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RESEARCH ARTICLE

Effects of both cold and heat stress on the liver of the giant spiny frog (*Quasipaa spinosa*): stress response and histological changes

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ABSTRACT

Ambient temperature-associated stress can affect normal physiological functions in ectotherms. To assess the effects of cold or heat stress on amphibians, giant spiny frogs (*Quasipaa spinosa*) were acclimated at 22°C followed by exposure to 5°C or 30°C for 0, 3, 6, 12, 24 and 48 h, respectively. Histological alterations, apoptotic index, generation of mitochondrial reactive oxygen species (ROS), antioxidant activity indices and stress-response gene expression in frog livers were subsequently determined. Results showed that many fat droplets appeared after 12 h of heat stress and the percentage of melanomacrophage centres significantly changed after 48 h at both stress conditions. Furthermore, the mitochondrial ROS levels were elevated in a time-dependent manner up to 6 h and 12 h in the cold and heat stress groups, respectively. The activities of superoxide dismutase, glutathione peroxidase and catalase were successively increased with increasing periods of cold or heat exposure, and their gene expression levels showed similar changes in both stress conditions. Most tested heat shock protein (HSP) genes were sensitive to temperature exposure, and the expression profiles of most apoptosis-related genes was significantly upregulated at 3 and 48 h under cold and heat stress, respectively. Apoptotic index at 48 h under cold stress was significantly higher than that under heat stress. Notably, lipid droplets, *HSP30*, *HSP70* and *HSP110* might be suitable bioindicators of heat stress. The results of these alterations at physiological, biochemical and molecular levels might contribute to a better understanding of the stress response of *Q. spinosa*, and perhaps amphibians more generally, under thermal stress.

KEY WORDS: Amphibian, Thermal stress, Histological alterations, Oxidative stress, Apoptosis, Stress response gene

INTRODUCTION

Understanding and predicting how global warming affects the structure and functioning of natural ecosystems is a key challenge of the 21st century (O'Gorman et al., 2014). The global atmospheric temperature is estimated to continue changing at a rapid rate and to increase by 1.8–4.0°C by the end of the century (Kennedy et al., 2016; Noyes et al., 2009). Owing to anthropogenically driven climate change, the numbers of extreme hot or cold days could increase, which is expected to have a particularly strong impact on ectothermic animals (Buckley et al., 2012; Li et al., 2015). As a key environmental factor in mediating homeostasis in ectotherms, ambient temperature not only affects factors associated with the living environment, such as water distribution (Buckley et al., 2012), germ transmission (Raffel et al., 2012) and the dissolution of toxic substances (Guo et al., 2018), but also acts as a stressor to organisms and affects normal larval development (Moyano et al., 2017), immune function (Maniero and Carey, 1997), tissue metabolism (Costanzo and Lee, 2008; Sinclair et al., 2012) and other physiological functions.

Low and high ambient temperatures can increase generation of reactive oxygen species (ROS) in ectotherms (Paital, 2016) and the overproduction of ROS can damage critical biomolecules such as DNA, proteins and lipids, consequently causing impaired cellular function and oxidative stress (Cheng et al., 2015, 2017). To counteract oxidative stress, all organisms are equipped with antioxidant defence systems, including antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and some non-enzymatic antioxidants (Dawson and Storey, 2016; Li et al., 2017). However, excessive stresses, such as thermal stress, beyond the specific tolerance levels may lead to oxidative damage and even cell apoptosis (Cheng et al., 2017; Curtin et al., 2002). Pro-apoptotic genes, including *Bax*, *Caspase-3*, *Caspase-8* and *Caspase-9*, and anti-apoptotic genes, such as *Bcl2* are involved in thermal stress-induced apoptosis (Gerber et al., 2016; Wang et al., 2012). There is evidence that gene expression of antioxidant enzymes might be induced by oxidative stress (Chai et al., 2017; Wu et al., 2017). Moreover, the heat shock response, as a protective mechanism against stress, protects the body from oxidative damage by increasing the expression levels of heat shock protein (HSP) genes (Heikkila, 2010; Ketola-Pirie and Atkinson, 1983; Rupik et al., 2011). These stress-response genes play an important role in maintaining general homeostasis and regulating organismal metabolism.

Ambient temperature changes, including extremes of low and high temperatures, may have different effects on ectotherms (Paital, 2016). Owing to their special sensitivity to temperature changes, amphibians could serve as model organisms to investigate the effects of cold or heat stress. Previous studies on the effects of cold stress on amphibians have shown the upregulation of freeze-responsive molecules (McNally et al., 2002; Storey and Storey, 2017; Wu et al., 2008), regulation of redox balance (Dawson and Storey, 2016, 2017; Sahoo and Kara, 2014), reduction of lymphocyte (Greenspan et al., 2017; Maniero and Carey, 1997) and synthesis of the cryoprotectants urea and glucose (Costanzo and Lee, 2008; Costanzo et al., 2013, 2015; Dieni et al., 2012), whereas heat stress upregulates the heat shock response (Heikkila, 2010; Young et al., 2009), antioxidant defence system (Freitas and Almeida, 2016) and the susceptibility of amphibians to harmful substances (Freitas et al., 2016; Gaitanaki et al., 2007). Furthermore,

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thermal stresses also can effectively induce histological alterations in the liver of other ectotherms, such as fat droplet accumulation (Liu et al., 2016), mitochondrial swelling (Xu et al., 2018), hepatocyte degeneration and necrosis (Ates et al., 2006). However, studies on the effects of both cold and heat stresses on the histology, apoptotic index, antioxidant activity indices and stress-response gene expression in amphibians remain obscure.

The giant spiny frog *Quasipaa spinosa* (David 1875) is distributed in China and Vietnam, particularly in southern and southeast parts of China (Hu et al., 2017). Owing to its narrow optimum temperature range (20–24°C), it could be a suitable heterothermic animal model to assess the impact of thermal stress, including cold and heat stress, on amphibians (Shu et al., 2017; Xie et al., 2017; Zheng and Liu, 2010). In the present study, to evaluate the resistance of frogs to cold (5°C) and heat (30°C) stress, histological and ultrastructural alterations of the liver were determined in *Q. spinosa*. Furthermore, apoptotic index, mitochondrial ROS generation, antioxidant activity indices and expression profiles of stress-response genes, including antioxidant-related genes, HSPs and apoptosis-related genes, were also investigated in the liver of *Q. spinosa*. The results of these alterations at physiological, biochemical and molecular levels would pave the way for a better understanding of the stress response and antioxidant mechanisms of *Q. spinosa* and even amphibians under thermal stress.

MATERIALS AND METHODS

Animals and temperature stress experiments

Healthy *Q. spinosa*, weighing 43.52±3.95 g, were obtained from a frog farm in Zhejiang province, China. Animals were maintained in tanks with well-aerated, dechlorinated tap water at 22°C for 2 weeks prior to the experiments. The frogs were fed with mealworm (*Tenebrio molitor*) once a day until 2 days before the experiments.

To assess the effects of extreme temperature stress on the frog, 72 healthy frogs were selected and randomly allocated to the cold (5°C) or heat (30°C) stress groups and treated for 0, 3, 6, 12, 24 and 48 h, respectively (6 frogs per group). Tanks containing *Q. spinosa* were transferred to an incubator (Bio Multi incubator; NK Systems, Osaka, Japan) for different temperature treatments. The individuals were anesthetized with 3-amino benzoic acid ethyl ester (MS-222, Sigma) before euthanization. Liver tissues were collected and immediately snap-frozen in liquid nitrogen and stored at −80°C until use.

Histological analysis

The liver fragments from 6 frogs in each group were fixed in 10% neutral buffered formalin for 24 h. After dehydration in graded ethanol concentrations and embedding in paraffin wax, sections were stained with Hematoxylin and Eosin (H&E), and prepared for light microscopy (DP80, Olympus).

The analysis of histological lesions was carried out by evaluating the congestion, enlargement of sinusoids, inflammatory cell infiltration, haemorrhage and changes in the number of melanomacrophage cells in hepatocytes according to Cakıcı (2015). The sections from each experimental organism were photographed and evaluated at ×200 magnification. To quantify the area occupied by melanomacrophages, 15 non-overlapping random regions from each section were analysed using Image Pro Plus 6.0 (Media Cybernetics, Inc.) software. Brown–black pigment cells were marked using the dropper tool and mean percentage of melanomacrophage area was calculated.

Ultrastructural observations

The liver samples from 6 frogs in each group were selected and fixed in 2.5% glutaraldehyde overnight at 4°C. The samples were washed three times (15 min each) with 0.1 mol l^{-1} phosphate buffer (pH 7.4). The livers were fixed in 1.5% osmic acid for 1 h and then washed again with 0.1 mol l^{-1} phosphate buffer. Thereafter, the samples were dehydrated using a 50–100% graded alcohol series, embedded in pure acetone, sliced into thin sections, and then stained with uranyl acetate and lead citrate. Finally, the samples were examined under a transmission electron microscope (JEM-1010, JEOL).

Terminal deoxynucleotidyl transferase deoxy-UTP nick end labelling (TUNEL) assay

The liver tissues sections from 6 frogs in each group were subjected to TUNEL assay (In Situ Cell Death Detection Kit and fluorescein, Roche Diagnostics) for the detection of cell apoptosis. Briefly, dewaxed and rehydrated tissue sections were treated with proteinase K for 15 min at room temperature, rinsed three times with phosphate-buffered saline (PBS) for 3 min each, and then stained with TUNEL reaction mixture, followed by being counterstained with DAPI (4′,6-diamidino-2-phenylindole). Finally, the sections were observed under a confocal microscope (Olympus FV3000 OSR).

To quantify the degree of hepatocyte apoptosis under cold and heat stresses, sections from each experimental organism were photographed and evaluated at ×200 magnification. Fifteen non-overlapping random regions from each section were analysed using ImageJ software (NIH, Windows version). TUNEL-positive cells stained green and nuclei stained blue were individually marked using the cell counter menu, and mean percentage of apoptotic cells (apoptotic index) was calculated.

Determination of mitochondrial ROS generation

The level of mitochondrial ROS was detected by using DCFH-DA (2′,7′-dichlorofluorescein diacetate) based on the method described by Zhang et al. (2013). Liver samples in 1.5 ml centrifuge tubes were mixed with 0.65% cold saline solution at a 1:9 ratio, homogenized on ice, and centrifuged at 1250 ***g*** and 4°C for 10 min, and the supernatant fluid was collected after centrifugation. Thereafter, 0.5 ml of the supernatant fluid was further centrifuged at 10,000 ***g*** and 4°C for 15 min. The precipitate, which was identified to be mitochondria (Pedram et al., 2006), was resuspended 1:19 (w/v) in 0.65% cold saline solution; then, 190 µl of suspension fluid was loaded with 10 µl of DCFH-DA (1 mmol l^{-1} prepared in DMSO) for enzyme labelling at 37°C for 30 min. The excitation light was set at 485 nm, and fluorescence intensity was measured as 528 nm.

Determination of antioxidant activity indices

The preparation and extraction of homogenates and supernatants fluid were performed as above. 10% of the liver homogenates fluid was diluted and maintained at 4°C until used for determination of antioxidant activity indices. Chemical assay kits were used to quantify the hepatic SOD, CAT, GPx and total antioxidant capacity (TAOC) (A001-3, A007-1, A005 and A015-2, Nanjing Jiancheng Bioengineering Institute, P. R. China) according to the instructions. The total protein content was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the liver tissue using RNAiso Plus (Takara) reagent and the purity and integrity were assessed using a

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NanoDrop 2000 spectrophotometer (Thermo Scientific) after agarose gel electrophoresis. All RNA samples were treated with gDNA Eraser (Takara) and used to synthesize cDNAs for real time PCR using PrimeScript™ RT reagent Kit (Takara).

Quantitative real-time PCR (qRT-PCR) analysis

The specific primers were designed with Primer Premier 6.0, and the sequences are shown in Table S1. The qRT-PCR was performed on the LightCycler 480 (Roche, Switzerland) using a SYBR Premix Ex Taq™ II kit (Takara). The amplification conditions of qRT-PCR were as follows: 30 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. PCR amplifications were performed in three duplicates for each sample. The relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All data were presented as the means±s.d. of each group. Statistically significant differences were calculated by one-way ANOVA with Bonferroni post-tests using SPSS software (version 19.0), and $P<0.05$ was considered statistically significant. The graphs were generated with GraphPad Prism 5.0. Ranking of genes by differential expression of HSP genes was analysed with a heat map using the R Programming Language version 3.4.2.

RESULTS

Effect of thermal stress on histology changes of liver

Morphological changes in the liver of the frog *Q. spinosa* under thermal stress are shown in Fig. 1 and Table S2. Histological examination of the liver from the control group showed that the architecture of blood sinusoids and hepatic cords, as well as many scattered melanomacrophages, were normal and present in the liver. All cells displayed clear cytoplasm and well-delineated cytoplasmic borders (Fig. 1A). In the cold stress group, observation of haemorrhage and congestion suggested that erythrocyte extravasation had occurred. Inflammatory cell infiltration was conspicuous and enlargement of sinusoids was also present at 48 h (Fig. 1B,C). Moreover, melanomacrophage centres (MMCs) showed a slight increase and aggregation at 12 h (Fig. 1B) but decreased significantly in morphology and quantity at 48 h (Fig. 1C). In the heat stress group, hemorrhage and congestion were ubiquitously observed. The hepatic sinusoids appeared dilated upon prolonged heat exposure (Fig. 1D,E). Furthermore, the number and size of MMCs increased markedly at 12 h (Fig. 1D) but these decreased significantly at 48 h (Fig. 1E).

Effect of thermal stress on ultrastructure changes of hepatic cell

The ultrastructure of hepatic cells under both cold and heat stress for 12 h is shown in Fig. 2. The liver from the control group exhibited a normal ultrastructure (Fig. 2A–C), displaying large and ovoid hepatocyte nucleus with centrally placed prominent nucleoli, numerous round or elongated mitochondria with well-developed cristae and matrix, normal rough endoplasmic reticulum, abundant glycogen and few lipid droplets. However, some ultrastructural abnormalities were observed in livers when frogs were exposed to cold stress (Fig. 2D–F). Degeneration and necrosis of hepatocytes were evident with an electron-lucent cytoplasm. Some organelles were covered by vacuoles, which were even scattered outside the hepatocyte. The lipid droplets were still present, but they were less numerous than in the control group. An increased number of melanomacrophages assembled into the regiment of the livers. Vacuoles with the contents were revealed on adjacent cells which had normal structure. Ultrastructural alterations were also observed in hepatocytes in the heat stress group (Fig. 2G–I). Many large electron-dense fat droplets, some of which were even larger than the nucleus, were present in hepatocytes and the nucleus was squeezed to the side of the cell. The mitochondria turned plump and appeared swollen with faint cristae. The endoplasmic reticulum was broken and glycogen practically disappeared.

Effect of thermal stress on the apoptosis of hepatic cell

Liver tissue samples were analysed by TUNEL assays to determine whether apoptosis was induced by thermal stress. As shown in Fig. 3, few apoptotic hepatocytes were observed in the control group. However, the number of TUNEL-positive cells significantly increased in both cold and heat stress groups. Moreover, the apoptotic index in the cold stress group was significantly higher than that in the heat stress group (Fig. 3D).

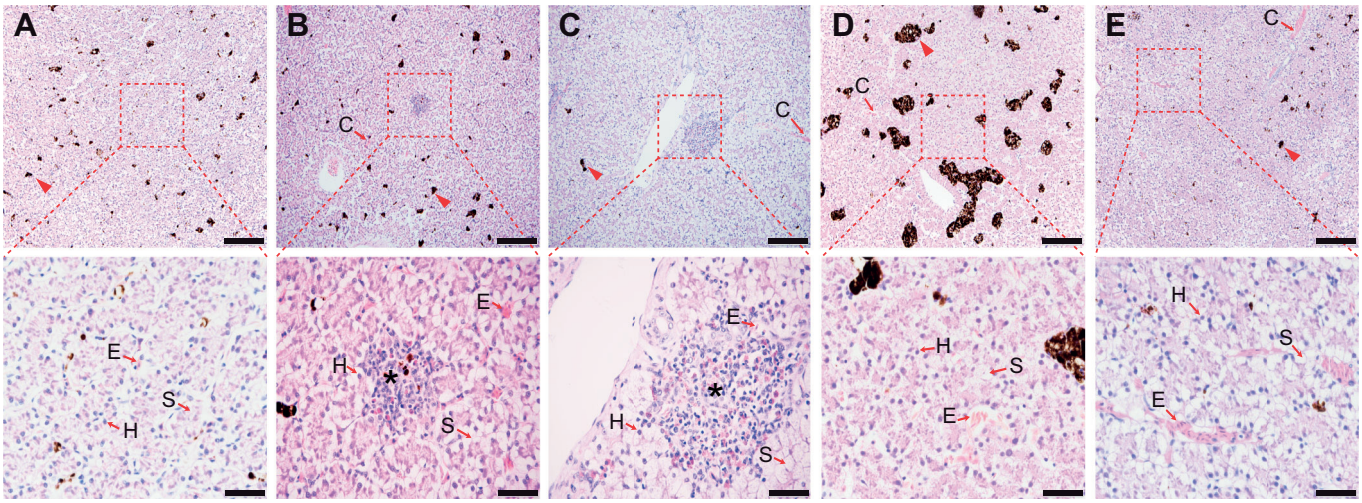

Fig. 1. Light micrographs of liver in the frog *Q. spinosa* under cold (5°C) and heat (30°C) stress for 12 h and 48 h. (A) Liver from control frog. (B,C) Livers from frogs exposed to 5°C for 12 h (B) or 48 h (C) . (D,E) Livers from frogs exposed to 30°C for 12 h (D) or 48 h (E) . The red arrowheads in top panel indicate melanomacrophages. Asterisks indicate inflammatory cell infiltration. C, congestion; E, erythrocyte; H, hepatocyte; S, sinusoids. Scale bars: 200 μm (top) and 50 μm (bottom).

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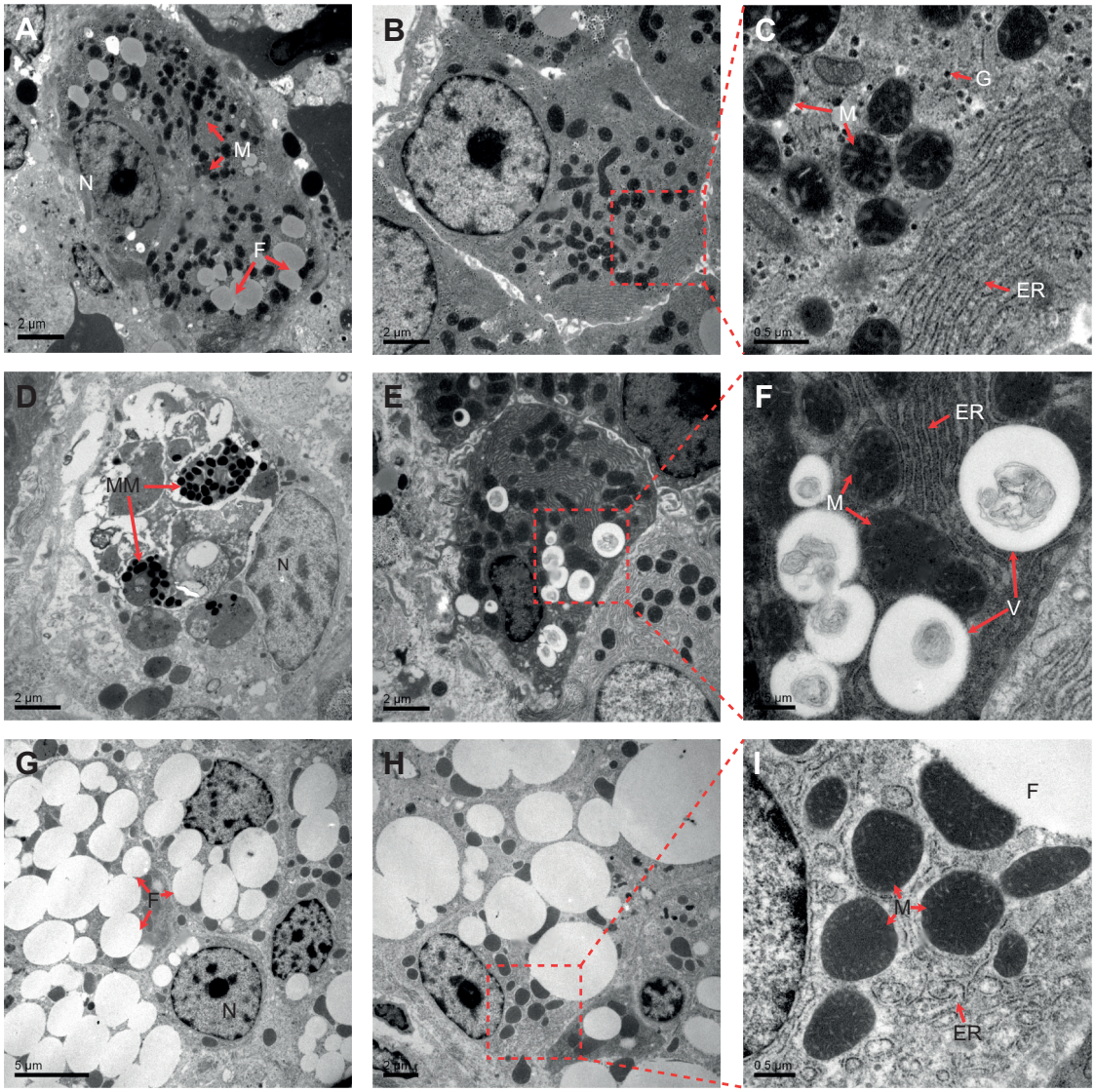

Fig. 2. Transmission electron micrographs of hepatic cells from *Q. spinosa* under cold and heat stress. Representative sections of control frog liver (A–C) and livers from frogs exposed to 5°C (D–F) or 30°C (G–I) for 12 h. F, fat; G, glycogen; M, mitochondria; N, nucleus; V, vacuole; ER, endoplasmic reticulum; MM, melanomacrophage.

Effect of thermal stress on liver antioxidant activity

Compared with the control group, the mitochondrial ROS levels were elevated in a time-dependent manner up to 6 h and 12 h in the cold and heat stress groups, respectively (Fig. 4). However, there were no significant differences in mitochondrial ROS levels among the time points mentioned above and other time intervals in both stress conditions. Moreover, the activity of SOD and GPx dramatically increased at 3, 6 and 12 h, and subsequently returned to baseline level after 24 h in the cold stress group, while activities in the heat stress group were significantly increased after 12 h and remained high at 24 h and 48 h (Fig. 5A,C). The activity of CAT increased significantly at 24 h in the cold stress group, while that in the heat stress group decreased markedly at 6 h and was subsequently upregulated significantly by 48 h (Fig. 5B). There was no significant difference of total antioxidant capacity (TAOC) level at different time periods in the cold stress group; however, the level of TAOC exhibited an overall trend of upregulation and increased significantly starting at 3 h under heat stress (Fig. 5D).

Effect of thermal stress on the expression of antioxidant-related genes

The mRNA expression levels of antioxidant-related genes are shown in Fig. 6. Expression of *SOD1* and *SOD3* increased significantly at 3 and 6 h in both cold and heat stress groups, respectively (Fig. 6A,C). *SOD2* expression significantly decreased at 3 and 12 h under heat stress while that in the cold stress group increased sharply after 24 h (Fig. 6B). *CAT* transcription level under heat stress decreased significantly at 6 h and was upregulated at 48 h, whereas that in the cold stress group increased significantly at 24 h (Fig. 6D). Finally, expression of *GPx* increased dramatically at 3 h in the cold stress group, and at 6 h in the heat stress group (Fig. 6E).

Effect of thermal stress on the expression of HSP genes

The expression levels of most HSP genes exhibited an overall trend of upregulation under thermal stress (Figs 7,8). In the heat stress group, expression of *HSP10*, *HSP40*, *HSP47*, *HSP60* and *HSP90*, were significantly increased at 3 h or 6 h and were maintained at a

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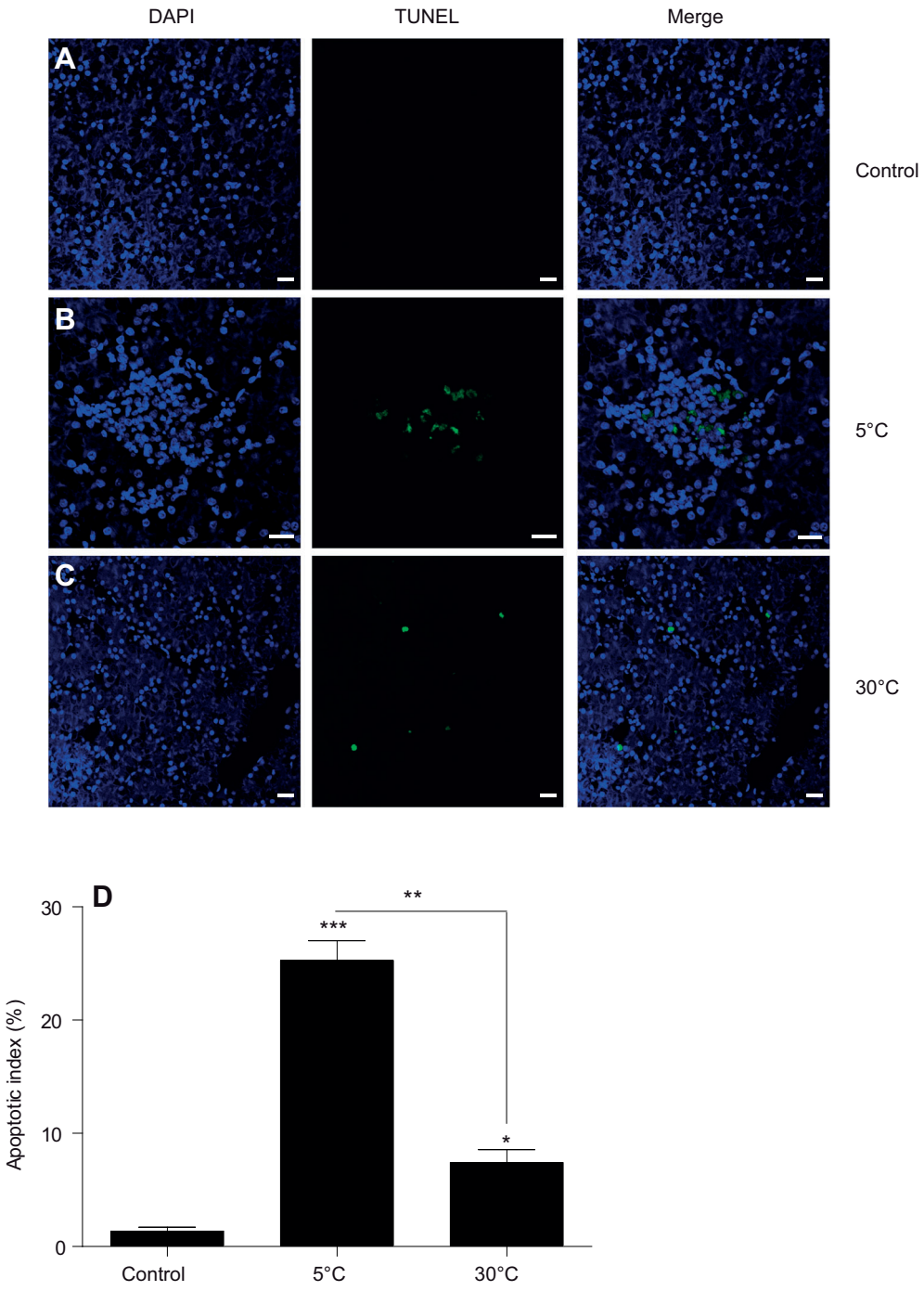

Fig. 3. Immunofluorescence analysis of thermal stress-induced apoptosis in the liver of *Q. spinosa*. The TUNEL detection kit was used to detect apoptotic cells (green). Nuclei are stained with DAPI (blue). (A) Liver from control frog. (B,C) Livers from frogs exposed to 5°C (B) or 30°C (C) for 48 h. (D) Apoptotic index of control, cold and heat stressed 48 h groups. Data are means±s.d. (n=6, *P<0.05 and ***P<0.001 compared with control group). Scale bars: 20 μm.

high level at 24 h or 48 h. The expression of three HSP genes, *HSP30*, *HSP70* and *HSP110*, which were clustered together in the heat map, was dramatically upregulated at 3 h and peaked at 6 h under heat stress. The expression of *GRP78* and *GRP94* peaked at 48 h in the heat stress group and at 6 h in the cold stress group. Most of the HSP genes were rapidly induced at 3 h or 6 h in the cold stress group, and their expression levels were generally lower than those in the heat stress group.

Effect of thermal stress on the expression of apoptosis-related genes

To determine whether apoptosis-related genes might be induced by thermal stress, the transcriptional level of these genes was detected by qRT-PCR (Fig. 9). Compared with the control group, most apoptosis-related genes, including *Bax*, *Caspase-3*, *Caspase-8* and *Caspase-9*, were significantly upregulated at 3 h and 48 h in the cold and heat stress groups (Fig. 9A,C–E). The expression of *Bcl-2* displayed a significant decrease in the cold and heat stress groups when compared with the control group (Fig. 9B).

DISCUSSION

The influence of global climate change on amphibians has been subject to an increasing amount of research in recent decades (Li et al., 2013). Because of their ectothermic nature, highly permeable skin, and complex life cycles, the survival, growth, activity and development of amphibians strongly depend on specific

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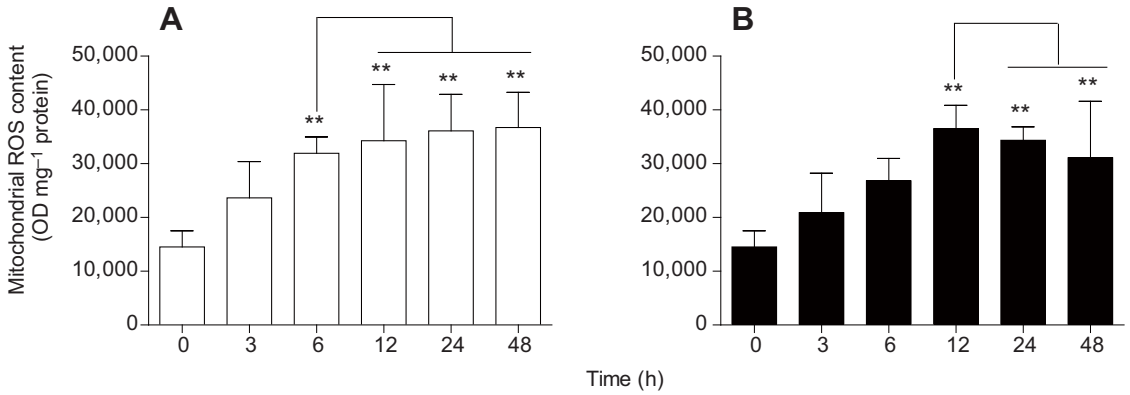

Fig. 4. Mitochondrial ROS generation in the liver of *Q. spinosa* under cold and heat stress at different time points. Mitochondrial ROS levels in frogs exposed to 5°C (A) and 30°C (B) for the indicated times. Values before treatment (0 h) serve as the control group. Data are means±s.d. (*n*=6; ***P*<0.01 compared with the control group).

temperature ranges, which are fundamentally affected by climate change (Üveges et al., 2016; Reading, 2007). In this study, we investigated the effects of both cold and heat stress on liver histology, apoptotic index, antioxidant activity indices and stress-response gene expression in the giant spiny frog *Q. spinosa*.

Histological and ultrastructural alterations were observed in the liver under thermal stress, and the hepatic cells were partially degenerated, necrotic and accompanied with increased apoptosis, which is consistent with results in the Chinese fire-belly newt *Cynops orientalis* (Wang et al., 2012) and rat (Ates et al., 2006; Qin et al., 2017). In this study, erythrocyte abnormalities, including hemorrhage and congestion, were present in liver tissues under cold and heat stress, which could be attributed to the destruction of hepatic erythrocytes and accumulation of newly produced erythrocytes in the liver (Maekawa et al., 2012). Furthermore, sinusoids were destroyed and inflammatory cell infiltration was also observed under thermal stress. These results suggest that thermal stress causes physiological changes in the giant spiny frog. We found marked ultrastructural alterations, including many large electron-dense fat droplets and swollen mitochondria, in hepatocytes under heat stress, which is consistent with the results in the Wuchang bream *Megalobrama amblycephala* (Liu et al., 2016). These results suggest that excessive ROS generation induced by heat stress can attack the phospholipid membrane, resulting in mitochondrial dysfunction and the accumulation of fat droplets, ultimately affecting the normal energy metabolism of the liver (Chai et al., 2017; Wu et al., 2017). Additionally, many vacuoles induced by cold stress were also observed in this study as also reported in other studies (Li et al., 2017; Yao et al., 2015; Zeng et al., 2014); these need to be studied further in the near future. Overall, a series of

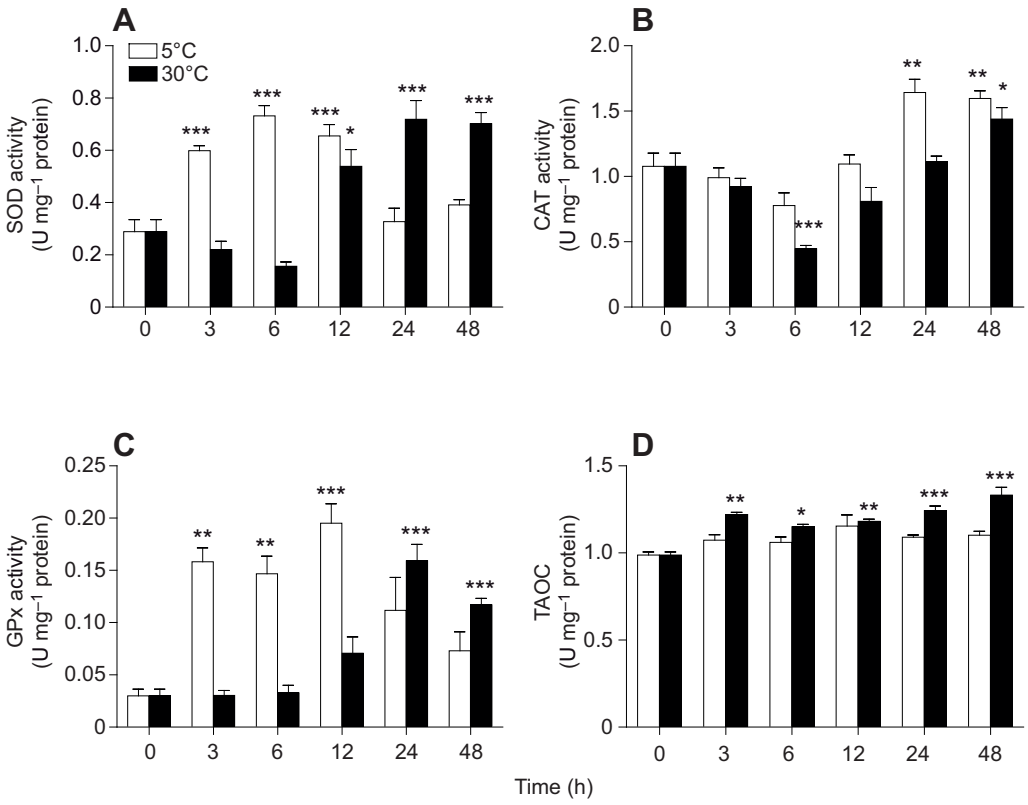

Fig. 5. Antioxidant activity indices in the liver of the frog *Q. spinosa* under cold and heat stress at different time points. Activity of SOD (A), CAT (B) and GPx (C) enzymes, and total antioxidant capacity (TAOC; D) in frogs exposed to 5°C (A) and 30°C (B) for the indicated times. Untreated frogs (0 h) serve as the control group. Data are means±s.d. (*n*=6, **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with control group).

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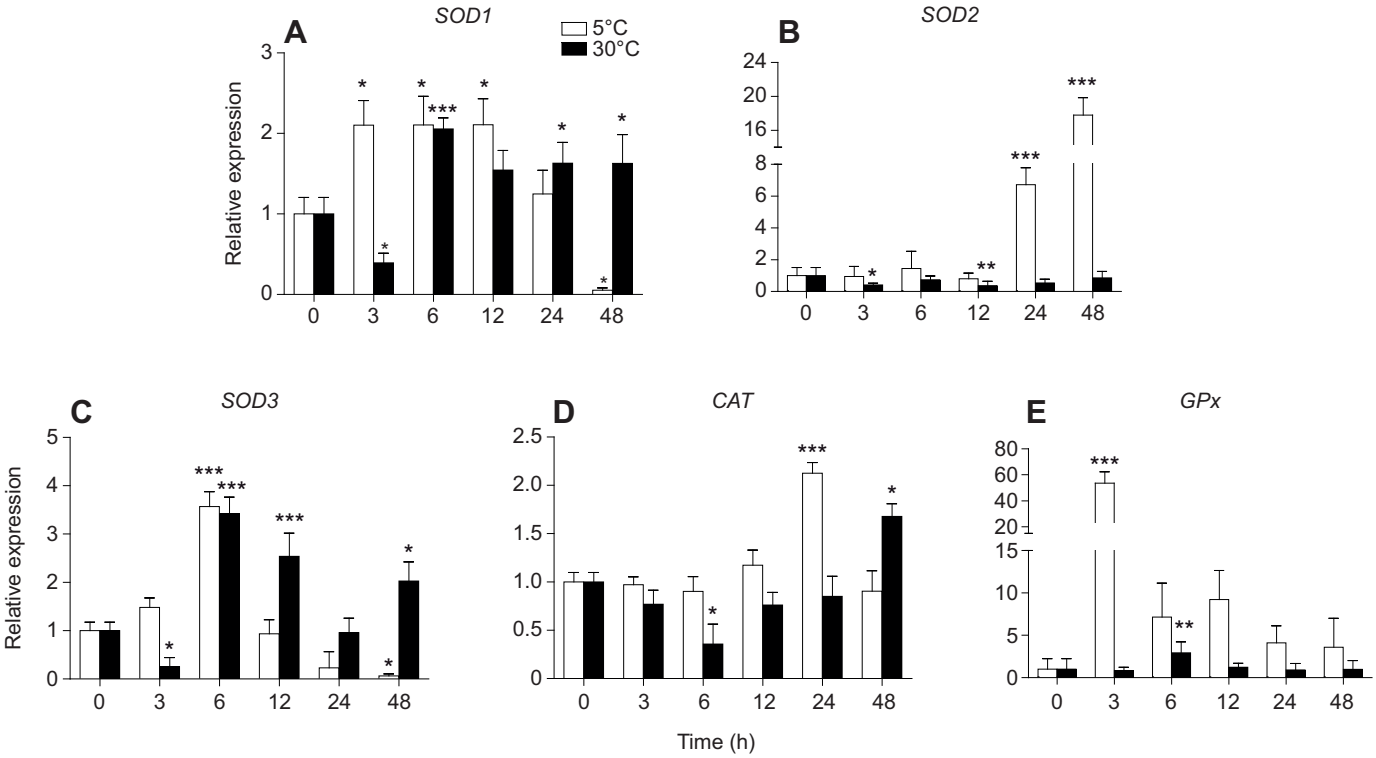

Fig. 6. Expression levels of antioxidant-related genes in the liver of *Q. spinosa* under cold and heat stress at different time points. *SOD1* (A), *SOD2* (B), *SOD3* (C), *CAT* (D) and *GPx* (E) gene expression in livers of frogs exposed to 5°C and 30°C for the indicated times. Untreated frogs (0 h) serve as the control. Gene expression at 0 h was set to 1.0 and levels of other time intervals were adjusted accordingly. Data are means±s.d. (n=6, *P<0.05, **P<0.01 and ***P<0.001 compared with control group).

histological and ultrastructural changes could be observed in the frog liver, indicating that a dysfunction state arises in hepatocytes under thermal stress. Notably, the increased lipid droplets could be used as biological indicators for the physiological changes under heat stress in *Q. spinosa* or even in amphibians more generally.

The MMCs, which are Kupffer cells in the liver, are focal accumulations of pigmented macrophages involved in the phagocytosis of apoptotic cells of poikilothermic vertebrates (Arciuli et al., 2012; van Wilpe and Groenewald, 2014; Purrello et al., 2001; Steinel and Bolnick, 2017). Several environmental stresses can affect the number and size of MMCs, leading to the disruption of physiological function (Ben Ameur et al., 2012; Ben Hamed et al., 2017; Chai et al., 2017; de Oliveira et al., 2016; Wu et al., 2017). MMCs in the liver were further analysed to detect whether thermal stress can cause physiological damage in *Q. spinosa*. Compared with the normal state, the number and size of MMCs increased at 12 h in both cold stress and heat stress groups. Results suggested that the MMC level as well as the phagocytic activity could be upregulated to potentially remove the debris produced by thermal stress, which is consistent with results reported in the mullet *Mugil cephalus* and sea bass *Dicentrarchus labrax* (Ben Ameur et al., 2012). However, the prevalence and

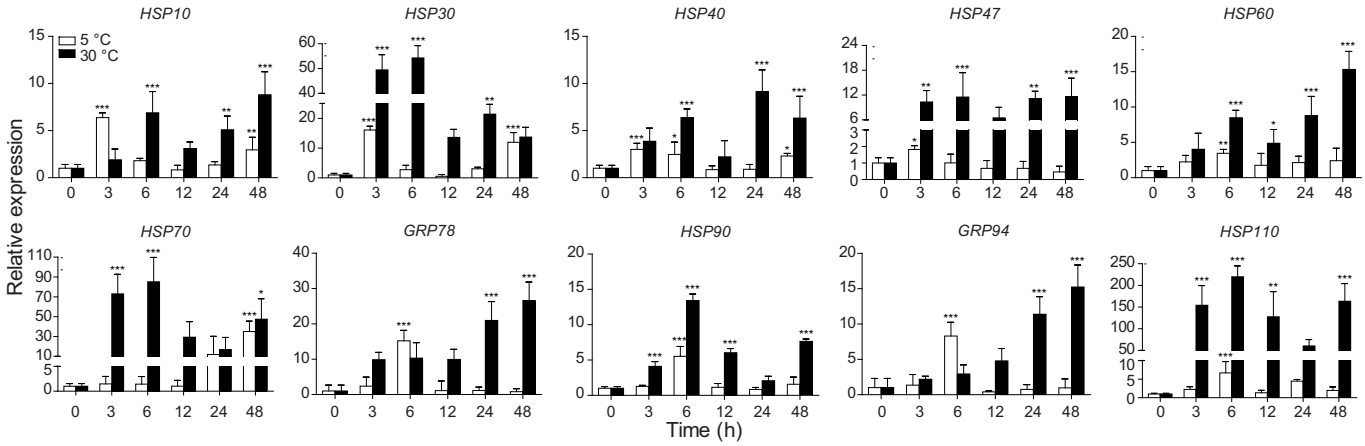

Fig. 7. Expression levels of HSP genes in the liver of *Q. spinosa* under cold and heat stress at different time points. Untreated frogs (0 h) serve as the control. Gene expression at 0 h was set to 1.0 and the levels of other time intervals were adjusted accordingly. Data are means±s.d. (n=6, *P<0.05, **P<0.01 and ***P<0.001 compared with control group).

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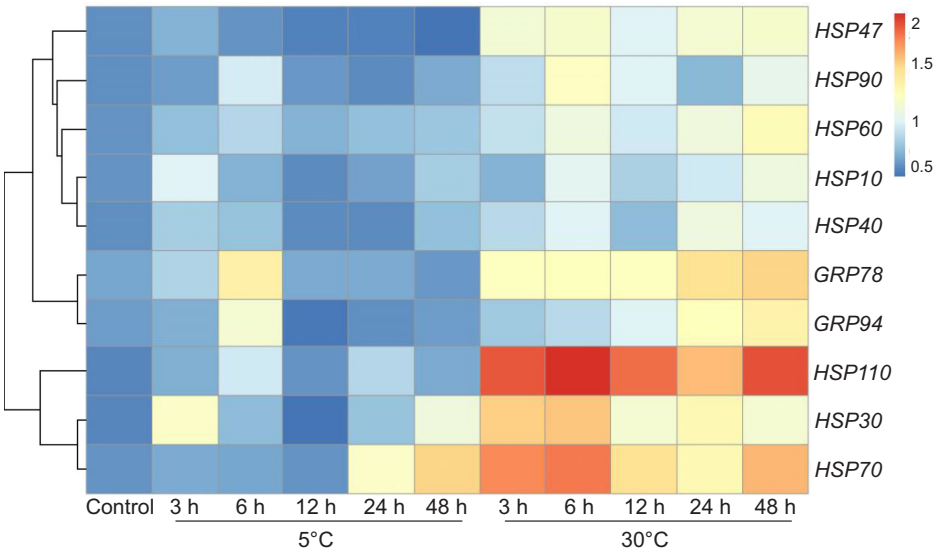

Fig. 8. Expression heat map of HSP genes in the liver of *Q. spinosa* under cold and heat stress at different time points. Untreated frogs (0 h) serve as the control. Hierarchical clustering was performed with each row and colour of each box indicates the gene expression level. The data were plotted after calculating base-10 logarithms for homogeneity (n=6).

intensity of MMCs decreased significantly at 48 h in both groups, which might be due to the decreased amount of melanosome and melanin caused by autophagy and apoptotic mechanisms, respectively (Barni et al., 2002). Notably, such novel findings on the alterations in liver MMCs might also serve as a biological indicator for the physiological changes of thermal stress-induced oxidative damage in *Q. spinosa* or even more widely in amphibians.

The liver is known to be a hub for metabolism, and mitochondria are considered to be the major site for cellular production of ROS (Kiss et al., 2011; Paital, 2016; Zhang et al., 2013). Results showed that both cold and heat stress can significantly increase the generation of mitochondrial ROS, which was consistent with results in other ectotherms, such as the euryhaline fish *Takifugu obscurus* (Cheng et al., 2015, 2017), the eurythermal bivalve *Mya arenaria* from a low-shore intertidal population (Abele et al., 2002) and the Chinese soft-shelled turtle *Pelodiscus sinensis* (Zhang et al., 2017b). To counteract the adverse effects of ROS, antioxidant enzymes can be activated to cope with oxidative stress (Cui et al., 2014). In this study, the activities of SOD and GPx in liver were significantly elevated with an increase of mitochondrial ROS

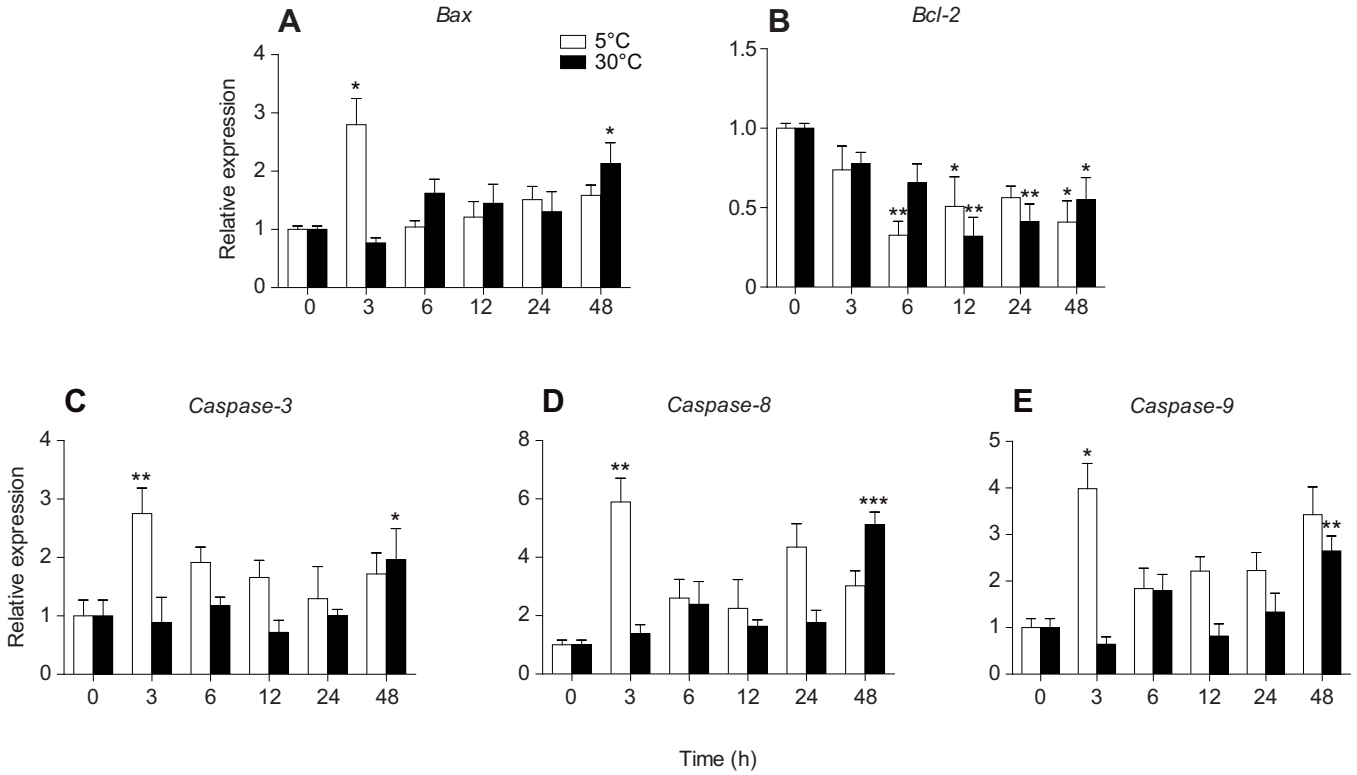

Fig. 9. Expression levels of apoptosis-related genes in the liver of *Q. spinosa* under cold and heat stress at different time points. *Bax* (A), *Bcl-2* (B), *Caspase-3* (C), *Caspase-8* (D) and *Caspase-9* (E) gene expression in livers of frogs exposed to 5°C and 30°C for the indicated times. Untreated frogs (0 h) serve as the control. Gene expression at 0 h was set to 1.0 and the levels of other time intervals are adjusted accordingly. Data are means±s.d. (n=6, *P<0.05, **P<0.01 and ***P<0.001 compared with control group).

generation, indicating that these enzymes are involved in eliminating the extra ROS generation induced by cold and heat stress, which is consistent with the results in other poikilotherms such as the pufferfish *Takifugu obscurus* (Cheng et al., 2015, 2017), the predatory mite *Neoseiulus cucumeris* (Zhang et al., 2014) and the whiteleg shrimp *Litopenaeus vannamei* (Xu et al., 2018; Zhou et al., 2010). Remarkably, the activity of GPx showed a quicker response to cold stress than heat stress, which could be due to the fact that GPx is considered to be a key antioxidant enzyme to reduce lipid hydroperoxide under cold stress (Dawson and Storey, 2017; Feidantsis et al., 2013; Sahoo and Kara, 2014). Moreover, the activities of SOD and GPx subsequently returned to baseline levels, whereas the CAT activity significantly increased after 24 h under cold stress. Furthermore, the CAT activity, which could be induced by high concentrations of ROS (Zhang et al., 2014), was maintained at a high level after 48 h under heat stress. These results indicate that these antioxidant enzymes might contribute to scavenge accumulated ROS and play important roles in protecting organisms from heat stress (Yang et al., 2010). Correspondingly, TAOC, an important biochemical indicator to evaluate the antioxidant capacity in organisms, was enhanced significantly after heat exposure. This demonstrates that the overall antioxidant capacity of the frog is improved under heat stress. However, there was no significant difference in TAOC between the cold stress group and control group, which is consistent with the results reported by other studies (Zhang et al., 2014; Zhao et al., 2014). Furthermore, the activity of CAT significantly declined after heat stress for 6 h as reported by other studies (Zhang et al., 2017a). All of these results remain to be investigated further in the near future.

Antioxidant enzyme activities, as well as their transcription levels, change dynamically when organisms are subjected to acute thermal stresses (Xu et al., 2018). In this study, the mRNA expression levels of *SOD1*, *SOD3*, *CAT* and *GPx* showed similar changes to the activity of the corresponding antioxidant enzymes at both stress conditions. Notably, expression of *SOD2* was sharply increased under cold stress but was reduced under heat stress. It may be that *SOD2* (*MnSOD*), which is located in the mitochondria, has a specific response to various degrees of destruction of mitochondria induced by temperature stress (Dawson et al., 2015; Gao et al., 2013). Overall, the antioxidant enzyme system in *Q. spinosa* was activated and could protect the liver from the thermal stress-induced oxidative stress (Meng et al., 2014).

Overgeneration of ROS can trigger the heat shock response and increase the expression of HSP genes under stress (Liu et al., 2016; Rupik et al., 2011). In this study, the expression patterns of HSP genes under thermal stress was investigated systematically. Most HSP gene expression was sensitive to thermal stress, but expression levels under heat stress were generally higher than those under cold stress. Notably, *GRP78* and *GRP94* were clustered together in the heat map, indicating that the acute hypothermia can activate the relevant anti-apoptotic protective response for *Q. spinosa* (Kiss et al., 2011; Nagasawa et al., 2013; Storey and Storey, 2007). Furthermore, *HSP30*, *HSP70* and *HSP110* could serve as biomarkers of heat stress, for their high sensitivity and expression levels at 3 and 6 h.

Apoptosis was observed in the liver tissue under thermal stress, which showed that both cold and heat stress can increase the rate of apoptosis in hepatocytes and disrupt the normal morphology of liver cells. This finding is consistent with results obtained in pufferfish (Cheng et al., 2015, 2017) and rat (Ates et al., 2006; Qin et al., 2017). Accordingly, acute thermal stress can induce the apoptosis of hepatocytes and cause the dysfunction of liver metabolism. Furthermore, overgeneration of ROS induced by thermal stresses can lead to the upregulation of pro-apoptotic genes including *Bax*, *Caspase-3*, *Caspase-8* and *Caspase-9* and the downregulation of anti-apoptotic genes such as *Bcl-2*. The expression patterns of apoptosis-related genes under cold stress responded faster than those under heat stress. Detailed investigations need to be conducted to determine why this occurs in the near future.

In conclusion, thermal stress caused physiological dysfunction and structural alterations in *Q. spinosa* liver. Simultaneously, thermal stress induced mitochondrial ROS generation and activated the antioxidant enzyme system at the biochemical level to potentially protect the liver from stress-induced oxidative stress. The increased expression of stress-related genes induced by thermal stress might contribute to the regulation of organismal metabolism. It was also found that lipid droplets and the HSP genes *HSP30*, *HSP70* and *HSP110*, might be suitable bioindicators of heat stress in *Q. spinosa*. The results of these alterations at physiological, biochemical and molecular levels help us to better understand the thermal stress response and can act as reference points for the biological response in amphibians against extreme ambient temperature changes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Z.-P.L.; Methodology: Z.-P.L.; Formal analysis: Z.-P.L., W.-B.G., D.-D.T.; Investigation: Z.-P.L., W.-B.G., D.-D.T.; Resources: Z.-P.L., Q.-H.Z., Y.-L.Z., C.W., L.-Z.W.; Writing - original draft: Z.-P.L., W.-B.G.; Writing - review & editing: Z.-P.L.; Supervision: M.-A.S.

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Table S1. The sequences of primers in this experiment.

Target gene	Primer sequence (5'-3')
Hsp10	F:CTCCCTTTATTTGACCGTGTCCTTGT
	R:CTTAGAGCCTTCTCCGACTGCTACTA
Hsp30	F:GAGGACGGAAGGAAGGAGACTGAT
	R:TTGGACATGGAGCACAGCACATC
Hsp40	F:CGTGAACGCCTCTGTTGAGATGG
	R:TACCGATGCTTGAGAGCCTCCTAC
Hsp47	F:AGTAGAGAAACTCCTGACCAGAGAACA
	R:CGTGGCTGACTTCCATGCTGATT
Hsp60	F:GTTGGTCTTCAGGTAGTTGCTGTCAA
	R:ACCTCTCCCACCTTTCCAAAGTCAT
Hsp70	F:ACCTCGGCACTACCTACTCCTGT
	R:CACCTGGTTCTTGGCAGCATCTC
Grp78	F:ACTGCTGCTGCCATTGCCTATG
	R:GCTGGTCAAAGTCTTCGCCTCC
Hsp90	F:GCCTGGAGCTTCCTGAAGATGA
	R:GCGGTTTGAGACGGTTACCTTT
Grp94	F:GGCACAAGCATACCAGACAGGA
	R:GACCGTAACGTGGCAGTCTCAA
Hsp110	F:TCCATCCCTTATCCACAGCCTCAG
	R:CCTCCACCTCTATCTTCTCTACCACAG
SOD1	F:ACTGCTGTGCGTCCGATGATG
	R:GGCGATAACACTAATGGCTGCGTAA
SOD2	F:GGTAGACCAAGGACGGACTGTCATAT
	R:GGAACATCCCAACGCAGGAACAA
SOD3	F:GAGGTGCTGCTGAATCTTGCCATA
	R:TCAGTCTTCAAGAGCAATTCACATCCA
CAT	F:TGGACGGATCATTCAGGAGGAGAC
	R:ACAAGAAGAGAACGATCAAGACCTACAC
GPx	F:GACCAATCACAGAGCCTCCTACAGA
	R:CCGCTTCGAGACCATCAACATCC
Bax	F:TGGAGTCTTCTTAGCTGGCGTTCT
	R:TTGTCAGTGCTGGAGTGAATTGGAAT
Bcl-2	F:ACGGCAATACTTAGGATGGTCTTGATG
	R:GTTGGATGGATGACTGACTATCTGAACA
Caspase-3	F:GCGTAATGGAACTGATGTAGATGCCTTA
	R:GTAGCCTTCCTCACCGTGACTCA
Caspase-8	F:ATCCAACTACTCAGCCAGAGAAGCA
	R:GTCGTTCTTGTTCATCCTAGCCTGTT
Caspase-9	F:GACTGCTGCCTTATCATCATCCTCTC
	R:GTGACCGTTGAACAAGTTGACGATTC
β-Actin	F:CCATGACAATACCAGTGGTACGACC
	R:ATGATATGGAGAAGATCTGGCATCA

Table S2. Histological lesions in the liver of *Q. spinosa* under the cold (5 °C) and heat (30 °C) stresses for 12 h and 48 h.

Histological lesions	Control	Cold stress		Heat stress	
		12 h	48 h	12 h	48 h
Congestion	0	2	3	1	3
Enlargement of sinusoids	0	0	2	0	1
Inflammatory cell infiltration	0	2	3	0	0
Hemorrhage	0	3	1	3	1
Number of melanomacrophages	1	2	0	3	0
Percentage of melanomacrophages area	6.59 ± 0.22	8.63 ± 0.18*	0.62 ± 0.12***	26.44 ± 0.58***	2.85 ± 0.16**

Note: Lesions were scored according to their severity (0: none; 1: mild; 2: moderate; and 3: severe). Data (percentage of melanomacrophages area) were shown as mean ± SD (n = 6, * $P < 0.05$ and ** $P < 0.01$ as compared with control group).