappears and conditions return to normal, Hsp30 separates from its bound denatured protein and restores its normal properties with the help of other ATP-dependent chaperones to protect cells from harm (Nakamoto & Vígh, 2007).

A number of studies have clearly demonstrated that this chaperone function of Hsp30 plays an important role in cellular homeostasis. The Hsp30 of *Xenopus* (*Xenopus laevis*) and *Rana* (*Rana catesbeiana*) could keep Luciferase (LUC) in a foldable state and inhibit the aggregation of LUC (Fernando & Heikkila, 2000; Abdulle, Mohindra, Fernando, & Heikkila, 2002; Heikkila, Kaldis, & Abdulle, 2006; Mulligan-Tuttle & Heikkila, 2007). The Hsp30 of *Rana* protected the activity of the restriction endonuclease PstI at high temperature (42°C) (Kaldis, Atkinson, & Heikkila, 2004). *E. coli bacteria, transformed with the hsp30C gene coding region, were more thermoresistant to a severe thermal challenge of 60°C than either nontransformed bacteria or those containing only the plasmid vector* (Fernando & Heikkila, 2000).

Most studies on the chaperone activity of *hsp30* gene are based on the *Xenopus* and *Rana*, and there are only a few studies on fish (Currie, Moyes, & Tufts, 2000; Zarate & Bradley, 2003; Kondo, Harano, Nakaya, & Watabe, 2004; Healy, Tymchuk, Osborne, & Schulte, 2010; Heikkila, 2017). Northern blot analysis revealed that the enhanced *hsp30* mRNA levels in cultured cells derived from caudal fin of goldfish (*Carassius auratus*) were only observed at 40°C, whereas *hsp70* mRNA was slightly accumulated at 35°C (Kondo *et al.*, 2004). Heat shock-induced *hsp30* mRNA accumulation was reported in rainbow trout (Currie *et al.*, 2000). The results showed that *hsp30* mRNA was not detected in control group (10°C), but incubation at 25°C for 3 h induced *hsp30* mRNA accumulation in all tissues (heart, liver, brain, red muscle and white muscle) except blood (Currie *et al.*, 2000). Heat shock induced the accumulation of *hsp30* mRNA in the muscle and gill of killifish (*Fundulus heteroclitus*), but the extent of up-regulation was greater in a high temperature tolerant southern subspecies compared to a less tolerant northern subspecies (Healy *et al.*, 2010). These results indicated that Hsp30 might have important functions under severe heat stress condition and *hsp30* mRNAexpression level was affected by different species and other factors.

The rainbow trout (*Oncorhynchus mykiss*), which is an economically important species of fish, is a cold-water species, and has a preference for temperatures between 12°C and 18°C with an upper lethal limit of 26°C (Kaya, 1978). Notably, due to habitat degradation and possible global and regional climate changes, many stocks of salmonids may experience temperatures approaching the upper lethal limit, particularly during the summer months. Therefore, exploring the mechanism of heat stress is very important to guide the practice of production. Previous studies have shown that Hsp30 accumulation is mainly at high temperature (Darasch, Mosser, Bols, & Heikkila, 1988; Ali et al, 1997; Quinn, McGowan, Cooper, Koop, & Davidson, 2011; Khan & Heikkila, 2014). Our previous study has shown that the key temperature point of occurring serious response in rainbow trout maybe 25°C (Zhou *et al.*, 2017). In order to understand the expression trend and molecular mechanism controlling of Hsp30 protein in response to heat stress with increasing time of high temperature stress in rainbow trout, we analyzed *hsp30* mRNA expression levels of rainbow trout in control group (CT, 18°C), heat stress (at 25°C for 2, 4, 8 and 12 h) and heat-stress recovering (HSR) from different tissues (heart, liver, gill, brain, spleen and head kidney) by using real-time quantitative polymerase chain reaction (qPCR) (Table 1).

Materials and Methods

One hundred rainbow trout individuals, with the same genetic background, which had an average body mass of 350±11 grams (approximately 10 months old), were obtained from the net cages in the Liujiaxia Reservoir fishing ground (Gansu province, Northwest China). They were maintained in fiberglass tanks with recycled water (18±0.2°C, 4 m^3/h) under a 12 h:12 h light: dark photoperiod, and the water was oxygenated day and night using oxygen increasing pump. The rainbow trout were initially acclimated at 18±0.2°C for 1 week prior to experiment and were fed by commercial pellets twice a day. All experiments complied with institutional guidelines and were approved by the Animal Experimentation Ethics Committee at Gansu Agricultural University, China.

After 1 week of acclimation, forty robust rainbow trout were selected and randomly assigned to five fiberglass tanks (8 individuals in each tank) with recycled water (18±0.2°C) under the same condition. Six groups

Table 1. Primers used for the real-time polymerase chain reaction

Gene	Accession No	Primer sequence	Nucleotide position	Product length (bp)
hsp30	U19370.1	F: 5′-CATCCTCTTTGACCATCCAA-3′ R: 5′-TGGGAAGACAGAGAAGAAGC-3′	229-384	156 bp
β-actin	AB196465.1	F: 5′-TGGGGCAGTATGGCTTGTATG-3′ R: 5′-CTCTGGCACCCTAATCACCTCT-3′	1624-1788	165 bp

F=forward primer; R=reverse primer

(five fish for each group) were compared in this study. Before the heat shock treatment, five fish were netted randomly from the tanks and used for the control group (CT, 18°C). Then, the water temperature was increased from 18°C to 25°C at a rate of 2.5°C/h by using a warm water machine and heating rods, and was held at 25 ± 0.2°C for 12 h in a recirculated and oxygenated water tank. After the temperature reached 25°C, four groups (five fish for each group) were randomly sampled from the tanks at 2, 4, 8 and 12 h, respectively. Finally, the fish still alive after heat stress were returned to a tank with recirculated water at 18°C for recovery, and samples were taken after 6 h of HSR. During the experiment, ten fish died after exposure to heat shock, among which two fish died at 25°C for 4 h, three fish died at 25°C for 8 h and five fish died at 25°C for 12 h.

Fish were quickly captured and anaesthetized by Tricaine mesylate (MS-222, 50 mg/L) until sedated. Then fish were disinfected with potassium permanganate (0.1%) and alcohol (75%) using a cotton swab. Tissue samples from the gill, liver, spleen, heart, head kidney and brain were quickly removed and transferred to 2-mL storage tubes which were immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction. All used materials were sterile and RNase free. The total time was less than 15 min from anaesthetizing to dissecting out all tissues from the fish.

Total RNA was isolated from each tissue sample using Trizol Reagent (TransGen, Beijing, China), according to the manufacturer's protocol. The final RNA pellet was dissolved in 30 µL of RNase-free water. RNA concentration and quality were verified by means of spectrophotometer (Implen, Nano Photometer® Pearl 360, Germany) measurements of optical density at 260 nm and RNA gel electrophoresis, respectively. First-strand complementary DNA (cDNA) was synthesized as per the manufacturer's instructions using Prime Script® RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). All cDNA was stored at -20°C until further use.

Cross-species qPCR primers for *hsp30* gene were designed based on consensus sequences in Chinook salmon (*Oncorhynchus tshawytscha*) (GenBank accession No. U19370.1). The expression of *β-actin* in a variety of animals and tissues is stable (Li *et al.*, 2010; Shekh, Tang, Niyogi, & Hecker, 2017) and shows no response to temperature (Ojima, Yamashita, & Watabe, 2005; Shi *et al.*, 2015), therefore rainbow trout *β-actin* (GenBank accession No. AB196465.1) was used as a reference gene in this study. Primers for qPCR reactions were designed using the Primer 5.0 program (http://www.premierbiosoft.com/) and synthesized from Takara Co., Ltd. The sequences of the primer pairs were as follows: *hsp30*-F: 5'-CATCCTCTTTGACCATCCAA-3', *hsp30*-R: 5'-TGGGAAGACAGAGAAGAAGC-3'; amplicon size was 156 base pairs. *β-actin*-F: 5'-TGGGGCAGTATGGCTTGTATG-3', *β-actin*-R: 5'-CTCTGGCACCCTAATCACCTCT-3'; amplicon size was 165 base pairs.

cDNAs were used as templates for the amplification of the reference (*β-actin*) and target (*hsp30*) genes. The qPCR was carried out in a 20 µL reaction volume containing 10 µL of SYBR® Premix Ex TaqTM II (TaKaRa), 0.4 µL of each primer (10 µM), 7.2 µL of RNase-free ddH_2O, and 2 µL of cDNA template (<100 ng). qPCRs were run in 96-well plates on a LightCycler® 480 Instrument II (Roche, Switzerland). The amplification conditions were as follows: 95°C for 30 s and 40 cycles of 95°C for 5 s, 50°C for 20 s and 72°C for 15 s. A melting curve analysis (95°C for 5 s and 60°C for 15 s) was performed after each qPCR run in order to verify the amplification specificity, which was indicated by a single peak.

qPCR cycle thresholds (Ct) were calculated as the average of triplicates (per tissue sample) for both the target and reference genes. *hsp30* mRNA expression level was measured in each individual specimen, and was normalized to that of the *β-actin* gene. The results of the relative *hsp30* mRNA expression level were analyzed by the comparison of $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001) and expressed as the mean ± S.E. (n = 3). In order to compare *hsp30* mRNA levels between different tissues and between different experimental groups, one-way analysis of variance (ANOVA) followed by LSD (Least Significant Difference) test at $P<0.05$ was performed using the SPSS 18.0 software (IBM, Armonk, NY, USA).

Results

In the control group (CT, 18°C), a low level of *hsp30* mRNA expression was observed in all tissues and the expression level of *hsp30* mRNA in the heart was significantly higher than liver, gill, spleen and head kidney (5.5-, 44-, 44- and 123.372-fold; P=0.016, 0.006, 0.006 and 0.005, respectively) except brain (P>0.05) (Figure 1). After heat stress at 25°C for 2 h, *hsp30* mRNA expression in the liver exhibited the largest increase and was significantly higher than that in heart, gill, brain, spleen and head kidney (1.261-, 3.284-, 4.244-, 6.165- and 23.904-fold; P=0.020, 0.000, 0.000, 0.000 and 0.000, respectively, Figure 1). After heat stress for 4 h, there was no significant difference between the heart and liver (P=0.917, Figure 1), but *hsp30* mRNA expression level in the heart was significantly higher than that in gill, brain, spleen and head kidney (15.794-, 20.403-, 15.238- and 64.208-fold; P=0.000, 0.000, 0.000 and 0.000, respectively, Figure 1). With increasing time of heat stress, the expression level of *hsp30* mRNA in the heart was still significantly higher than those of other tissues after high temperature stress for 8 h and 12 h, and after 6 h of HSR (18°C) (P<0.05, Figure 1).

After heat stress at 25°C, *hsp30* mRNA expression levels in all tissues were significantly increased at 2 h exposure to elevated water temperatures than control group (P<0.05, Figure 2). Then, *hsp30* mRNA expression level in the brain at 4 h started to decline sharply than 2

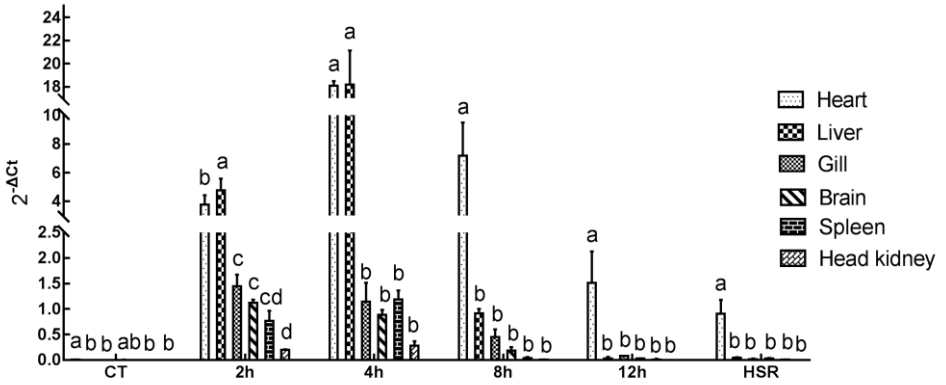

Figure 1. Comparison of *hsp30* mRNA expression levels in different tissues at the same time point, HSR=heat stress recovering. Different letters mean significant differences (P<0.05).

h (P=0.000), and *hsp30* mRNA expression in the gill remained the same significant level between 2 h and 4 h (Figure 2). While the expression levels of *hsp30* mRNA in the heart, liver, spleen and head kidney at 4 h were significantly higher than 2 h (4.806-, 3.832-, 1.541- and 1.418-fold; P=0.000, 0.000, 0.000 and 0.014, respectively, Figure 2). At 8 h after heat stress, *hsp30* mRNA expression levels in the heart, gill and brain were still significantly higher than those in the control group (1631.932-, 4837.316- and 98.526-fold; P=0.000, 0.013 and 0.001, respectively). Finally, *hsp30* mRNA expression levels in all tissues returned to the control level at 12 h and HSR (P>0.05, Figure 2).

Compared with other tissues, *hsp30* mRNA expression levels were highest in the heart and liver after 2-4 h of heat stress (Figure 1). However, the most significant changes of *hsp30* mRNA after 2-4 h of heat stress were observed in the gill where they were significantly higher than those in the control group by approximately 15668.243- and 11452.125-fold (P=0.000 and 0.000, respectively, Figure 2). The levels of *hsp30* mRNA began to increase after 2 h of heat stress in heart, liver, spleen and head kidney, and then they reached a maximal level after 4 h of heat stress which was significantly increased when compared to the control group (4110.750-, 22741.375-, 10118.206- and 7810.872-fold; P=0.000, 0.000, 0.000 and 0.000, respectively, Figure 2). The highest expression level of *hsp30* mRNA after 2 h of heat stress was recorded in the liver as shown in Figure 1.

Discussion

A study of adult rainbow trout has shown that *hsp30* mRNA was not detected in all tissues (heart, liver, brain, white muscle (wm), red muscle (rm) and blood) under control condition (10°C) through Northern blot (Currie *et al.*, 2000). In this study, *hsp30* mRNA in all tissues (heart, liver, gill, brain, spleen and head kidney) had begun to synthesize at 18°C, indicating that the fish body has already started a heat stress reaction at 18°C. Our results showed that *hsp30* mRNA expression levels after heat stress at 25°C for 2 h were up-regulated in heart, liver, gill, brain, head kidney and spleen, which is consistent with the previous research of Currie *et al.* (2000) which showed that *hsp30* mRNA was increased with heat shock at 25°C for 3 h in all tissues (heart, liver, brain, white muscle (wm) and red muscle (rm)) with the exception of blood. These results suggested that *hsp30* mRNA expression in rainbow trout is induced by heat stress. Interestingly, our results showed that *hsp30* mRNA expression in all tissues was down-regulated significantly after heat stress at 25°C for 8 h and returned to the control level after heat stress at 25°C for 12 h, which suggested that the tissues were damaged (Zhou *et al.*, 2017) and the *hsp30* mRNA synthesis was affected. If the heat stress is beyond certain intensity, it may lead to abnormalities of the cell membrane structure and proteins. In addition, it can lead to a change of the distribution of *hsp30* within the cell, thus reducing the heat endurance of cells. In the HSR group, *hsp30* mRNA expression in all tissues returned to the control level, indicating that the physiological function of organism gradually recovered after heat stress.

Previous study has shown that *hsp30* mRNA accumulation was induced primarily in the heart and red muscle of rainbow trout at 25°C for 3 h, and heart, red muscle and liver had the highest levels of *hsp30* mRNA (Currie *et al.*, 2000). In our study, *hsp30* mRNA expression level in the heart at 18°C was significantly higher than liver, gill, spleen and head kidney, and heart and liver had the highest levels of *hsp30* mRNA after heat stress at 25°C for 2-4 h and the level of *hsp30* mRNA in the heart was still significantly higher than other tissues at 25°C for 8-12 h. The heart is the most important organ in the circulation system of the fish body. Under the heat stress, the metabolism of the fish body and the energy metabolism are strengthened, which leads to the increase of the heart rate, the decrease of the hemoglobin binding oxygen, and the hypoxia of the tissues of the body (Clark, Sandblom, Cox, Hinch, & Farrell, 2008). It is inferred that the heart is first

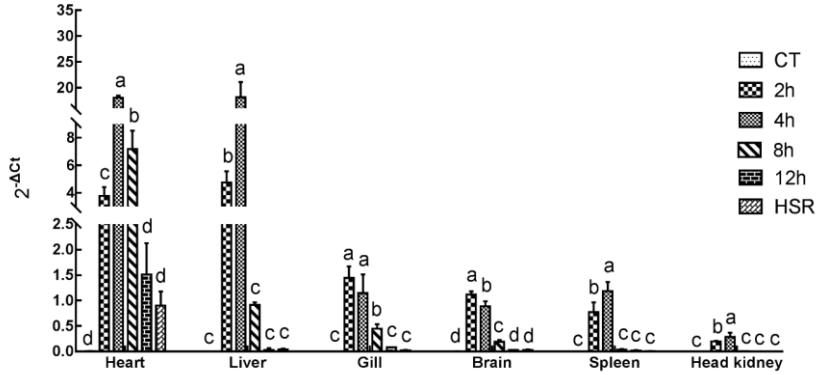

Figure 2. Expression level changes of *hsp30* mRNA in the same tissue of control group (CT), heat-stressed, and heat-stress recovering (HSR) fish. Different letters mean significant differences (P<0.05).

affected and protected under the heat stress, so Hsp30 protein can protect the heart timely for a longer time than the other tissues. A prominent induction of *hsp30* gene synthesis was clearly evident in the liver of fish inhabiting a lake receiving the discharges from a thermal power plant (Wang, Xu, Sheng, & Zheng, 2007). Thermal stress forces fish to divert energy from growth to maintenance, including to processes that help minimize thermal damage such as the induction of Hsps and reorganization of plasma membranes (Iwama, Thomas, Forsyth, & Vijayan, 1998; Farkas, Fodor, Kitajka, & Halver, 2001). Hsp30 has been found to be primarily plasma membrane-associated in both membrane fractionation and immunofluorescence studies (Panaretou & Piper, 1992). Plasma membrane H^+-ATPase activity is an immediate physiological response of cells subjected to heat stress and consumes a substantial fraction of the ATP generated by the cell, a usage that will be increased by H^+-ATPase stimulation occurring with Hsp30-inducing stress (Wu, Dawe, & Aris, 2000). Hsp30 might provide an energy conservation role that limits excessive ATP consumption by plasma membrane H^+-ATPase during prolonged stress exposure or glucose restriction (Piper, Ortiz-Calderon, Holyoak, Coote, & Cole, 1997). The liver is the important metabolic organ to maintain glucose and energy homeostasis of an organism, especially under stress conditions. In this study, the *hsp30* mRNA expression level in the liver was significantly higher than that in the gill, brain, spleen and head kidney during heat stress (2 and 4 h), indicating that the liver was damaged seriously than other tissues and the heat stress was strongest during 2~4 h of high temperature. It showed that Hsp30 might play a significant role in dealing with heat-induced damage in the liver. Therefore, the organism have to up-regulate the expression of *hsp30* mRNA. In addition, Hsp30 can play a role of molecular chaperone to help denatured protein refolding (Nakamoto & Vígh, 2007). It can be inferred that the expression levels of *hsp30* mRNA in the heart and liver are tissue-specific, which is the same as previous study (Cheng, Liu, Zhang, & He, 2007).

Previous studies have shown that the gill of Chinook salmon (*Oncorhynchus tshawytscha*) and killifish (*Fundulus heteroclitus*) is more sensitive than other organs to change of water environment (Healy *et al.*, 2010; Tomalty *et al.*, 2015). Heat shock-induced accumulation of *hsp30* mRNA was found in the gill of Atlantic salmon (*Salmo salar*), which were transferred from 15°C to 26°C for 30 min (Zarate & Bradley, 2003). In the present study, *hsp30* mRNA expression in the gill was significantly up-regulated (15668.243-fold) at 25°C for 2 h compared with the control group (25°C), then significantly down-regulated at 8 h than 4 h, and finally return to control group level at 12 h. These results indicated that gill, which is the first organ to contact environment at high temperatures, is more sensitive and faster than heart, liver, brain, spleen and head kidney to response heat stress. The brain is part of the central nervous system and heat stress-induced Hsps in the central nervous system has been largely demonstrated, and heat stress can increase the heat tolerance of the brain (Currie & White, 1983; Brown, 1990; Nowak, Bond, & Schlesinger, 1990; Kiang & Tsokos, 1998; Kagawa, 2004). In our study, during heat stress, the trend of *hsp30* expression in brain was the same as that in gill, but the fold change of *hsp30* mRNA was significantly lower than that in gill, which indicated that the thermotolerance of brain is lower than gill.

The spleen is the largest lymphoid organ found in fish in addition to being a hematopoietic tissue (Press & Evensen, 1999; Mulero, García-Ayala, Meseguer, & Mulero, 2007), and head kidney of fish is a hematopoietic organ, which is the same as spleen. They have important functions in implementing appropriate immune responses which are necessary for a homeostatic state. During the whole period of heat stress, the expression levels of *hsp30* mRNA in spleen and head kidney have been lower than that in heart, which showed that it is the same as *hsp60* mRNA expression (Shi *et al.*, 2015). So it can be inferred that the spleen and head kidney are less affected by the exposure to high temperature in the body thereby

indicating their phenotypic plasticity in response to chronic stressors in order to maintain homeostasis.

In this study, we have found that *hsp30* mRNA expression regulations which provide protection and thermotolerance in organisms during heat stress like other sHsps studies. Rainbow trout have already had a heat stress response at 18°C, then the expression of *hsp30* mRNA was significantly up-regulated in all tissues after heat stress at 25°C for 2 h, but significantly down-regulated after heat stress for 8~12 h. These results indicated that *hsp30* mRNA expression is regulated by negative feedback, and Hsp30 can provide some protection in heat stress, but it is only within a certain range. The fish responded to heat stress in a tissue-specific manner, which indicates that Hsp30 has tissue-related functions and may even become a potential stress marker.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No. 31460687). We thank Dr. Noe Gomez (Department of Animal Sciences Washington State University, USA) for his careful revisions of the manuscript. The first two authors contributed equally to this work and should be considered co-first authors.

References

Abdulle, R., Mohindra, A., Fernando, P., & Heikkila, J.J. (2002). *Xenopus* small heat shock proteins, Hsp30C and Hsp30D, maintain heat- and chemically denatured luciferase in a folding-competent state. *Cell Stress & Chaperones*, 7(1), 6-16. http://dx.doi.org/10.1379/1466 1268(2002)007<0006:XSHSPH>2.0.CO;2

Ali, A., Fernando, P., Smith, W.L., Ovsenek, N., Lepock, J.R., & Heikkila, J.J. (1997). Preferential activation of HSF-binding activity and *hsp70* gene expression in *Xenopus* heart after mild hyperthermia. *Cell Stress & Chaperones*, 2(4), 229-237.

Bagnéris, C., Bateman, O.A., Naylor, C.E., Cronin, N., Boelens, W.C., Keep, N.H., & Slingsby, C. (2009). Crystal structures of α-crystallin domain dimers of αB-crystallin and Hsp20. *Journal of Molecular Biology*, 392(5), 1242-1252. http://dx.doi.org/10.1016/j.jmb.2009.07.069

Basha, E., O'Neill, H., & Vierling, E. (2012). Small heat shock proteins and α-crystallins: dynamic proteins with flexible functions. *Trends in Biochemical Sciences*, 37(3), 106-117. http://dx.doi.org/10.1016/j.tibs.2011.11.005

Brady, J.P., Garland, D., Duglas-Tabor, Y., Robison, W.G., Groome, A., & Wawrousek, E.F. (1997). Targeted disruption of the mouse αA-crystallin gene induces cataract and cytoplasmic inclusion bodies containing the small heat shock protein αB-crystallin. *Proceedings of the National Academy of Sciences of the United States of America*, 94(3), 884-889. http://dx.doi.org/10.1073/pnas.94.3.884

Brown, I.R. (1990). Induction of heat shock (stress) genes in the mammalian brain by hyperthermia and other traumatic events: A current perspective. *Journal of Neuroscience Research*, 27(3), 247-255. http://dx.doi.org/10.1002/jnr.490270302

Chen, R., Dai, R., Duan, C., Liu, Y., Chen, S., Yan, D., … Li, H. (2011). Unfolded protein response suppresses cisplatin-induced apoptosis via autophagy regulation in human hepatocellular carcinoma cells. *Folia Biologica*, 57(3), 87-95. http://www.ncbi.nlm.nih.gov/pubmed/21888831

Cheng, P., Liu, X., Zhang, G., & He, J. (2007). Cloning and expression analysis of a HSP70 gene from Pacific abalone (*Haliotis discus hannai*). *Fish & Shellfish Immunology*, 22(1-2), 77-87. http://dx.doi.org/10.1016/j.fsi.2006.03.014

Clark, T.D., Sandblom, E., Cox, G.K., Hinch, S.G., & Farrell, A.P. (2008). Circulatory limits to oxygen supply during an acute temperature increase in the Chinook salmon (*Oncorhynchus tshawytscha*). *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 295(2), R1631-R1639. http://dx.doi.org/10.1152/ajpregu.90461.2008

Currie, R.W., & White, F.P. (1983). Characterization of the synthesis and accumulation of a 71-kilodalton protein induced in rat tissues after hyperthermia. *Canadian Journal of Biochemistry and Cell Biology*, 61(6), 438-446. http://dx.doi.org/10.1139/o83-059

Currie, S., Moyes, C.D., & Tufts, B.L. (2000). The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: *in vivo* and *in vitro* comparisons. *Journal of Fish Biology*, 56(2), 398-408. http://dx.doi.org/10.1006/jfbi.1999.1166

Darasch, S., Mosser, D.D., Bols, N.C., & Heikkila, J.J. (1988). Heat shock gene expression in *Xenopus laevis* A6 cells in response to heat shock and sodium arsenite treatments. *Biochemistry and Cell Biology-Biochimie et Biologie Cellulaire*, 66(8), 862-870. http://dx.doi.org/10.1139/o88-098

Farkas, T., Fodor, E., Kitajka, K., & Halver, J.E. (2001). Response of fish membranes to environmental temperature. *Aquaculture Research*, 32(8), 645-655. http://dx.doi.org/10.1046/j.1365-2109.2001.00600.x

Fernando, P., & Heikkila, J.J. (2000). Functional characterization of *Xenopus* small heat shock protein, Hsp30C: the carboxyl end is required for stability and chaperone activity. *Cell Stress & Chaperones*, 5(2), 148-159. http://dx.doi.org/10.1379/1466-1268(2000)005<0148:FCOXSH>2.0.CO;2

Goplen, D., Bougnaud, S., Rajcevic, U., Bøe, S.O., Skaftnesmo, K.O., Voges, J., … Bjerkvig, R. (2010). αB-crystallin is elevated in highly infiltrative apoptosis-resistant glioblastoma cells. *American Journal of Pathology*, 177(4), 1618-1628. http://dx.doi.org/10.2353/ajpath.2010.090063

Guzzo, J. (2012). Biotechnical applications of small heat shock proteins from bacteria. *International Journal of Biochemistry & Cell Biology*, 44(10), 1698-1705. http://dx.doi.org/10.1016/j.biocel.2012.06.007

Hanazono, Y., Takeda, K., Oka, T., Abe, T., Tomonari, T., Akiyama, N., … Miki, K. (2013). Nonequivalence observed for the 16-meric structure of a small heat shock protein, SpHsp16.0, from *Schizosaccharomyces pombe*. *Structure*, 21(2), 220-228. http://dx.doi.org/10.1016/j.str.2012.11.015

Hasanuzzaman, M., Nahar, K., Alam, M.M., Roychowdhury, R., & Fujita, M. (2013). Physiological, biochemical, and

molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences*, 14(5), 9643-9684. http://dx.doi.org/10.3390/ijms14059643

Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H.E., Chen, S., … Buchner, J. (1999). Hsp26: a temperature-regulated chaperone. *EMBO Journal*, 18(23), 6744-6751. http://dx.doi.org/10.1093/emboj/18.23.6744

Healy, T.M., Tymchuk, W.E., Osborne, E.J., & Schulte, P.M. (2010). Heat shock response of killifish (*Fundulus heteroclitus*): candidate gene and heterologous microarray approaches. *Physiological Genomics*, 41(2), 171-184. http://dx.doi.org/10.1152/physiolgenomics.00209.2009

Heikkila, J.J. (2003). Expression and function of small heat shock protein genes during *Xenopus* development. *Seminars in Cell & Developmental Biology*, 14(5), 259-266. http://dx.doi.org/10.1016/j.semcdb.2003.09.022

Heikkila, J.J. (2017). The expression and function of *hsp30*-like small heat shock protein genes in amphibians, birds, fish, and reptiles. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 203, 179-192. http://dx.doi.org/10.1016/j.cbpa.2016.09.011

Heikkila, J.J., Kaldis, A., & Abdulle, R. (2006). Analysis of molecular chaperones using a *Xenopus* oocyte protein refolding assay. In X.J. Liu (Eds.), *Xenopus Protocols* (pp. 213-222). Clifton, New Jersey, Humana Press., 489 pp.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., & Vijayan, M.M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, 8(1), 35-56. http://dx.doi.org/10.1023/A:1008812500650

Jakob, U., Gaestel, M., Engel, K., & Buchner, J. (1993). Small heat shock proteins are molecular chaperones. *Journal of Biological Chemistry*, 268(3), 1517-1520. http://www.jbc.org/content/268/3/1517.short

Kagawa, N. (2004). A drastic reduction in the basal level of heat-shock protein 90 in the brain of goldfish (*Carassius auratus*) after administration of geldanamycin. *Zoological Science*, 21(11), 1085-1089. http://dx.doi.org/10.2108/zsj.21.1085

Kaldis, A., Atkinson, B.G., & Heikkila, J.J. (2004). Molecular chaperone function of the *Rana catesbeiana* small heat shock protein, hsp30. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 139(2), 175-182. http://dx.doi.org/10.1016/j.cbpb.2004.08.006

Kaya, C.M. (1978). Thermal resistance of rainbow trout from a permanently heated stream, and of two hatchery strains. *The Progressive Fish-Culturist*, 40(40), 138-142. http://dx.doi.org/10.1577/1548-8659(1978)40[138:TRORTF]2.0.CO;2

Khan, S., & Heikkila, J.J. (2014). Distinct patterns of HSP30 and HSP70 degradation in *Xenopus laevis* A6 cells recovering from thermal stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 168(2), 1-10. http://dx.doi.org/10.1016/j.cbpa.2013.10.026

Kiang, J.G., & Tsokos, G.C. (1998). Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacology & Therapeutics*, 80(2), 183-201. https://dx.doi.org/10.1016/S0163-7258(98)00028-X

Kondo, H., Harano, R., Nakaya, M., & Watabe, S. (2004). Characterization of goldfish heat shock protein-30 induced upon severe heat shock in cultured cells. *Cell Stress & Chaperones*, 9(4), 350-358. http://dx.doi.org/10.1379/CSC-55R.1

Li, H., Liu, S., He, C., Gao, X., & Yuan, X. (2013). Identification of a small HSP gene from hard clam *Meretrix meretrix* and its potential as an environmental stress biomarker. *Aquatic Biology*, 18(3), 243-252. http://dx.doi.org/10.3354/ab00503

Li, Z., Yang, L., Wang, J., Shi, W., Pawar, R.A., Liu, W., … Zhang, Y. (2010). *β-Actin* is a useful internal control for tissue-specific gene expression studies using quantitative real-time PCR in the half-smooth tongue sole *Cynoglossus semilaevis* challenged with LPS or *Vibrio anguillarum*. *Fish & Shellfish Immunology*, 29(1), 89-93. http://dx.doi.org/10.1016/j.fsi.2010.02.021

Livak, K.J., & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25(4), 402-408. http://dx.doi.org/10.1006/meth.2001.1262

Marvin, M., O'Rourke, D., Kurihara, T., Juliano, C.E., Harrison, K.L., & Hutson, L.D. (2008). Developmental expression patterns of the zebrafish small heat shock proteins. *Developmental Dynamics*, 237(2), 454-463. http://dx.doi.org/10.1002/dvdy.21414

Morrow, G., & Tanguay, R.M. (2012). Small heat shock protein expression and functions during development. *International Journal of Biochemistry & Cell Biology*, 44(10), 1613-1621. http://dx.doi.org/10.1016/j.biocel.2012.03.009

Mulero, I., García-Ayala, A., Meseguer, J., & Mulero, V. (2007). Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture*, 268(1), 244-250. http://dx.doi.org/10.1016/j.aquaculture.2007.04.046

Mulligan-Tuttle, A., & Heikkila, J.J. (2007). Expression of the small heat shock protein gene, *hsp30*, in *Rana catesbeiana* fibroblasts. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 148(2), 308-316. http://dx.doi.org/10.1016/j.cbpa.2007.04.022

Nakamoto, H., & Vígh, L. (2007). The small heat shock proteins and their clients. *Cellular and Molecular Life Sciences*, 64(3), 294-306. http://dx.doi.org/10.1007/s00018-006-6321-2

Nowak, T.S., Bond, U., & Schlesinger, M.J. (1990). Heat shock RNA levels in brain and other tissues after hyperthermia and transient ischemia. *Journal of Neurochemistry*, 54(2), 451-458. http://dx.doi.org/10.1111/j.1471-4159.1990.tb01893.x

Ojima, N., Yamashita, M., & Watabe, S. (2005). Quantitative mRNA expression profiling of heat-shock protein families in rainbow trout cells. *Biochemical and Biophysical Research Communications*, 329(1), 51-57. http://dx.doi.org/10.1016/j.bbrc.2005.01.097

Panaretou, B., & Piper, P.W. (1992). The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase. *European Journal of Biochemistry*, 206(3), 635-640. http://dx.doi.org/10.1111/j.1432-1033.1992.tb16968.x

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P., & Cole, M. (1997). Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H^+-ATPase. *Cell Stress & Chaperones*, 2(1), 12-24.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC31297 6

Prakash, M.K.C., Thomas, R.R., Reddy, M.K., & Mohandas, S. (2013). Evidence for molecular evolutionary conservedness of small heat-shock protein sequence in solanaceaeous crops using *in silico* methods. *Journal of Horticultural Sciences*, 8(1), 82-87.

Press, C.M., & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology*, 9(4), 309-318. https://dx.doi.org/10.1006/fsim.1998.0181

Quinn, N.L., McGowan, C.R., Cooper, G.A., Koop, B.F., & Davidson, W.S. (2011). Identification of genes associated with heat tolerance in Arctic charr exposed to acute thermal stress. *Physiological Genomics*, 43(11), 685-696. http://dx.doi.org/10.1152/physiolgenomics.00008.2011

Ratajczak, E., Stróżecka, J., Matuszewska, M., Ziętkiewicz, S., Kuczyńska-Wiśnik, D., Laskowska, E., & Liberek, K. (2010). IbpA the small heat shock protein from *Escherichia coli* forms fibrils in the absence of its cochaperone IbpB. *FEBS Letters*, 584(11), 2253-2257. http://dx.doi.org/10.1016/j.febslet.2010.04.060

Rogalla, T., Ehrnsperger, M., Preville, X., Kotlyarov, A., Lutsch, G., Ducasse, C., … Gaestel, M. (1999). Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress/tumor necrosis factor α by phosphorylation. *Journal of Biological Chemistry*, 274(27), 18947-18956. http://dx.doi.org/10.1074/jbc.274.27.18947

Shekh, K., Tang, S., Niyogi, S., & Hecker, M. (2017). Expression stability and selection of optimal reference genes for gene expression normalization in early life stage rainbow trout exposed to cadium and copper. *Aquatic Toxicology*, 190, 217-227. http://dx.doi.org/10.1016/j.aquatox.2017.07.009

Shi, H., Liu, Z., Zhang, J., Kang, Y., Wang, J., Huang, J., & Wang, W. (2015). Effect of heat stress on heat-shock protein (Hsp60) mRNA expression in rainbow trout *Oncorhynchus mykiss*. *Genetics and Molecular Research*, 14(2), 5280-5286. http://dx.doi.org/10.4238/2015.May.18.20

Tomalty, K.M.H., Meek, M.H., Stephens, M.R., Rincón, G., Fangue, N.A., May, B.P., & Baerwald, M.R. (2015). Transcriptional response to acute thermal exposure in juvenile Chinook salmon determined by RNAseq. *G3-Genes Genomes Genetics*, 5(7), 1335-1349. http://dx.doi.org/10.1534/g3.115.017699

Tóth, M.E., Szegedi, V., Varga, E., Juhász, G., Horváth, J., Borbély, E., … Sántha, M. (2013). Overexpression of Hsp27 ameliorates symptoms of Alzheimer's disease in APP/PS1 mice. *Cell Stress & Chaperones*, 18(6), 759-771. http://dx.doi.org/10.1007/s12192-013-0428-9

Wang, Y., Xu, J., Sheng, L., & Zheng, Y. (2007). Field and laboratory investigations of the thermal influence on tissue-specific Hsp70 levels in common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 148(4), 821-827. http://dx.doi.org/10.1016/j.cbpa.2007.08.009

Wu, K., Dawe, J.H., & Aris, J.P. (2000). Expression and subcellular localization of a membrane protein related to Hsp30p in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta-Biomembranes*, 1463(2), 477-482. http://dx.doi.org/10.1016/S0005-2736(99)00255-2

Zarate, J., & Bradley, T.M. (2003). Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture*, 223(1–4), 175-187. http://dx.doi.org/10.1016/S0044-8486(03)00160-1

Zhou, Y., Liu, Z., Xia, B., Kang, Y., Wang, Y., & Liu, X. (2017). Effect of continuing heat stress on the liver tissue morphology of rainbow trout (*Oncorhynchus mykiss*) (in Chinese with English abstract). *Journal of Gansu Agricultural University*. 52(6), 1-5. http://dx.doi.org/10.13432/j.cnki.jgsau.2017.06.001