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Effects of acute and chronic chromium stress on the expression of heat shock protein genes and activities of antioxidant enzymes in larvae of *Orthetrum albistylum*☆

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A B S T R A C T

The dragonfly species *Orthetrum albistylum*, can accumulate heavy metals from its aquatic environment and thus serves as a biological indicator for monitoring and evaluating water quality. Heat shock proteins (HSPs) play important biological roles in resistance to various types of environmental stress. The full-length cDNA sequences of the heat shock cognate (*hsc*) 70 and heat shock protein (*hsp*) 70 genes were cloned from *O. albistylum* larvae. Relative levels of expression of *hsc70* and *hsp70* in the head, epidermis, midgut, and adipose tissue were measured by qRT-PCR after chronic and acute contamination of 5–8 instar larvae with chromium (Cr) solution, and under control conditions. Activities of superoxide dismutase (SOD) and catalase (CAT) in chronically contaminated larvae were also measured. Phylogenetic analysis revealed that the cloned *hsc70* and *hsp70* genes were highly homologous to known HSP70 family members reported in other insects. The mRNA levels of *hsc70* and *hsp70* did not differ significantly in various larval tissues. Under chronic chromium stress, *hsc70* and *hsp70* expression were upregulated to a maximum and then downregulated; *hsp70* mRNA levels were higher than those of *hsc70* at all concentrations of chromium. Under acute chromium stress, *hsc70* expression was inhibited at low chromium concentrations and upregulated at chromium concentrations higher than 125 mg/L. However, *hsp70* expression was higher than that in the control group and markedly higher than that of *hsc70*. Changes in SOD and CAT activities displayed consistent trends for different chronic chromium concentrations, first increasing and then decreasing over time. Collectively, these findings demonstrated the response of the HSP family of genes and antioxidant enzymes following exposure to heavy metal stress, as well as their potential applicability as biomarkers for monitoring environmental pollutants.

1. Introduction

Heavy metals are hazardous pollutants introduced into the environment through multiples industrial activities, including discharge of wastewater (Korbahti et al., 2011). Heavy metal pollution incidents have occurred frequently in recent years. Such contamination has significant detrimental effects on ecosystems and modifies the physical and chemical properties of water, thereby affecting aquatic organisms (Cardoso et al., 2011; Das et al., 2011; Tovar-Gómez et al., 2012). Chromium (Cr) is widely used in industrial production, including chromium plating, metal processing, printing and dyeing, leather tanning, and paint production. It is the second most abundant heavy metal pollutant, ranked only after lead (Gao and Xia, 2011). Chromium can affect algal morphology, respiration, chlorophyll synthesis, and normal growth and development (Corradi and Gorbi, 1993). It also influences growth, development, and physiological and biochemical indices of fish (Oner et al., 2009; Perumalsamy, 2013). Some heavy metals may also be converted into highly toxic metal compounds that are transmitted and accumulate through the food chain, causing harm to human health by inducing immune deficiency diseases, reproductive disorders, and

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abnormal fetal development (Stankovic and Jovic, 2012; Cinnirella et al., 2014; Krupskaya and Zvereva, 2014; Zhang et al., 2016). Monitoring and preventing heavy metal pollution in water have become important topics in environmental pollution research.

Aquatic insects are currently one of the main biological indicators used for monitoring and evaluating water quality, thus constituting a new field for the practical application of entomology (Cadmus et al., 2020). Dragonflies spend 95% of their lives in water, are ecologically diverse, abundant, and widely distributed; they also accumulate heavy metals from their habitat (water). Dragonflies are suitable bioindicators for evaluating the ecological conditions of river corridors (Golfieri et al., 2016). Jeremiason et al. (2016) tested the use of MeHg in dragonfly larvae as an indicator of MeHg levels in a range of aquatic systems, including 16 river/stream sites and two lakes. *Orthetrum albistylum* (*O. albistylum*) is a common species of dragonfly that is widely distributed and its larvae can be easily obtained. This species was therefore selected as the experimental animal for the current study.

After Cr^{6+} enters a cell, it is reduced to Cr^{4+}, Cr^{5+}, and finally Cr^{3+}, which induces the production of reactive oxygen radicals (ROS) (Shi et al., 1999; O'Brien et al., 2003). Excessive ROS levels can cause damage to DNA, protein, and lipid, resulting in oxidative stress (Nordberg and Arnér, 2001; Patlolla et al., 2009).

Heat shock proteins (HSPs) are a family of highly conserved proteins present in all organisms, from bacteria to humans, that act as molecular chaperones for other proteins (Feder and Hofmann, 1999; Joly et al., 2010; Mjahed et al., 2012). HSPs help proteins fold or correct misfolding by partially unfolding when organisms face environmental stresses including heavy metal pollution, heat shock, and pathogenic infection (Rivera et al., 2005; Finka et al., 2016). The heat shock protein 70 (HSP70) family is the most highly conserved and largest member of the stress protein family. HSP70s are also the most sensitive and important proteins involved in stress responses and are present in all tissues and organs. HSP70s play an important role in maintaining the homeostasis of cell proteins and are frequently used in research as indicators of stress responses. The HSP70 family is comprised of 21 proteins divided into four types: HSP70, heat shock cognate (HSC) 70, glucose-regulated protein (GRP) 78, and GRP75 (Kregel, 2002; Wadhwa et al., 2002). Among them, *hsp70* and *hsc70* have been the most widely studied because of their important biological function in resistance to various types of stress (Bukau and Horwich, 1998; Bukau et al., 2000; Wang et al., 2022). Many *hsp70* and *hsc70* genes and their functions have been identified in aquatic organisms (Planello et al., 2010; Jing et al., 2013; Haap et al., 2016; Zeeshan et al., 2017). However, little is known about the activity of these genes, especially *hsc70*, in response to molting or the presence of heavy metals, including chromium.

Heavy metal ions, chemical pesticides, oils, and other substances can affect or block normal cell metabolism in the body, including respiratory and electron transport chains and enzymatic reactions, and induce the production of ROS (Zandalinas and Mittler, 2018). In response to certain biological or abiotic conditions, cellular ROS levels increase and activate cell apoptosis and autophagy. It is suggested that ROS mediate different cell-to-cell signaling pathways that control responses to wounds, pathogens, or abiotic stress (Schieber and Chandel, 2014; Reczek and Chandel, 2015). To reduce ROS-induced cellular damage, ROS levels are balanced by the activity of antioxidant enzymes (Slaninova et al., 2009; Lushchak, 2011), including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione S-transferase, and peroxidase (POD) (Lushchak, 2011). The production and removal of cellular ROS mainly relies on the coordinated regulation of SOD, CAT, and POD, which form a protective antioxidant enzyme system in insects (Felton and Summers, 1995). The activity of antioxidant enzymes in aquatic organisms is regulated through feedback adjustments to protect the organism from oxidative damage and enhance its resistance to adverse environments including heavy metal stress and organic pollution (Jasinska et al., 2015; Özok, 2020). Antioxidant enzyme activity can be induced or inhibited by many factors; thus, antioxidant enzymes are often used as biomarkers to monitor environmental pollutants (Uner et al., 2001; Chen et al., 2007; Main et al., 2010; Stohs and Bagchi, 1995).

We assessed the potential effects of chromium exposure on HSP gene expression in *O. albistylum* larvae. We cloned *hsc70* and *hsp70* from *O. albistylum* larvae, evaluated the levels of expression of *hsc70* and *hsp70* in larval tissues and analyzed the mRNA expression patterns of *hsc70* and *hsp70* under acute and chronic chromium stress. We also analyzed the enzyme activities of SOD and CAT in *O. albistylum* larvae under chronic chromium stress. Our results provide a deeper understanding of the roles of *hsc70, hsp70* and antioxidant enzymes under adverse growth conditions. This study supports the applicability of the HSP family and antioxidant enzymes as biomarkers for monitoring heavy metal pollution in aquatic environments.

2. Materials and methods

2.1. Experimental animals

O. albistylum larvae were obtained from the Hangzhou Normal University (Zhejiang Province, China) and immediately transferred to laboratory tanks. The larvae were maintained at 23 ± 1 °C with a photoperiod of 14 h light and 10 h dark for 1 week before initiating the experiment. The tank water was changed daily and the larvae were fed *Chironomid* larvae during the acclimation and experimental periods.

2.2. Cloning hsc70 and hsp70

Total RNA was extracted from eighth instar *O. albistylum* larvae using Trizol® reagent (Invitrogen, Shanghai, China), and 1 μg RNA was used to synthesize cDNA using the PrimeScript RT Kit with the gDNA Eraser kit (TaKaRa Bio, Dalian, China). Based on the conserved sequences of

Table 1
Primer sequences used in this study.

Primer name	Nucleotide sequences (5′–3′)	Sequence information
Hsc70-F	ACVACITACTCBTGYGTBGG	For partial sequence of *hsc70*
Hsc70-R	CGACYTCTTCVATIGTDGGICC	
Hsp70-F	GTNCCNGCBTACTTCAAYGA	For partial sequence of *hsp70*
Hsp70-R	ACAGCYTCATCHGGRTTGAT	
Hsc70-3′F	ATCTCTGACGCTGACAGGAAGACCAC	5′-RACE
Hsp70-3′F	GGAGGAGTTCAAGAAGAAGCAC	
Hsc70-5′R	TCCAGTTCCCTTTTTATCTAGACCAT	3′-RACE
Hsp70-5′R	CAAAGTCCTCACCACCCAAGT	
actin-QF	ATCCTCCGTCTCGACTTGG	Quantitative real-time PCR
actin-QR	TGATGTCACGCACGATTTCT	
Hsc70-QF	AAGTCAAGTCCACCGCTG	Quantitative real-time PCR
Hsc70-QR	GTACGCTTTGCCCTCTCA	
Hsp70-QF	CCCAAGCCAGTATTGAGATCG	Quantitative real-time PCR
Hsp70-QR	GTTAAGTTCCTTGCCATTGAAG	

hsc70 and *hsp70* from closely related species, degenerate primers (Table 1) were designed to amplify *hsc70* and *hsp70* cDNA fragments from *O. albistylum*. After cloning the partial sequences, the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm their identities. The thermal cycling parameters for PCR (AG22331, Eppendorf, Hamburg, Germany) amplification were as follows: one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 48 °C for 1 min, 72 °C for 15 s, and a final extension step at 72 °C for 10 min. Based on the two *hsp70* gene fragments, specific primers were designed and used to obtain full-length cDNA sequences using Rapid Amplification of cDNA ends (RACE) technology and a SMARTTM kit (TaKaRa Bio), according to the manufacturer's protocols. The resulting PCR products were separated by electrophoresis on 1% agarose gels, and cDNA fragments of interest were purified using a DNA gel extraction kit (Omega, Hangzhou, China). Purified DNA was ligated into the pMD18-T vector (TaKaRa Bio) and subjected to Sanger sequencing. Full-length cDNA sequences were assembled using the SeqMan sequence assembly software (Gao et al., 2016).

2.3. Sequence and phylogenetic analysis

The nucleic acid sequences of *hsc70* and *hsp70* from *O. albistylum* were queried for similarity using the National Center for Biotechnology Information (NCBI) database. Multiple *hsc70* and *hsp70* sequences belonging to *O. albistylum* and other insects were aligned using ClustalW (Thompson et al., 2002). A neighbor-joining phylogenetic tree was constructed using Mega 5.2 software and evaluated using 1000 bootstrap replications.

2.4. Expression of hsc70 and hsp70 genes in different tissues

Total RNA was extracted from the head, epidermis, midgut, and adipose tissue as described in Section 2.2. After cDNA synthesis, the relative expression of *hsc70* and *hsp70* genes was detected by quantitative real-time PCR (qRT-PCR) (CFX96, Bio-Rad, CA, USA), as described in Section 2.10.

2.5. Chronic exposure to chromium

Potassium dichromate ($K_2Cr_2O_7$, Purity >99.8%, National Pharmaceutical Reagents, Shanghai, China) was selected to prepare a chromium solution of the required concentration in accordance with the Chinese National Standards GB/T 602–2002, 'Chemical reagent-Preparations of standard solutions for impurity' and GB7467-87 'Water quality-Determination of Chromium (VI)-1,5 Diphenylcarbohydrazide spectrophotometric method', to verify the required concentration of the solution.

Globally, permissible levels of chromium in drinking water and effluent vary. In the USA, the Clean Water Act (CWA) set effluent limits for various pollutants, including heavy metals, in surface waters. The Safe Drinking Water Act (SDWA) mandates maximum contaminant levels (MCLs) for heavy metals in drinking water of 0.1 mg/L for chromium. In the European Union, the Water Framework Directive (WFD) established environmental quality standards for pollutants, including heavy metals, in surface water. The REACH regulations address the registration, evaluation, and authorization of chemicals, including heavy metals, within the EU market, and the permissible limit for chromium in drinking water is 0.05 mg/L. Japan enforces the Water Pollution Control Law, setting standards for heavy metals in effluents, with the permissible limit for chromium in drinking water of 0.05 mg/L. China's Water Pollution Prevention and Control Action Plan aims to control heavy metal pollution in water bodies. China's standard for drinking water quality (GB5749-2006) limits hexavalent chromium in drinking water to 0.05 mg/L and the standard for integrated wastewater discharge (GB8978-1996) stipulates that the content of hexavalent chromium in the sewage discharged by all sewage units shall not exceed 0.5 mg/L.

Chronic chromium concentrations of 0, 0.05, 0.5 and 1.289 mg/L were selected to reflect the Chinese standards and 1/100 of the 96-h half-lethal concentration (128.9 mg/L, unpublished data) obtained from a preliminary experiment. Fifth-to eighth-instar *O. albistylum* larvae (n = 20) were placed in plastic tubes containing each of the concentrations of the chromium solution. The tubes were maintained in a laboratory incubator. Six larvae from each treatment were randomly sampled on days 1, 3, 5, 7, and 9. The larvae were immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. All experiments were repeated three times.

2.6. Acute exposure to chromium

Acute concentrations of chromium (0, 25, 30, 50, 75, 100, 125, 150, 200, 300, and 400 mg/L) were determined in preliminary experiments (unpublished data). Six larvae from each group were randomly sampled after 96 h. All experiments were repeated three times. The preparation method for the chromium solution was the same as that described in Section 2.5.

2.7. Determination of SOD activity

SOD activity was determined using the superoxide dismutase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Whole larval homogenate was prepared by adding nine times the volume of normal saline to a specific weight of tissue, followed by homogenization on ice. For the treatment group, 20 μL of homogenate supernatant was mixed with 20 μL of enzyme working solution and 200 μL of substrate application solution. For the control group, the homogenate supernatant was replaced with 20 μL of double distilled water. For the control group, the homogenate supernatant and enzyme working solution were replaced with 20 μL double distilled water and 20 μL enzyme diluent, respectively. The control group contained 20 μL tissue homogenate supernatant, 20 μL enzyme diluent, and 200 μL substrate application solution. Each group was evenly mixed and incubated at 37 °C for 20 min, and absorbance was determined at 450 nm using an enzyme-labeled instrument (Infinite 200 PRO, TECAN, Männedorf, Switzerland). The SOD inhibition rate and activity were calculated as follows:

SOD inhibition rate (%) = [(control group absorbance value - blank control group absorbance value) - (measurement group absorbance value - measurement blank group absorbance value)]/(control group absorbance value - control blank group absorbance value) × 100%

SOD activity (U/mg protein) = SOD inhibition rate/50% (reaction system/dilution multiple)/protein concentration of the sample to be tested.

2.8. Determination of CAT activity

CAT activity was determined using a catalase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Whole larval homogenates were prepared by adding nine times the volume of normal saline to a specific weight of tissue. For the treatment group, 0.05 mL diluted homogenate supernatant was mixed well with 1 mL of reagent 1 and 0.1 mL of reagent 2 (both reagents preheated at 37 °C) and heated at 37 °C for 1 min, and then 1 mL of reagent 3 and 0.1 mL of reagent 4 were added. For the control group, 1 mL of reagent 1 was added to 0.1 mL of reagent 2 (both reagents preheated at 37 °C) and heated at 37 °C for 1 min, and then 1 mL of reagent 3 and 0.1 mL of reagent 4 were added. The absorbance of each sample was measured at 405 nm. The CAT activity was calculated as follows:

CAT activity (U/mg protein) = (optical density value of control group - optical density value of determination group) × 271 × 1/(60 ×

sample size)/protein concentration of sample to be tested.

2.9. Determination of protein concentration

A BCA protein assay kit (TaKaRa Bio, Kyoto, Japan) was used to determine the protein content of each sample.

2.10. qRT-PCR

Total RNA was extracted from *O. albistylum* larvae of each treatment group, and cDNA was synthesized as described in Section 2.2. The expression of *hsc70* and *hsp70* genes obtained from cloning was estimated with qRT-PCR using a CFX96TM system with the SYBR® Premix Ex TaqTM kit (TaKaRa Bio, Kyoto, Japan). qRT-PCR was performed in a total reaction volume of 20 μL containing 1 μL cDNA template, 1 μL (10 μmol/μL) of each primer, 7 μL RNase-free and DNase-free water, and 10 μL SYBR. Gene expression data were normalized to the housekeeping gene actin (obtained from a preliminary experiment) as an internal control. Primers for actin, *hsc70* and *hsp70* genes of *O. albistylum* were designed to target their unique regions; each primer pair nucleotide sequence is shown in Table 1. The target amplification efficiency was identical to the reference amplification at each annealing temperature. The thermal cycling parameters were as follows: 94 °C for 5 min (initial denaturation), 40 cycles at 94 °C for 15 s, 40 cycles at 58–60 °C for 30 s, and 40 cycles at 65 °C for 30 s; the fluorescence signal was collected at 59 °C. The relative expression of genes was quantified using the $2^{-\triangle\triangle CT}$ method (Schmittgen and Livak, 2008).

2.11. Statistical analysis

Data normality and homogeneity were evaluated based on three replicates for each treatment concentration and tissue sampling site. Statistical analyses were conducted using IBM SPSS Statistics v20 software (IBM Corp., Armonk, NY, USA). Multiple comparisons of means were conducted using Duncan's test. Differences between means were considered statistically significant at $P < 0.05$. A two-way analysis of variance (ANOVA) was used to evaluate the effects of time and chromium concentration on gene expression.

3. Results

3.1. Cloning and phylogenetic analysis of hsc70 and hsp70

The length of the *hsc70* open reading frame was 1938 bp, encoding a protein with 645 amino acids. The 3′UTR was followed by a poly (A) tail. A potential consensus signal sequence for polyadenylation, AATAAA, was located 15 bp upstream of the poly (A) tail. The molecular weight of HSC70 was approximately 70.58 kDa with a theoretical isoelectric point of 5.82. As predicted by MotifScan analysis, three conserved characteristic motifs of the HSP70 family (IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK) were located at amino acid positions 9–16, 197–210, and 334–348, respectively. Similarly, the EEVD domain occupies the C-terminus of the protein. The special ATP/GTP-binding site AEAYLGK (aa 131–137) and the non-organellar consensus motif RARFEEL (aa 299–305) were found in *hsc70* (Text. S1). An intermediate fragment of 850 bp was obtained for the cloned *hsp70*, which also contained two signature sequences from the HSP70 family: IFDLGGGTFDVSIL (aa 112–125) and IVLVGGSTRIPK (aa 249–263). In addition, a special ATP/GTP binding site, AEAYLGK (aa 46–52), and the non-organellar consensus motif RARFEEL (aa 214–220) were found in *hsp70* (Text. S2).

BLAST comparison of cloned *hsc70* and *hsp70* in the NCBI GenBank database revealed that the cloned *hsc70* was highly homologous (87–90% identity) with the *hsc70* genes of other insects, especially Hemiptera, Lepidoptera, and Hymenoptera (Table S1). Comparatively, the cloned *hsp70* shared 93–95% identity with *hsp70* genes of Lepidoptera, Orthoptera, and Hemiptera insects (Table S2).

Multiple sequence alignment indicated that the deduced amino acid sequences of *hsc70* and *hsp70* in *O. albistylum* were highly similar to the *hsp70* amino acid sequences of other insects. A phylogenetic tree was constructed based on the deduced amino acid sequences of *hsc70* and *hsp70* for different species, which revealed that the cloned *hsc70* sequence did not align with those of other closely-related species (Fig. S1A) and that the cloned *hsp70* sequence of *O. albistylum* belonged to the same branch as *Cryptocercus punctulatus* (Fig. S1B).

3.2. Tissue expression patterns of hsc70 and hsp70

The relative mRNA expression of *hsc70* and *hsp70* in the four larval tissues (head, epidermis, midgut, and adipose tissue) is shown in Fig. 1, indicating no significant differences ($P > 0.05$) in expression of these genes among the different tissues.

3.3. Effect of chronic chromium stress on hsc70 and hsp70 expression

Toxicological experiments were conducted to understand the expression patterns of *hsc70* and *hsp70* under chronic chromium stress (Fig. 2). The relative expression of *hsc70* did not differ across treatments on day 1 ($P > 0.05$); however significant differences were observed among the four chromium treatments at other times ($P < 0.05$). On day 9, the relative expression of *hsc70* at each chromium concentration was downregulated compared with that in the control group, and the downregulated range decreased with increasing chromium concentration (Fig. 2A). At the same concentration and different treatment times, the relative expression of *hsc70* showed was initially upregulation followed by downregulation. Maximum upregulation was attained on day 3 at a chromium concentration of 1.289 mg/L (3.62 times that in the 0 mg/L control group) (Fig. 2A).

At the same treatment time but different concentrations, the expression of *hsp70* varied significantly throughout the experiment ($P < 0.05$) and was higher in the treatment groups than the control group. Expression of *hsp70* reached a maximum on day 9 in the 1.289 mg/L treatment group (3.52 times that of the 0 mg/L control group) (Fig. 2B). The expression of *hsp70* in the control and 0.05 mg/L group was upregulated as treatment time increased, while the expression of *hsp70* at the higher chromium concentrations (0.5 and 1.289 mg/L) was initially upregulated, followed by a period of downregulation and then upregulation.

The results of the two-way ANOVA demonstrated that exposure duration significantly influenced the expression of *hsc70* ($P < 0.05$), but not the expression of *hsp70* ($P > 0.05$). Chromium concentration did not significantly influence *hsc70* expression ($P > 0.05$), but significantly affected *hsp70* expression ($P < 0.05$) (Fig. 2).

3.4. Effects of chronic chromium stress on SOD and CAT activities

SOD activity was significantly higher in the chronic chromium treatment groups than in the control groups on the 1st, 7th, and 9th days ($F_{15,30} = 7.221$, $P < 0.05$). SOD activity at chronic chromium concentrations of 0 and 0.05 mg/L first increased and then decreased, reaching a maximum on the 7th day, while that of the 0.5 and 1.289 mg/L treatment groups first decreased, then increased, followed by a decrease on the 3rd and 7th days after treatment. SOD activity in the 0.5 mg/L treatment group was highest on the 5th day, and subsequently decreased with time (Fig. 3A).

CAT activity was significantly higher in the treatment groups than that in the control groups on the 1st, and 9th days ($F_{7,14} = 22.211$, $P < 0.05$). CAT activity was not significantly different between treatment and control groups on day 3, 5 and 7 ($F_{11,24} = 1.989$, $P > 0.05$). CAT activity in the 0 and 0.05 mg/L treatment groups first increased and then decreased, whereas that in the 0.5 and 1.289 mg/L treatment groups first decreased, then increased, then decreased, and then increased (Fig. 3B).

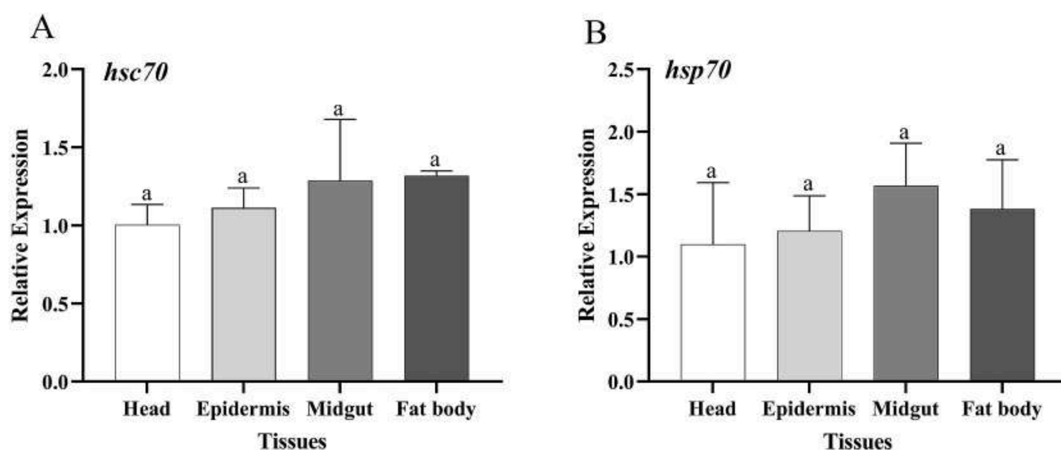

Fig. 1. Relative expression in different tissues of *Orthetrum albistylum* larvae: (A) Relative expression of *hsc70*; (B) Relative expression of *hsp70*. Data are presented as means ± SD (n = 3). Bars with different letters indicate significant differences among different tissues ($P < 0.05$) using Duncan's test, α = 0.05.

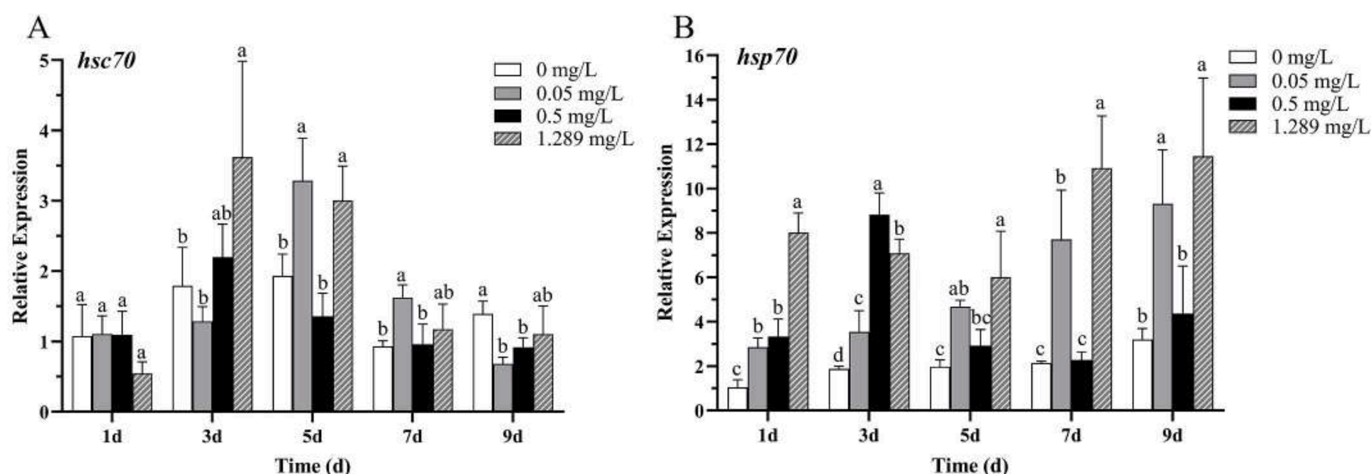

Fig. 2. Relative expression in *Orthetrum albistylum* under chronic chromium stress. (A) Relative expression of *hsc70*; (B) Relative expression of *hsp70*. Data are presented as means ± SD (n = 3). Bars with different letters indicate significant differences among different treatments at the same exposure time ($P < 0.05$) using Duncan's test, α = 0.05, a > b > c > d.

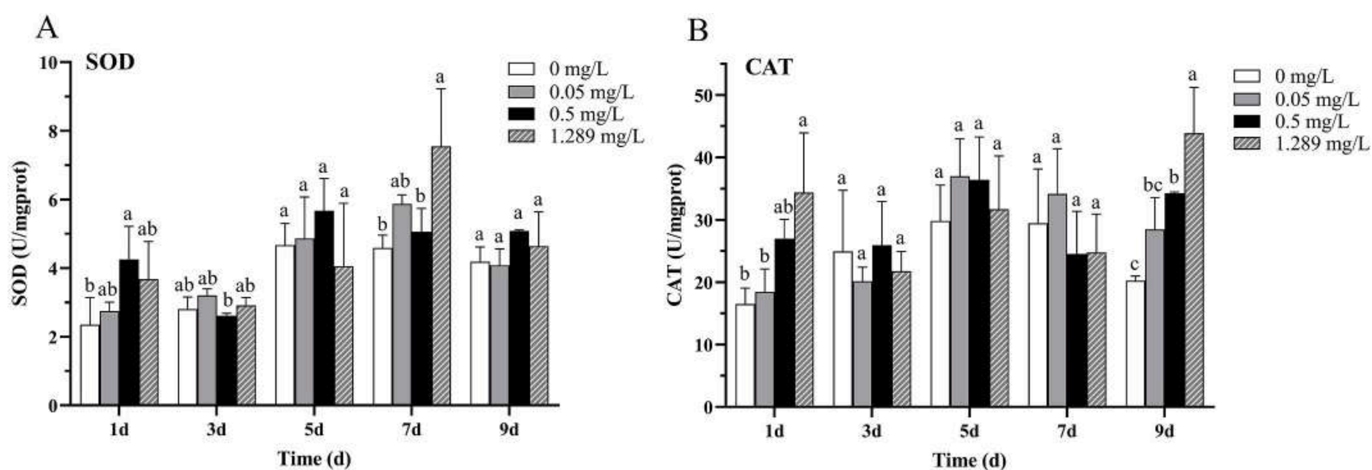

Fig. 3. Enzyme activities in *Orthetrum albistylum* under chronic chromium stress: (A) Superoxide dismutase (SOD); (B) Catalase (CAT). Data are presented as means ± SD (n = 3). Bars with different letters indicate significant differences among different treatments at the same exposure time ($P < 0.05$) using Duncan's test, α = 0.05, a > b > c > d.

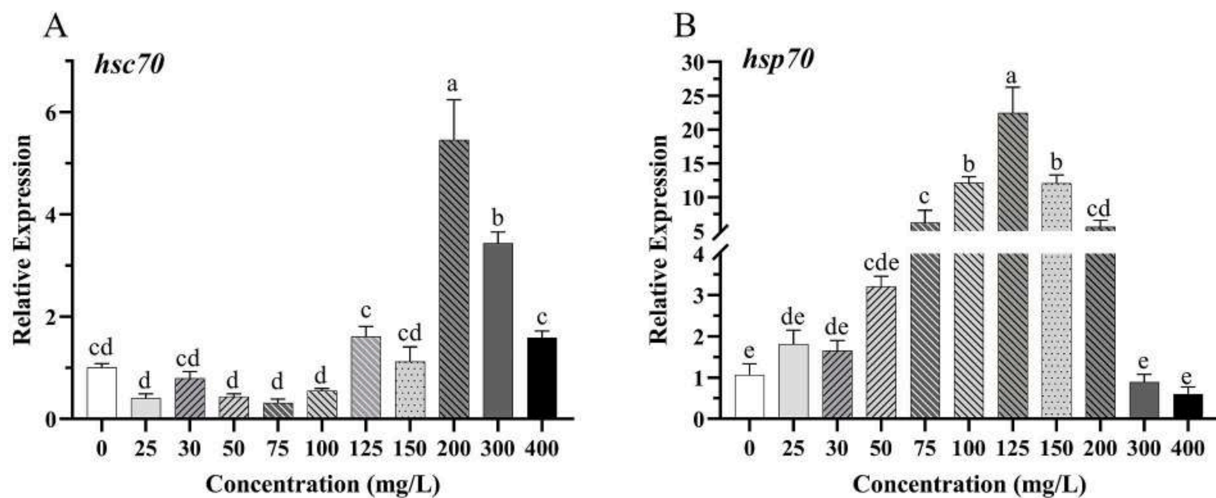

Fig. 4. Relative expression in *Orthetrum albistylum* under acute chromium stress for 96 h: (A) Relative expression of *hsc70*; (B) Relative expression of *hsp70*. Data are presented as means ± SD (n = 3). Bars with different letters indicate significant differences among different treatments ($P < 0.05$) using Duncan's test, α = 0.05, a > b > c > d.

3.5. Effects of acute chromium stress on hsc70 and hsp70 expression

There were significant differences ($P < 0.05$) in the mRNA expression of *hsc70* and *hsp70* when challenged with acute levels of chromium (Fig. 4), with marked changes with increasing concentrations of chromium. Maximum expression of *hsc70* in the 200 mg/L and 125 mg/L treatment groups was 5.46 times and 22.44 times that of the 0 mg/L control group, respectively. The expression of *hsc70* in the 25, 30, 50, 75, and 100 mg/L treatment groups was slightly lower than that in the control group. The relative expression of *hsp70* was significantly upregulated in the 75–200 mg/L treatment groups, but not in the 300 and 400 mg/L treatment groups.

4. Discussion

4.1. Cloning and phylogenetic analysis of hsc70 and hsp70

HSPs participate in DNA and protein repair and assist in cell replacement (Del Razo et al., 2001; Somasundaram et al., 2019). *hsp70* is an important member of the HSP family, regulating not only the growth and development of organisms, but also helping organisms survive unfavorable environmental conditions (King and MacRae, 2015). The mRNA sequences encoding *hsc70* and *hsp70* are important for predicting the biological functions of these proteins in *O. albistylum* larvae. In the present study, *hsc70* and *hsp70* were cloned from *O. albistylum* larvae. As expected, typical characteristics of *hsp70* family members were observed in the deduced amino acid sequences of *hsc70* and *hsp70* (Text. S1). The EEVD motif at the C-terminus of *hsc70* indicates location in the cytoplasm (Tan et al., 2022). These sequences were similar to the *hsp70* gene sequences of *Spodoptera exigua*, *Ericerus pela*, *Onthophagus chinensis*, and *Harmonia axyridis* (Jiang et al., 2012; Liu et al., 2014; Zhang et al., 2015; Shen et al., 2016), thus confirming that the cloned gene belonged to the HSP70 family. The two cloned genes displayed high homology with those of other insects (Tables S1 and 2, Fig. S1).

4.2. Tissue expression patterns of hsc70 and hsp70

HSPs are generally highly expressed in the midgut of insects and are responsive to environmental stresses (Kanakala et al., 2019; Singh and Lakhotia, 2000). We also found that the expression of *hsc70* and *hsp70* was highest in the midgut and lowest in the head of *O. albistylum* (Fig. 1). Expression of *hsc70-4* is highest in the female adults and expression of *AcHsp70Ba* and *AcHsp70-2* is highest in the heads of male adults of *Arma chinensis* (Meng et al., 2022).

4.3. Effect of chronic chromium stress on hsc70 and hsp70 expression

The expression of HSP70s is related to the stress state of an organism. Therefore, the HSP70 family has been extensively used in molecular ecotoxicology (Hu et al., 2019; Nadeau et al., 2001). HSP70s use ATP hydrolysis to unfold and solubilize stable protein aggregates, converting functional alternatively folded or toxic misfolded polypeptides into harmless, protease-degradable, or biologically active native proteins (Finka et al., 2016). Heavy metals can induce increased expression of *hsp70*, to evade the misfolding of existing proteins and newly synthesized peptides (Tamás et al., 2018; Maresca et al., 2020). In the present study, *hsc70* expression in larvae of *O. albistylum* under chronic chromium stress was increased on Day 3 and Day 5 of stress, and was highest on Day 9 of stress, indicating temporality in the response of *hsc70* expression to heavy metal stress (Fig. 2). This temporality has been observed in similar studies. For example, *hsc70* expression in *Palaemon carinicauda* was significantly increased after 24 h of exposure to Cadmium, but did not change significantly at other treatment times (Gao et al., 2016). The results suggest that, similar to *hsp70*, *hsc70* may be induced in response to environmental stressors, with *hsc70* being highly expressed in early and middle stress periods and *hsp70* after longer periods of stress. These two expression modes of *hsc70* and *hsp70* have been reported in other studies. Expression of *hsp70*, but not *hsc70*, in *Chironomus ripariusz* and *Litopenaeus vannamei* was regulated by cadmium stress (Planello et al., 2010; Qian et al., 2012). When organisms are affected by pollution, they require more resources, leading to energy deficiency, and reducing the organism's ability to cope with multiple stresses. Under such conditions, reduced *hsp70* expression can save energy (Matić et al., 2023). The subsequent increase in *hsp70* expression is presumed to be due to a further readjustment to chromium stress. However, other studies have demonstrated that heavy metal stress only promotes *hsc70* expression. For example, *hsc70* expression was higher in the digestive glands of *Mytilus galloprovincialis* after Cr^{6+} stress than in the control group (Franzellitti and Fabbri, 2005). Unlike *hsp70*, which can be induced to a 100-times higher expression under stress conditions than under normal conditions, *hsc70* is a constitutive gene whose expression is very low and only increases several times after induction, even when stimulated by external stimuli (Chuang et al., 2007). This conclusion is consistent with the results of the present study.

4.4. Effects of chronic chromium stress on SOD and CAT activities

In the current study, ROS are considered to be typical markers of environmental adversity. An imbalance of heavy metal ions in organisms increases ROS production, leading to oxidative stress, DNA damage and protein modifications (Jomova and Valko, 2011). SOD and CAT play crucial roles (Kim et al., 2014). For example, under oxidative stress conditions caused by chromium stress, the activities of SOD and CAT in *Oxya chinensis* increased with degree of stress (Li et al., 2005), with similar findings observed for *Caenorhabditis elegans* (González-Manzano et al., 2012). In the present study, under chronic chromium stress, the activity of SOD and CAT initially increased and subsequently decreased with increasing duration of stress (Fig. 3). This may be because larvae exposed to heavy metals produce a large amount of ROS, which induces an increased activity of SOD and CAT enzymes *in vivo* to remove free radicals. However, the scavenging ability of free radicals becom 1996es saturated over time, preventing the complete elimination of free radicals. This is similar to the recently reported response of *Gambusia affinis* to Cu and Zn stress (Wang et al., 2022). The present study showed that the activity of SOD and CAT was lower at higher compared to lower chromium concentrations after the same period of stress (Fig. 3), consistent with changes in antioxidant enzyme levels reported by Lee and Lee (2000). Excessive ROS levels may lead to tissue damage and reduce the enzymatic activity of SOD and CAT.

4.5. Effects of acute chromium stress on hsc70 and hsp70 expression

The harmful effects of chromium on organisms are due to the excessive production of ROS, which further affects the integrity of lipids and proteins and can induce oxidative DNA damage (Santos-Escobar et al., 2014). In the present study acute chromium stress caused a significant increase in the expression level of *hsp70* in the larvae, and this increase was significantly higher than that of *hsc70* (Fig. 4). *hsc70* acts to protect organisms and cellular components from damage by reestablishing the biological function of hsp70 as a molecular chaperone. Matsuda et al. (2010) reported that higher *hsp70* expression suppresses the formation of UVB-induced 8-oxo-dG by decreasing the level of ROS in transgenic mice. Our results showed that the mRNA expression of *hsp70* was highest at a chromium concentration of 125 mg/mL; the expression level of *hsp70* was 22.44 times that of the control group and much higher than the maximum expression of *hsc70* (5.46 times that of the control group at 200 mg/mL) (Fig. 4). Most eukaryotic genes reduce mRNA splicing under stressful conditions, resulting in decreased gene expression (Shaul, 2017). The expression of *hsc70* is affected by the presence of introns, whereas *hsp70* does not contain introns and can be translated normally. The expression of *hsp70* was significantly different under a low level of chromium stress (75 mg/L) (Fig. 4), while the expression of *hsc70* increased significantly only when the larvae were stressed with high concentrations of chromium (200 mg/L). The different expression patterns of *hsc70* and *hsp70* indicate a synergistic relationship in the repair of denatured proteins. Increased expression of heat stress genes is considered a way to protect organisms from additional toxicity. Given the response of HSP70 to heavy metal stress, the expression of *hsp70* and *hsc70* may prove to be a useful molecular indicator for water quality monitoring.

5. Conclusions

This study examined the effects of acute and chronic chromium stress on heat shock protein genes and antioxidant enzyme activities of *O. albistylum* larvae in the laboratory. Overall, our results indicate that the expression of the HSP gene and the activity of antioxidant enzymes in *O. albistylum* larvae can be used as biomarkers to evaluate the health of freshwater ecosystems. *O. albistylum* larvae can be used as a biological indicator of chromium pollution and for qualitative analysis of chromium pollution in water; however, they cannot be used to determine the quantitative relationship with the concentration of heavy metals in water, and further research is needed. The complex nature by which organisms adapt to external stresses suggests that many other genes and enzymes are likely to be involved in adaptation. Further research is needed to fully understand the mechanisms of adaptation of juvenile dragonflies to heavy metal pollution in aquatic ecosystems. In this study, only a part of the sequence of *hsp70* was cloned, and the full-length cDNA of *hsp70* was obtained by RT-PCR combined with RACE for further study and analysis. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are known protective enzymes in insects (Somasundaram et al., 2019; Di et al., 2021; Berenbaum and Calla, 2021; Liang et al., 2022). The effects of these three enzymes on resistance to heavy metal stress in *O. albistylum* larvae should be further studied. For example, enzymatic activity occurs under acute heavy metal stress. Metallothionein (MT) is associated with the presence of heavy metals, hence the cDNA sequence of MT could be cloned and its expression examined in *O. albistylum* larvae under heavy metal stress. Subsequent analysis of differences in gene expression in different tissues and organs, and the relationship between gene expression and heavy metal enrichment determined by immunohistochemistry and frozen section technology, would provide further insights into the adaptive mechanism of resistance to heavy metal stress in *O. albistylum* larvae.

CRediT authorship contribution statement

Tingting Ma: Formal analysis, Data curation, Writing – review & editing. **Yanjuan Ding:** Validation, Formal analysis, Writing – original draft, preparation. **Fengjiao Xu:** Methodology, Data curation, Writing – original draft, preparation. **Chen Zhang:** Validation. **Min Zhou:** Methodology. **Ya Tang:** Data curation. **Yanrong Chen:** Validation. **Yating Wen:** Validation. **Rufei Chen:** Formal analysis. **Bin Tang:** Conceptualization, Methodology. **Shigui Wang:** Conceptualization, Validation, Formal analysis, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122712.

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