Fish Physiol Biochem (2014) 40:1801–1816
DOI 10.1007/s10695-014-9969-9

Two HSPs gene from juvenile Amur sturgeon (*Acipenser schrenckii*): cloning, characterization and expression pattern to crowding and hypoxia stress

Meng Ni · Haishen Wen · Jifang Li · Meili Chi · Yuanyuan Ren · Zhifei Song · Houmeng Ding

Received: 12 May 2014 / Accepted: 30 July 2014 / Published online: 13 August 2014

Abstract In this study, the cDNA sequences of *HSP70* and *HSP90* were isolated from the special chondr-ganoid scale, Amur sturgeon, for the first time. Homology analysis indicated that amino acid sequences of *HSP70* and *HSP90* shared high identity with other species (82.68–99.07 and 90.19–98.07 %, respectively). The tissue expression analysis showed that the as*HSP70* and as*HSP90* mRNA were ubiquitously expressed in all the examined tissues under unstressed condition. The expression pattern of *HSP70* and *HSP90* under chronic (crowding) and acute (hypoxia) stress was examined by q-PCR in liver, spleen and kidney. Results showed that stocking density could significantly influence the expression of *HSP70* at day 20 and/or day 40. In contrast to stocking density, levels of *HSP70* transcripts indicated a remarkable increase in all examined tissues after hypoxia stress. *HSP90* levels in liver and spleen increased significantly in high stocking density. By comparison, significant increase of as*HSP90* in kidney was only found in high stocking density at day 40. Similar to *HSP70*, the levels of *HSP90* transcripts showed significant increases after hypoxia stress except the transcript of liver in H2 group 6 h after hypoxia. The assessment of as*HSP70* and as*HSP90* mRNA levels under crowding and hypoxia stresses indicated that as*HSP70* and as*HSP90* gene might be good indicators of stressful situations for Amur sturgeon. Taking serum globulin and electrolytes account, we suggest that crowding and hypoxia stress can result in considerable stress for Amur sturgeon.

Keywords *Acipenser schrenckii* · Heat shock protein 70 · Heat shock protein 90 · Stocking density · Hypoxia

Introduction

Molecular chaperones are major cell constituents in all organisms under benign conditions, and they are essential to ensure proper folding and intracellular localization of newly synthesized polypeptides (Feder and Hofmann 1999). The demand for molecular chaperones is increased under stressful conditions (Bukau and Horwich 1998; Sørensen et al. 2003). Heat shock proteins (HSPs), a subset of the molecular chaperones, are the proteins known to help organisms to modulate stress response and protect organisms from environmentally induced cellular damage (Kiang and Tsokos 1998; Lindquist and Craig 1988). HSPs synthesis has been shown to be up-regulated in response to a wide variety of environmental insults such as crowding, hypoxia, exogenous hormones,

M. Ni · H. Wen (✉) · J. Li · M. Chi · Y. Ren · Z. Song · H. Ding
Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao, Shandong 266003, People's Republic of China
e-mail: wenhaishen@ouc.edu.cn

heavy metal exposure and bacterial infection (Ming et al. 2010; Deane and Woo 2010; Yue et al. 2011; Ma et al. 2012; Mu et al. 2013). The expression of HSPs gene can lead to protecting cells against harmful conditions by binding and refolding of damaged proteins (Su et al. 2010). The HSP members are usually grouped according to their homology and molecular weights, and divided into three main families: small *HSPs, HSP40, HSP60, HSP70, HSP90* and *HSP110* (Joly et al. 2010). Among the HSPs families, *HSP70*s and *HSP90* are the most conserved and most extensively studied proteins which play important roles in the cell as molecular chaperones. The HSP70 family is necessary for membrane translocation, degradation and protein folding (Gething and Sambrook 1992), while the HSP90 family is involved in steroid receptor formation and protein folding (Pratt 1997). HSPs could also function as potent activators of the innate immune system (Weigl et al. 1999; Wallin et al. 2002) and act as cytokine to make an immunoregulatory effect on the host's immune system (Tsan and Gao 2004).

The aquatic environment is a very complex system in which stocking density, temperature, salinity, pollutant content, parasite and oxygen vary greatly. Among them, stocking density and hypoxia are two major chronic or acute stressors in aquaculture and organism often evolved a variety of physiological alterations to cope with the crowding and/or hypoxia environment (Montero et al. 1999; Ellis et al. 2002; Martinovic et al. 2009). Stocking density could directly influence physiology, welfare and behavior of farmed fish (Schreck et al. 1997; North et al. 2006). In recent years, there are many studies have been conducted in fish in order to understand the role of stocking density in physiology of aquatic organisms (Gornati et al. 2004b; Schram et al. 2006; Imorou et al. 2007). Many reports showed that the synthesis of HSPs could be induced under crowding conditions (Gornati et al. 2004a; Salas-Leiton et al. 2010). Dissolved oxygen is also one relevant factor to consider in aquatic environment. The physiological effects of hypoxia have also been studied extensively in aquatic organisms (Wu 2002; Douxfils et al. 2012). Heat shock proteins could be induced under hypoxia condition in some aquatic organisms, such as *Sebastes schlegeli* and *Haliotis diversicolor* (Mu et al. 2013; Huang et al. 2014). Due to the importance of these two adverse environment factors, it is crucial to elucidate the molecular mechanisms under these stressors to warrant an optimal welfare of Amur sturgeon.

Sturgeons are notable ancient fish species with unique properties: They have low evolutionary scale, and all living representatives of sturgeons are listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Bemis et al. 1997; Ludwig 2008). Amur sturgeon, *Acipenser schrenckii*, distributed throughout the Amur River (an international boundary between China and Russia). Now, it is among the most important cultivated sturgeon species in China (Zhuang et al. 2002). In recent years, there are some studies have been conducted in Amur sturgeon (Zhuang et al. 2002; Yang et al. 2011; Ni et al. 2013). However, the effects of stocking density and hypoxia on the heat shock proteins expression of Amur sturgeon have not been reported.

In the present study, the full-length cDNA of *HSP70* and *HSP90* from Amur sturgeon (*Acipenser schrenckii*) was cloned for the first time. The gene expression profiles for *HSP70* and *HSP90* in different tissues were showed using RT-PCR. In addition, this is the first study to clearly define molecular regulation of *HSP70* and *HSP90* gene in this ancient fish, acclimated to crowding and hypoxia stress. To correlate the heat shock protein response with aspects of stress and immune physiology, serum levels of globulin and electrolytes were also reported.

Methods and materials

Fish and holding conditions

The experiment took place in a commercial farm (Shandong Xunlong Sci-Tech Co., Ltd). Prior to the start of the experiment, fish were initially acclimated to experimental conditions for 2 weeks. During both the adaptation period and experimental period, pelleted feed (Ningbo Tech-Bank, containing 42 % of crude protein) was provided. Experimental ponds were supplied with freshwater from a system equipped with mechanical filters. Mean values of water parameters were temperature at 18.2 ± 1.8 °C, O_2 at 8.5 ± 1.2 mg/l, TAN at 0.015 ± 0.002 mg/l and pH at 7.84 ± 0.08 (Mean ± SEM).

Table 1 Primers used for cloning and mRNA expression analysis

Primers	Sequence (5′–3′)	Position	Usage
RT-PCR			
HSP70-1F	GATGTGTCCATCCTGACTATCgargayggnat	721–752	Amplification of cDNA fragment
HSP70-1R	CACAGTCATCACTCCTCCAgcngtytcdat	1,312–1,341	Amplification of cDNA fragment
HSP70-2F	TTACGGAGCTGCTGTGcargcngcnat	1,215–1,241	Amplification of cDNA fragment
HSP70-2R	CTTGGTGATGATAGGGttgcanacyttytc	1,903–1,932	Amplification of cDNA fragment
HSP90-1F	CCCTGCTTTTCATTCCACGAC	1,071–1,091	Amplification of cDNA fragment
HSP90-1R	AGCGGTTGGACACTGTGACTT	1,809–1,829	Amplification of cDNA fragment
HSP90-2F	TCCCAGTTTATCGGCTACC	692–710	Amplification of cDNA fragment
HSP90-2R	GGAGATGTTCAAGGGCAAG	1,234–1,252	Amplification of cDNA fragment
RACE			
HSP70-5-R1	CATCTTGGCATCACGGAGGGACTTC	1,059–1,083	5′-RACE
HSP70-5-R2	CTTTCACAGGCGGTGCGGAGACG	889–911	Nested 5′-RACE
HSP70-3-R1	CACCATCACCAACGACAAAGGACGC	1,608–1,632	3′-RACE
HSP70-3-R2	TGCCCTGGAGTCCTACGCTTTCAAC	1,725–1,749	Nested 3′-RACE
HSP90-5-R1	GGGGTAGCCGATAAACTGG	694–712	5′-RACE
HSP90-5-R2	GCAACCAGGTAGGCAGAGTA	476–495	Nested 5′-RACE
HSP90-3-R1	CATCCTCCTGTTTGAGACTGC	2,044–2,064	3′-RACE
HSP90-3-R2	CGCTTGAAGATCCTCAGACTCAC	2,087–2,107	Nested 3′-RACE
qPCR			
HSP70-eF	GGTCCAGTCCGATATGAAGCAC	351–372	RT-PCR and qPCR
HSP70-eR	CGTTGGTCACAGACTTTCCGAG	508–529	RT-PCR and qPCR
HSP90-eF	TGCGATACCACAGCTCTCAGTC	1,443–1,464	RT-PCR and qPCR
HSP90-eR	GTAGATCACCTCAAAGCCACGC	1,594–1,615	RT-PCR and qPCR
18S-eF	GCCACACGAGATGGAGCA	–	Reference primer
18S-eR	CCTGTCGGCGAAGGGTAG	–	Reference primer

Treatments and protocols

Chronic stress

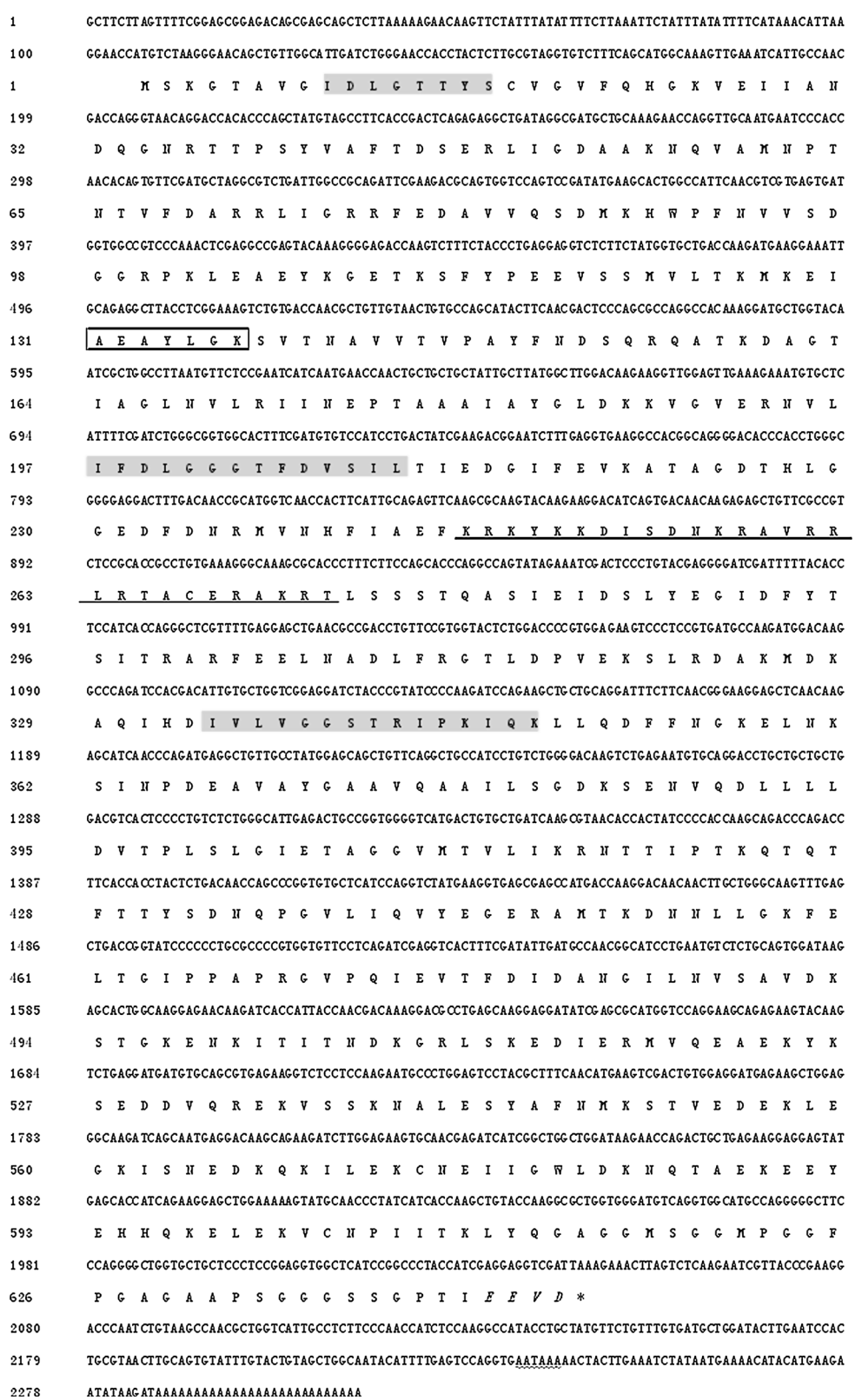
After the adaptation period, fish (42.0 ± 2.3 g) were randomly distributed into three stocking densities (LSD = 3.7 kg/m^3, MSD = 6.9 kg/m^3 and HSD = 9.3 kg/m^3, respectively). The experiment was conducted in 9 square concrete ponds (4.4 × 4.4 × 0.45 m^3) for a period of 60 days. Samplings were performed on triplicated groups every 20 days with a sample size of 4 fish in each pond. The fish were anaesthetized in tricaine methane sulfonate (MS-222) and sampled within 20 min.

Acute stress

Nine 100-l indoor tanks were used in this experiment. There were three experimental groups (three replicate tanks in each group): normal group (N, 6 mg/l), hypoxia group 1 (H1, 3 mg/l) and hypoxia group 2 (H2, 1 mg/l). Nitrogen gas bubbling was used to quickly reduce the DO concentrations. Fish were euthanized, and blood sampled and tissue sampled at 0.5, 1, 3 and 6 h post-stress.

Total RNA extraction and reverse transcription (RT)

Total RNA was extracted using RNAiso reagent (Takara, Japan) following the manufacturer's protocols. RNA concentration was quantified in a Nucleic acid analyzer, Biodropsis BD-1000 (OSTC, China), and sample integrity was evaluated in a 1.5 % agarose gel electrophoresis. Then, first-strand cDNA was synthesized with 1 μg total RNA from each sample using random primers and Reverse Transcriptase M-MLV (Takara, Japan) in a 20-μl reaction.


1 GCTTCTTAGTTTTCGGAGCGGAGACAGCGAGCAGCTCTTAAAAAGAACAAGTTCTATTTATATTTTCTTAAATTCTATTTATATTTTCATAAACATTAA
100 GGAACCATGTCTAAGGGAACAGCTGTTGGCATTGATCTGGGAACCACCTACTCTTGCGTAGGTGTCTTTCAGCATGGCAAAGTTGAAATCATTGCCAAC
1 M S K G T A V G I D L G T T Y S C V G V F Q H G K V E I I A N
199 GACCAGGGTAACAGGACCACACCCAGCTATGTAGCCTTCACCGACTCAGAGAGGCTGATAGGCGATGCTGCAAAGAACCAGGTTGCAATGAATCCCACC
32 D Q G N R T T P S Y V A F T D S E R L I G D A A K N Q V A M N P T
298 AACACAGTGTTCGATGCTAGGCGTCTGATTGGCCGCAGATTCGAAGACGCAGTGGTCCAGTCCGATATGAAGCACTGGCCATTCAACGTCGTGAGTGAT
65 N T V F D A R R L I G R R F E D A V V Q S D M K H W P F N V V S D
397 GGTGGCCGTCCCAAACTCGAGGCCGAGTACAAAGGGGAGACCAAGTCTTTCTACCCTGAGGAGGTCTCTTCTATGGTGCTGACCAAGATGAAGGAAATT
98 G G R P K L E A E Y K G E T K S F Y P E E V S S M V L T K M K E I
496 GCAGAGGCTTACCTCGGAAAGTCTGTGACCAACGCTGTTGTAACTGTGCCAGCATACTTCAACGACTCCCAGCGCCAGGCCACAAAGGATGCTGGTACA
131 A E A Y L G K S V T N A V V T V P A Y F N D S Q R Q A T K D A G T
595 ATCGCTGGCCTTAATGTTCTCCGAATCATCAATGAACCAACTGCTGCTGCTATTGCTTATGGCTTGGACAAGAAGGTTGGAGTTGAAAGAAATGTGCTC
164 I A G L N V L R I I N E P T A A A I A Y G L D K K V G V E R N V L
694 ATTTTCGATCTGGGCGGTGGCACTTTCGATGTGTCCATCCTGACTATCGAAGACGGAATCTTTGAGGTGAAGGCCACGGCAGGGGACACCCACCTGGGC
197 I F D L G G G T F D V S I L T I E D G I F E V K A T A G D T H L G
793 GGGGAGGACTTTGACAACCGCATGGTCAACCACTTCATTGCAGAGTTCAAGCGCAAGTACAAGAAGGACATCAGTGACAACAAGAGAGCTGTTCGCCGT
230 G E D F D N R M V N H F I A E F K R K Y K K D I S D N K R A V R R
892 CTCCGCACCGCCTGTGAAAGGGCAAAGCGCACCCTTTCTTCCAGCACCCAGGCCAGTATAGAAATCGACTCCCTGTACGAGGGGATCGATTTTTACACC
263 L R T A C E R A K R T L S S S T Q A S I E I D S L Y E G I D F Y T
991 TCCATCACCAGGGCTCGTTTTGAGGAGCTGAACGCCGACCTGTTCCGTGGTACTCTGGACCCCGTGGAGAAGTCCCTCCGTGATGCCAAGATGGACAAG
296 S I T R A R F E E L N A D L F R G T L D P V E K S L R D A K M D K
1090 GCCCAGATCCACGACATTGTGCTGGTCGGAGGATCTACCCGTATCCCCAAGATCCAGAAGCTGCTGCAGGATTTCTTCAACGGGAAGGAGCTCAACAAG
329 A Q I H D I V L V G G S T R I P K I Q K L L Q D F F N G K E L N K
1189 AGCATCAACCCAGATGAGGCTGTTGCCTATGGAGCAGCTGTTCAGGCTGCCATCCTGTCTGGGGACAAGTCTGAGAATGTGCAGGACCTGCTGCTGCTG
362 S I N P D E A V A Y G A A V Q A A I L S G D K S E N V Q D L L L L
1288 GACGTCACTCCCCTGTCTCTGGGCATTGAGACTGCCGGTGGGGTCATGACTGTGCTGATCAAGCGTAACACCACTATCCCCACCAAGCAGACCCAGACC
395 D V T P L S L G I E T A G G V M T V L I K R N T T I P T K Q T Q T
1387 TTCACCACCTACTCTGACAACCAGCCCGGTGTGCTCATCCAGGTCTATGAAGGTGAGCGAGCCATGACCAAGGACAACAACTTGCTGGGCAAGTTTGAG
428 F T T Y S D N Q P G V L I Q V Y E G E R A M T K D N N L L G K F E
1486 CTGACCGGTATCCCCCCTGCGCCCCGTGGTGTTCCTCAGATCGAGGTCACTTTCGATATTGATGCCAACGGCATCCTGAATGTCTCTGCAGTGGATAAG
461 L T G I P P A P R G V P Q I E V T F D I D A N G I L N V S A V D K
1585 AGCACTGGCAAGGAGAACAAGATCACCATTACCAACGACAAAGGACGCCTGAGCAAGGAGGATATCGAGCGCATGGTCCAGGAAGCAGAGAAGTACAAG
494 S T G K E N K I T I T N D K G R L S K E D I E R M V Q E A E K Y K
1684 TCTGAGGATGATGTGCAGCGTGAGAAGGTCTCCTCCAAGAATGCCCTGGAGTCCTACGCTTTCAACATGAAGTCGACTGTGGAGGATGAGAAGCTGGAG
527 S E D D V Q R E K V S S K N A L E S Y A F N M K S T V E D E K L E
1783 GGCAAGATCAGCAATGAGGACAAGCAGAAGATCTTGGAGAAGTGCAACGAGATCATCGGCTGGCTGGATAAGAACCAGACTGCTGAGAAGGAGGAGTAT
560 G K I S N E D K Q K I L E K C N E I I G W L D K N Q T A E K E E Y
1882 GAGCACCATCAGAAGGAGCTGGAAAAAGTATGCAACCCTATCATCACCAAGCTGTACCAAGGCGCTGGTGGGATGTCAGGTGGCATGCCAGGGGGCTTC
593 E H H Q K E L E K V C N P I I T K L Y Q G A G G M S G G M P G G F
1981 CCAGGGGCTGGTGCTGCTCCCTCCGGAGGTGGCTCATCCGGCCCTACCATCGAGGAGGTCGATTAAAGAAACTTAGTCTCAAGAATCGTTACCCGAAGG
626 P G A G A A P S G G G S S G P T I F F V D *
2080 ACCCAATCTGTAAGCCAACGCTGGTCATTGCCTCTTCCCAACCATCTCCAAGGCCATACCTGCTATGTTCTGTTTGTGATGCTGGATACTTGAATCCAC
2179 TGCGTAACTTGCAGTGTATTTGTACTGTAGCTGGCAATACATTTTGAGTCCAGGTGAATAAAAACTACTTGAAATCTATAATGAAAACATACATGAAGA
2278 ATATAAGATAAAAAAAAAAAAAAAAAAAAAAAAAA

◀**Fig. 1** cDNA and deduced amino acid sequence of the *HSP70* gene in Amur sturgeon. The initiation codon and termination codon are indicated in *bold*, *HSP70* family signatures are highlighted as *shaded regions*, ATP/GTP-Binding Site Motif A is real-line boxed, nuclear localization signal sequence is *underlined*, RNA instability motifs is marked with a *wavy line*, consensus sequence EEVD at the C-terminus is indicated in *italics*, and the nucleotides and amino acids are numbered along the *left margin*

Isolation and amplification of *HSP70* and *HSP90* cDNA

In order to isolate the two HSPs cDNA, four cDNA fragments of *HSP70* and *HSP90* were first obtained from PCR amplification, respectively. PCR was carried out in a final volume of 25 μl containing 2 μl of cDNA from liver tissue, 2.5 μl of 10× reaction buffer, 2 μl of a 10 mM dNTP mix, 0.5 μl of 25 μM solution of each primer (Table 1) and 0.2 μl of Taq polymerase (Takara, Japan). PCR products were isolated from a 1.5 % agarose gel electrophoresis, then cloned into pGEM-T vector (Tiangen, China), followed by propagation in *E.coli* DH5α, and subsequently sequenced using the ABI3730XL sequencer to give at least threefold coverage.

SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA) was used for 3′ and 5′ ends RACE PCR according to the manufacturer's protocol. PCR products which have corresponding predicted length were isolated, purified, cloned into vector and then sequenced as described above. BLASTN searches were used to verify gene identity and determine similarities with other vertebrates.

Sequence analysis

The sequence analysis of two HSPs cDNA sequences was performed using some online softwares. The open reading frames (ORF) of the cDNA were determined using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Theoretical protein molecular mass and isoelectric points were predicted using compute pI/Mw tool. Molecular Biology Server was used for scanning all known PROSITE motifs based on PROSITE database. Percent identities of proteins motifs between Amur sturgeon and other species were calculated using ClustalW2. Scans for known amino acid motifs were performed against PROSITE and Pfam databases. *HSP70* and *HSP90* amino acid sequences from various fishes were gained from GenBank. Multi-sequences with deduced amino acid sequences for *HSP70* and *HSP90* of Amur sturgeon were aligned using Clustal X (version 1.83) (Thompson et al. 1994). MEGA 4.0 software package (Tamura et al. 2007) was used to construct and analyze phylogenetic tree using the neighbor-joining method (Saitou and Nei 1987) with 1,000 bootstrap trials.

Tissue-specific expression of *HSP70* and *HSP90* genes

The expression profiles of *HSP70* and *HSP90* mRNA in different tissues were studies through semiquantitative RT-PCR, including liver, heart, spleen, kidney, stomach, intestine, brain, pituitary, muscle, gill, fin and adipose tissue of Amur sturgeon. Total RNA of those tissues was extracted and reverse-transcribed as described above. The 18S rRNA (internal control gene) primers and specific primers are listed in Table 1. After separating on 1.5 % agarose gels, the PCR products were electrophoresed and analyzed by Chemiluminescent and Fluorescent Imaging System (SAGECREATION, China).

HSP70 and *HSP90* genes transcripts in different stocking density and dissolved oxygen

Real-time quantitative PCR (q-PCR) was used for studying the expression pattern of *HSP70* and *HSP90* in crowding and hypoxia stress. Q-PCR was performed with the SYBR Premix Ex Taq (Takara, Japan) on Roche 480 light cycler system. PCR amplification of 18S ribosomal RNA was applied as internal control to normalize the concentration of templates. Annealing temperatures and cycle number were optimized as follows: 2 min denaturing step at 94 °C, 35 cycles (for *HSP70* and *HSP90*) or 25 cycles (for 18S ribosomal RNA) of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C. The melting curve was finally determined during a slow temperature elevation from 62 to 99 °C (3 °C min^{-1}). Specific primers and internal control gene (18S) primers are listed in Table 1. The relative expression levels of gene were calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2002).

Serum biochemical indexes

Blood samples were collected from the caudal vein using a 1-ml plastic syringe. Then the blood samples

Table 2 Amino acid identities of *HSP70* and *HSP90* between Amur sturgeon and other fish and tetrapod

Species	*HSP70*		*HSP90*	
	Accession number	Score	Accession number	Score
Acipenser ruthenus	AEK81529.1	99.07	AFA25806.1	98.07
Solea senegalensis	BAI67713.1	88.39	BAF92790.1	92.28
Oryzias latipes	NP_001098385.1	89.27	XP_004083479.1	91.02
Dicentrarchus labrax	AAR01102.2	90.4	AAQ95586.1	91.72
Oncorhynchus mykiss	NP_001117704.1	94.58	NP_001117703.1	91.7
Salmo salar	NP_001135156.1	89.01	NP_001117004.1	91.97
Danio rerio	AAH56709.1	85.69	NP_571385.2	91.45
Paralichthys olivaceus	AAC33859.1	92.88	AAO92751.1	91.17
Oreochromis niloticus	ACI25099.1	85.0	XP_003446339.1	91.03
Xenopus laevis	NP_001121147.1	84.37	NP_001086624.1	90.3
Mus musculus	AAC84168.1	83.96	NP_032328.2	90.19
Homo sapiens	NP_005518.3	82.68	NP_031381.2	90.19

were clot at 4 °C for 4–6 h, centrifuged at 3,000 *g* for 10 min and stored at −20 °C for subsequent analysis. Serum globulin and electrolytes including calcium (Ca), inorganic phosphore (P) and magnesium (Mg) were determined with an automated biochemistry analyzer (Mindray BS180, China) according to Rehulka's method (Rehulka 2000).

Statistics

The relevant values in this study were analyzed through a one-way ANOVA followed by Duncan's multiple range tests. Statistical significance was considered as $P < 0.05$. All data were presented as mean ± standard error of mean (SEM).

Results

Isolation and characterization of as*HSP70* cDNA

The isolated full-length cDNA of as*HSP70* consisted of 2,313 base pairs (bp), including an open reading frame (ORF) encoding deduced proteins of 646 amino acid residues with a calculated molecular weight of 71.24 kDa and theoretical isoelectric point of 5.25 (Fig. 1). The deduced protein included 82 positively charged residues (Arg and Lys) and 96 negatively charged residues (Asp and Glu), with a net negative charge. The 5′ UTR was located 105 bp upstream of the putative start codon (ATG), and the 3′ UTR (267 bp) with a canonical polyadenylation signal sequence AATAAA was followed by a poly (A) tail. The sequences have been submitted to GenBank with an accession number JQ991596. The full length of nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. Amino acid sequence analysis indicated that as*HSP70* contained a cytoplasmic characteristic motif EEVD (Demand et al. 1998) and three signature sequences of *HSP70* family: IDLGTTYS (residues 9–16), IFDLGGGTFDVSIL (residues 197–210) and IVLVGGSTRIPKIQK (residues 334–348). Putative bipartite nuclear targeting sequence (KRKFKKDITDNKRAVRR) was also observed in the as*HSP70* using the online software of PSORT II.

The coding sequence and translated protein were readily aligned to the known fish HSP70. ClustalW2 results revealed that percent identities of HSP70 proteins motifs between Amur sturgeon and other species were 82.68–99.07 % (Table 2). Based on the amino acid sequences of Amur sturgeon HSP70 along with other HSP70 family member sequences from other species, a phylogenetic tree was constructed using the program of MEGA 4. As expected, the sequence homology implicated that as*HSP70* was more similar to fish *HSP70*, especially in Starlet (*Acipenser ruthenus*), than the tetrapod *HSP70* (Fig. 3a).

Isolation and characterization of as*HSP90* cDNA

The cDNA sequence of the as*HSP90* gene was deposited in GenBank under accession number JX477807. The full-length cDNA of as*HSP90* was

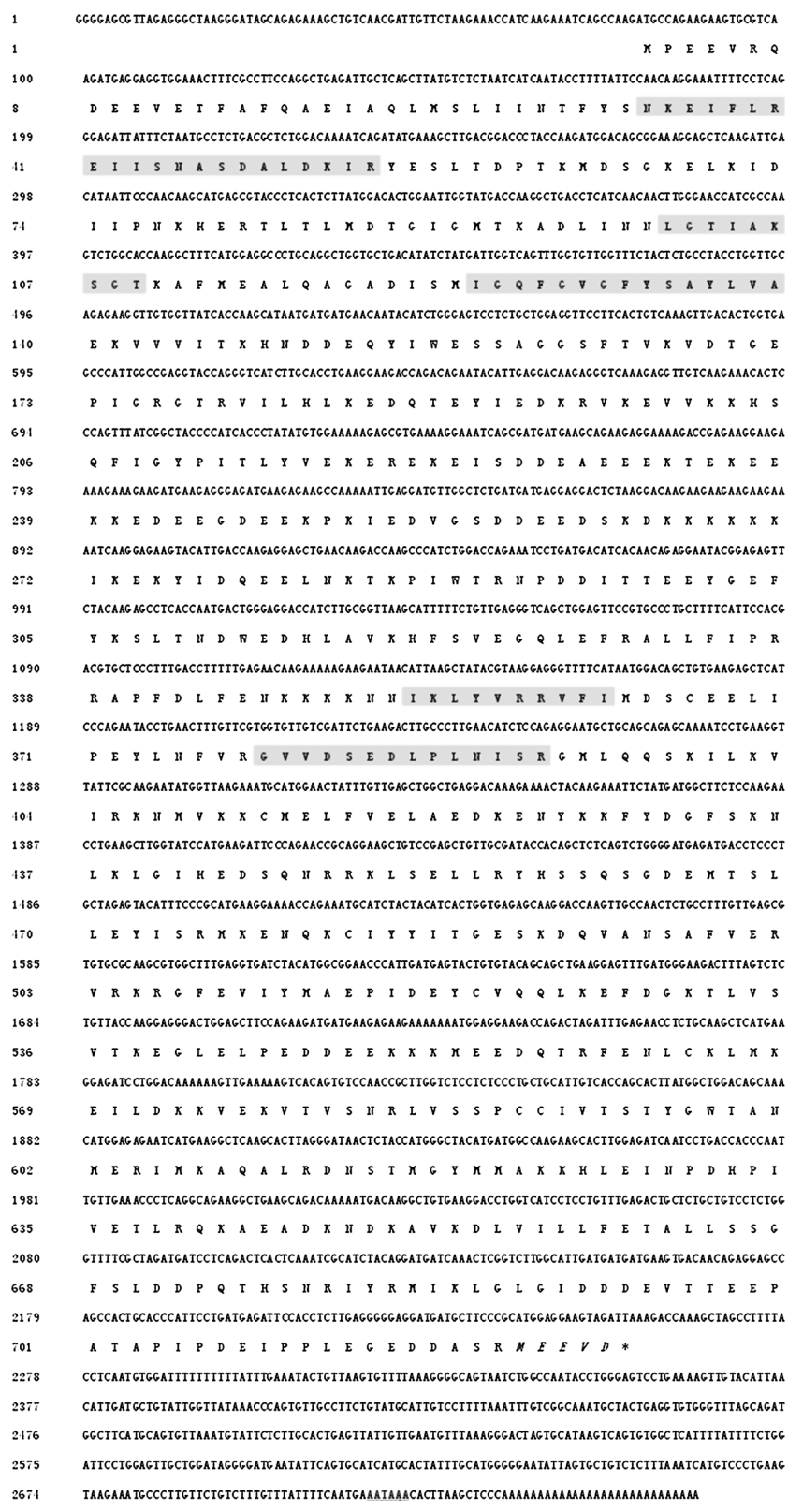


Fig. 2 cDNA and deduced amino acid sequence of the *HSP90* gene in Amur sturgeon. *HSP90* family signatures are highlighted as *shaded regions* (positions 34–54, 101–109, 125–139, 353–362 and 379–392), RNA instability motifs are marked with a *wavy line*, consensus sequence MEEVD at the C-terminus is indicated in *italics*, the stop codon is indicated by a *star*, and the nucleotides and amino acids are numbered along the *left margin*

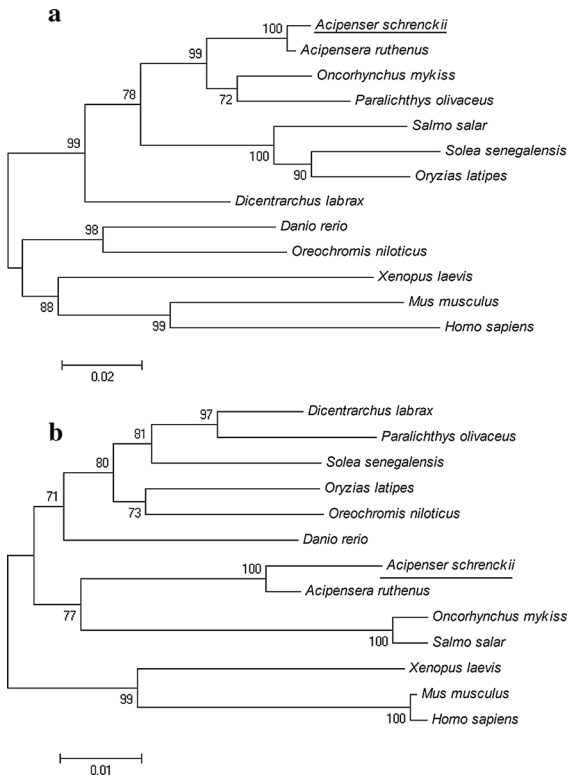


Fig. 3 Phylogenetic tree based on amino acid sequences for *HSP70* and *HSP90* in vertebrates. The tree was generated by MEGA 4.0 software using the neighbor-joining method, following Clustal X. *Scale bar* indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (1000 replicates)

of 2,760 bp, including a 5′-terminal untranslated region (UTR) of 79 bp, a 3′-terminal UTR of 503 bp with a poly (A) tail and an open reading frame (ORF) of 2,178 bp. as*HSP90* encoded a polypeptide of 725 amino acids with a predicted molecular mass of 83.66 kDa and theoretical isoelectric point of 4.94. The full length of nucleotide sequence and the deduced amino acid sequence are shown in Fig. 2. Amino acid sequence analysis indicated the existence of all five conserved amino acid blocks distinctive of the HSP90 protein family described by Gupta (1995): NKEIFLRELISN–[SA]–SDALDKIR (residues 34–54), LGTIA–[KR]–SGT (residues 101–109), IGQFGVGFYSA–[YF]–LVA–[ED] (residues 125–139), IKLYVRRVFI (residues 353–362) and GVVDS–[ED]–DLPLN–[IV]–SRE (residues 379–392). The C-terminal consensus cytosolic motif, MEEVD, appeared at position 721–725.

ClustalW2 results revealed that percent identities of asHSP90 proteins motifs between Amur sturgeon and other species were 90.19–98.07 % (Table 2). Phylogenetic analysis, based on deduced amino acids, was applied in order to determine the evolutionary position of as*HSP90*. Similar to as*HSP70*, as*HSP90* clustered with the *HSP90* of *Acipenser ruthenus*, and then, they clustered to *Oncorhynchus mykiss*, *salmo salar* and other fish species on a branch of fish *HSP90* (Fig. 3b).

Expression of *HSP70* and *HSP90* genes in different tissues of unstressed Amur sturgeon

Primers *HSP70*-eF, *HSP70*-eR and *HSP90*-eF, *HSP90*-eR were applied to determine specific tissue expression of as*HSP70* and as*HSP90*, respectively. as*HSP70* expressed in all examined tissues. as*HSP70* mRNA was abundant in liver, spleen, kidney, brain and gill, and moderate in heart, stomach, intestine, pituitary, muscle and fin. The lowest *HSP70* expression was found in adipose tissue (Fig. 4b). as*HSP90* also displayed a wide distribution. It expressed strongly in liver, spleen and kidney, and moderate in heart, intestine, pituitary, muscle and gill. Similar to as*HSP70*, the lowest transcripts of as*HSP90* were also detected in adipose tissue (Fig. 4c). The levels of 18S rRNA were used to normalize the PCR products to obtain quantitative results and were found at a similar intensity in all tissues studied.

Expression of *HSP70* gene under different stocking density and dissolved oxygen

HSP70 transcript levers in liver, spleen and kidney of Amur sturgeon under different stocking densities were detected by qPCR. The samples at day 0 were used as calibrators for qPCR. The transcript levels in liver reached the highest level at day 40 and then decreased at day 60 in any of the three stocking densities (Fig. 5a). Similar changes of as*HSP70* transcript were also observed in the spleen and kidney. In liver, the *HSP70* transcript showed the highest levels in high stocking density at day 20 and day 40. However, in spleen, significant difference was only found at day 40 (Fig. 5b). In addition, the *HSP70* transcript levels of kidney in high and medium stocking density were significantly higher than those in low stocking density group at day 20 and day 40 ($P < 0.05$, Fig. 5c).

Compared to normal group, the levels of *HSP70* transcript in H1 and H2 group indicated a remarkable increase in spleen after hypoxia stress (Fig. 6b). The

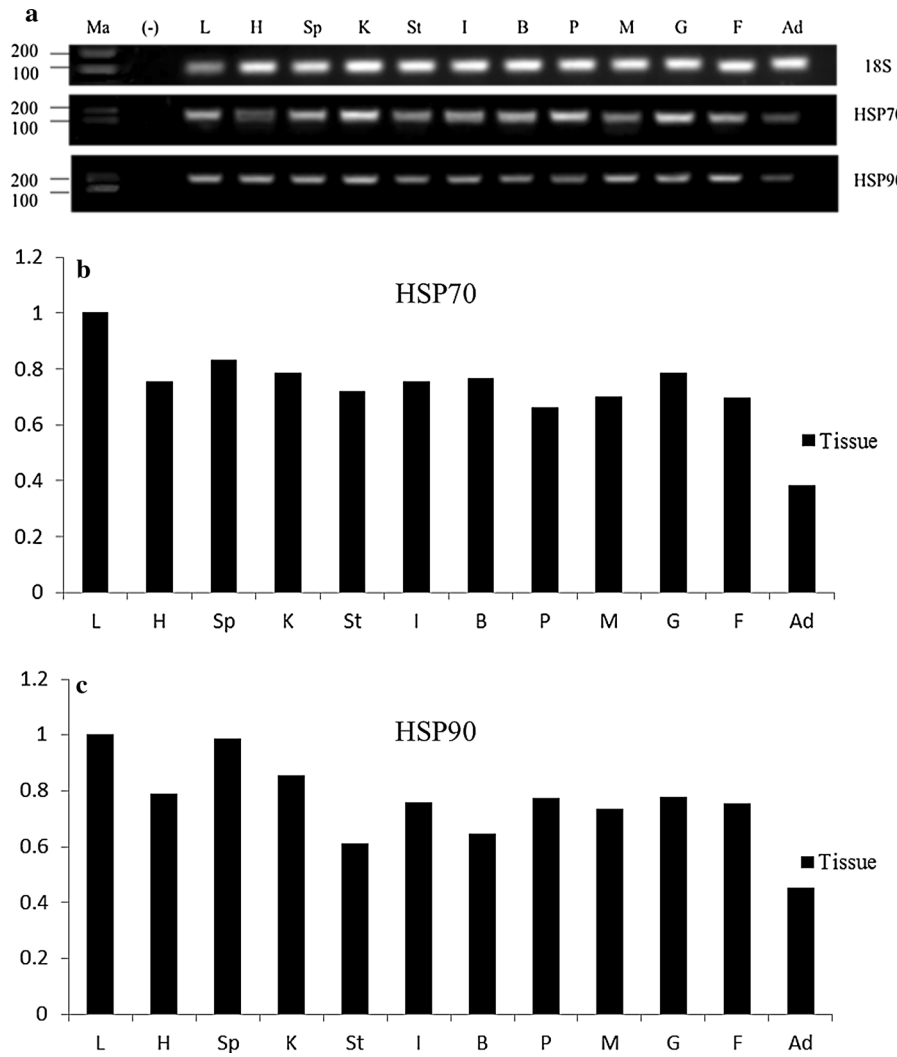


Fig. 4 RT-PCR expression analysis of *HSP70* and *HSP90* in tissues of Amur sturgeon. PCR amplification of *18S* ribosomal RNA was used as an internal control. *Ma* marker, (−): mRNA not reversed transcribed, *L* liver, *H* heart, *Sp* spleen, *K* kidney, *St* stomach, *I* intestine, *B* brain, *P* pituitary, *M* muscle, *G* gill, *F* fin, *Ad* adipose tissue

levels of *HSP70* transcript in liver were similar to those in spleen except that there was a decline in H2 group at 6 h after hypoxia stress (Fig. 6a). In kidney, *HSP70* also showed a significant increase in H1 and H2 group. Moreover, *HSP70* transcripts in H2 group were also significantly higher than those in H1 group (Fig. 6c).

Expression of *HSP90* gene under different stocking density and dissolved oxygen

During 60-day crowding stress treatment, the transcripts of as*HSP90* in liver and spleen were increased gradually throughout the experiment, and reached the highest at day 60 (Fig. 7a, b). However, for kidney, as*HSP90* mRNA increased at first, then peaked at day 40 and finally decreased to a lower level at day 60 (Fig. 7c). With regard to stocking density, *HSP90* levels in liver and spleen in high stocking density increased significantly compared to those of other groups ($P < 0.05$). By comparison, significant increase of as*HSP90* in kidney was only found in high stocking density at day 40 ($P < 0.05$).

In the hypoxia stress experiment, the levels of *HSP90* transcript in H1 and H2 group showed significant increases in all the three tissues after hypoxia stress except the transcript of liver in H2 group 6 h after hypoxia (Fig. 8). In addition, *HSP90* transcripts in H2 group were also significantly higher than those in H1 group at 0.5 and 1 h after hypoxia. However, at 6 h after hypoxia, the highest transcript was found in H1 group.

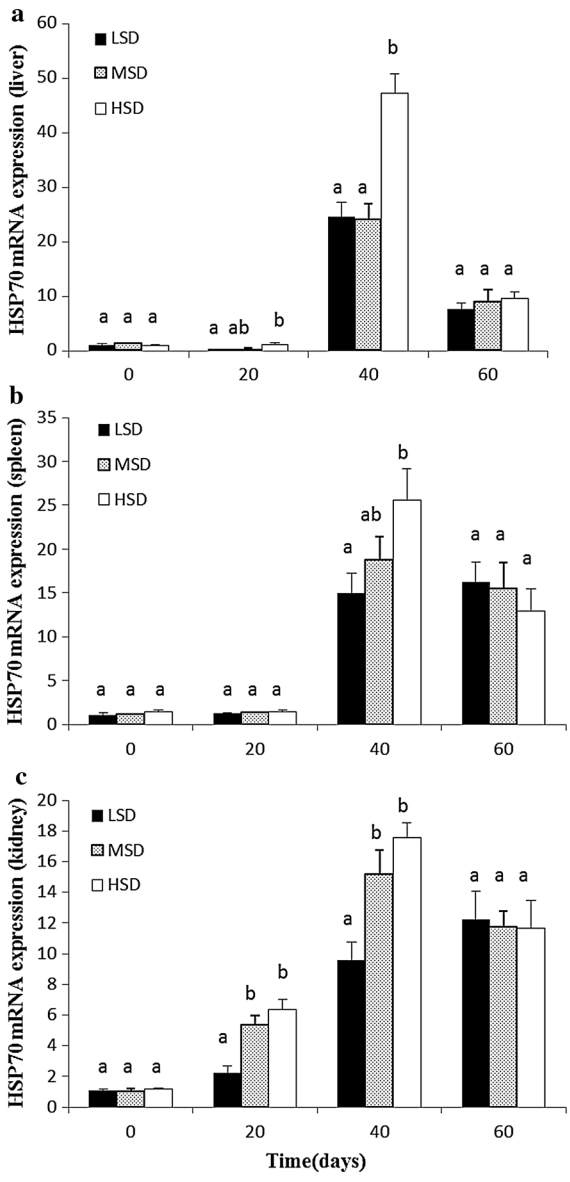


Fig. 5 mRNA expression of as*HSP70* in liver (**a**), spleen (**b**) and kidney (**c**) in different stocking densities. Expression of 18 s rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. *Different letters* in the same time point indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

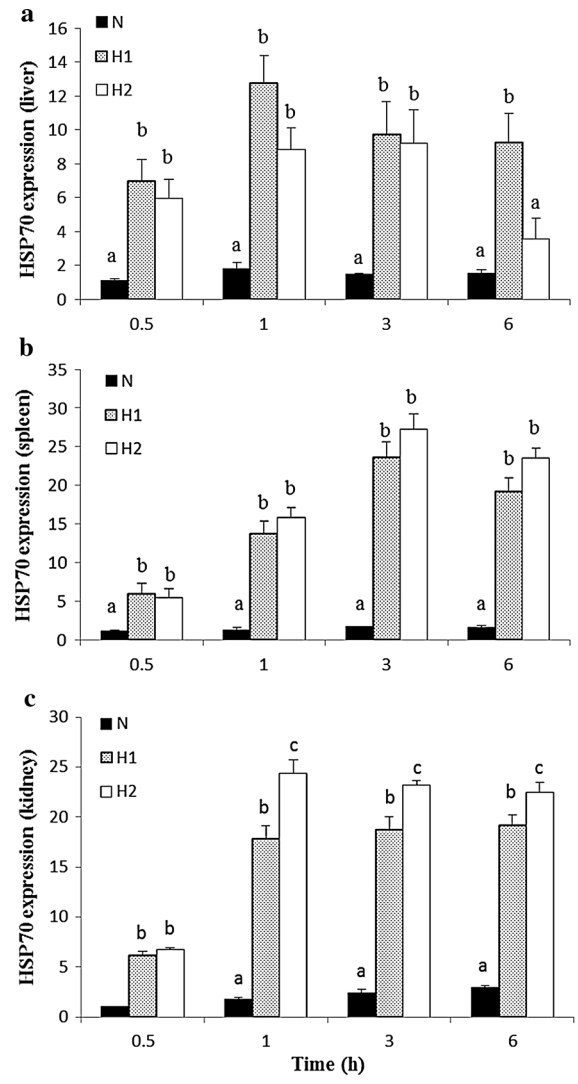


Fig. 6 mRNA expression of as*HSP70* in liver (**a**), spleen (**b**) and kidney (**c**) in different dissolved oxygen. Expression of 18 s rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. *Different letters* in the same time point indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

Levels of serum biochemical indexes under different stocking density and dissolved oxygen

Serum electrolytes in different stocking densities including calcium, magnesium and phosphorus are shown in Table 3. Serum calcium revealed an up-regulate trend with the increase of the stocking density. Similarly, the maximum inorganic phosphorus was found in high stocking density. However, the level of magnesium appeared to be stable during the whole stocking density experiment. In addition, serum globulin was significantly higher in high stocking density than those in other groups (Table 3).

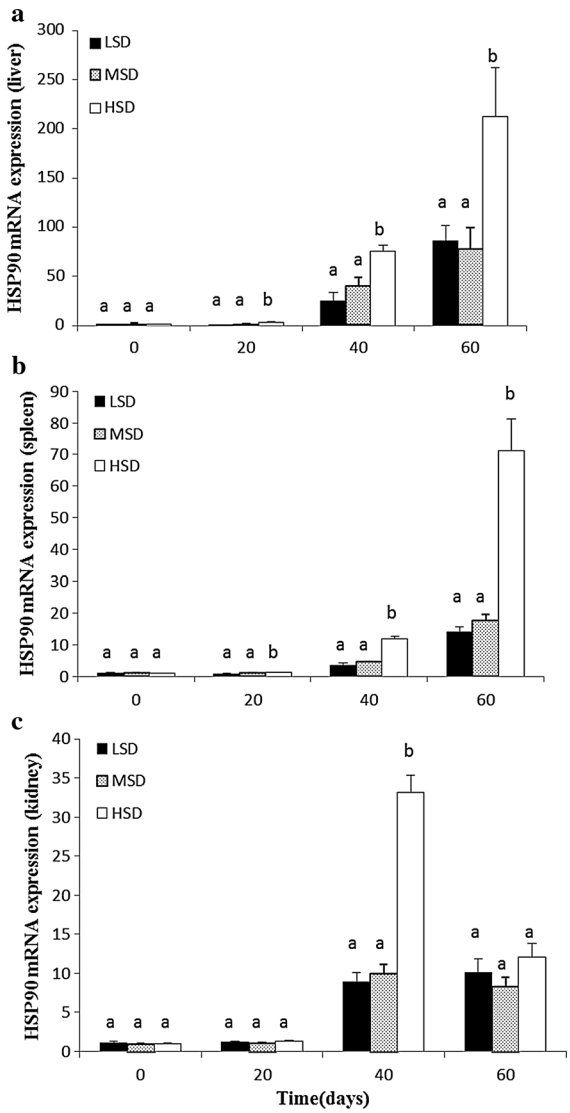


Fig. 7 mRNA expression of as*HSP90* in liver (**a**), spleen (**b**) and kidney (**c**) in different stocking densities. Expression of 18 s rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. *Different letters* in the same time point indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

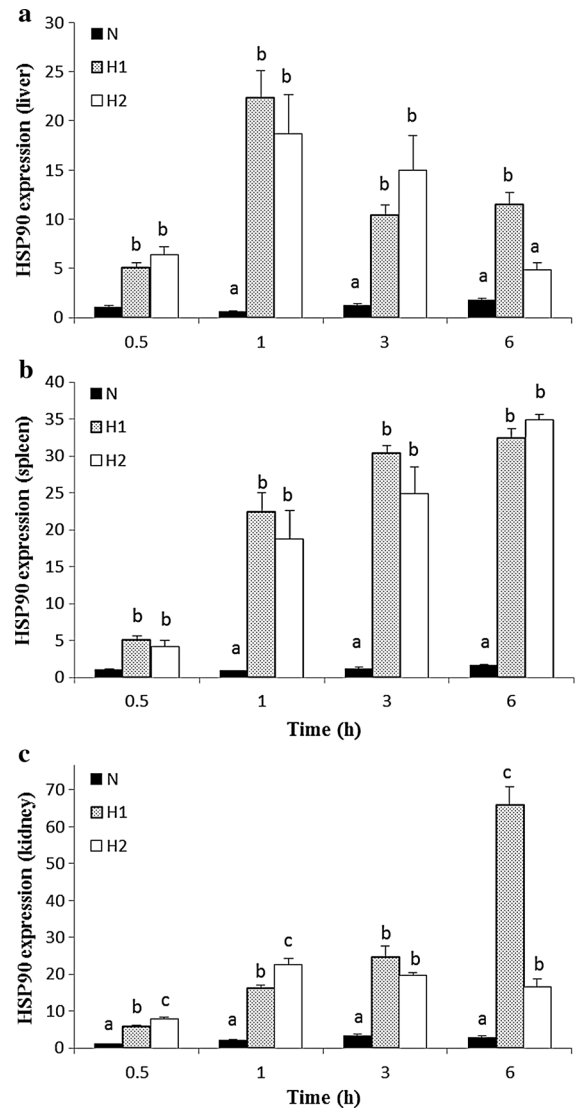


Fig. 8 mRNA expression of as*HSP90* in liver (**a**), spleen (**b**) and kidney (**c**) in different dissolved oxygen. Expression of 18 s rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. *Different letters* in the same time point indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

In the hypoxia stress experiment, serum calcium increased significantly at 1, 3 and 6 h in H2 group (Fig. 9a), while the inorganic phosphorus only increased at 6 h (Fig. 9b). There was no evident change in content of magnesium (Fig. 9c). Similar to stocking density, hypoxia stress caused a systemic rise in serum levels of globulin at 6 h (Fig. 10).

Discussion

As members of the stress-related protein, HSP70 and HSP90 are involved in resistance to environment stress. In this study, full lengths of as*HSP70* and as*HSP90* cDNA sequence were cloned from Amur sturgeon liver for the first time. The typical

Table 3 Levels of serum globulin and electrolytes (calcium, magnesium and phosphorus) of Amur Sturgeon in different stocking densities at the end of the experiment

Parameter	Stocking densities		
	LSD	MSD	HSD
Ca	0.85 ± 0.11^{a}	1.40 ± 0.02^{b}	1.63 ± 0.15^{b}
Mg	0.86 ± 0.09	0.86 ± 0.04	0.96 ± 0.06
P	3.50 ± 0.20^{a}	3.68 ± 0.09^{a}	4.30 ± 0.20^{b}
G	12.12 ± 0.85^{a}	12.12 ± 1.34^{a}	16.60 ± 1.06^{b}

Ca calcium, *Mg* magnesium, *P* phosphorus, *G* globulin. *LSD* low stocking density, *MSD* medium stocking density, *HSD* high stocking density

Data are presented as mean ± SEM. Different letters indicate statistical differences ($P < 0.05$) between groups

characteristics of HSPs also existed in as*HSP70* and as*HSP90*, including signature sequences and canonical features of the HSP family protein domains. Three signature sequences for HSP70 were identified in asHSP70 protein, which was the same as in *Phascolosoma esculenta* and *Sciaenops ocellatus* (Su et al. 2010; Dang et al. 2010). In addition, all five signature sequences existed in asHSP90 protein, which was same to typical characteristics of HSP90 (Gupta 1995). Moreover, the consensus amino acid sequences, EEVD and MEEVD located at the 3′-terminus, were identified in asHSP70 and asHSP90, respectively. Members of the HSPs family are highly conserved. Both HSP70 and HSP90 proteins shared remarkable homologies with their counterparts in other species. The highly conserved amino acid sequences between species may indicate that there are similar functions of HSPs in vertebrates and invertebrates (Alfredo et al. 2000).

The tissue expression analysis showed that the as*HSP70* and as*HSP90* were ubiquitously expressed in all the examined tissues suggesting that asHSPs were synthesized under unstressed conditions. In particularly, spleen and kidney are major lymphoid organs of fishes (Mulero et al. 2007). The high expression pattern of as*HSP70* and as*HSP90* in spleen and kidney indicated that as*HSP70* and as*HSP90* might be involved in immune response (Pockley 2003). The results were in agreement with previous studies in *Sebastes schlegeli* (Mu et al. 2013).

To maintain the internal environment and homeostasis, fish develop a defensive cellular stress response. The mechanism mainly depends on changes in

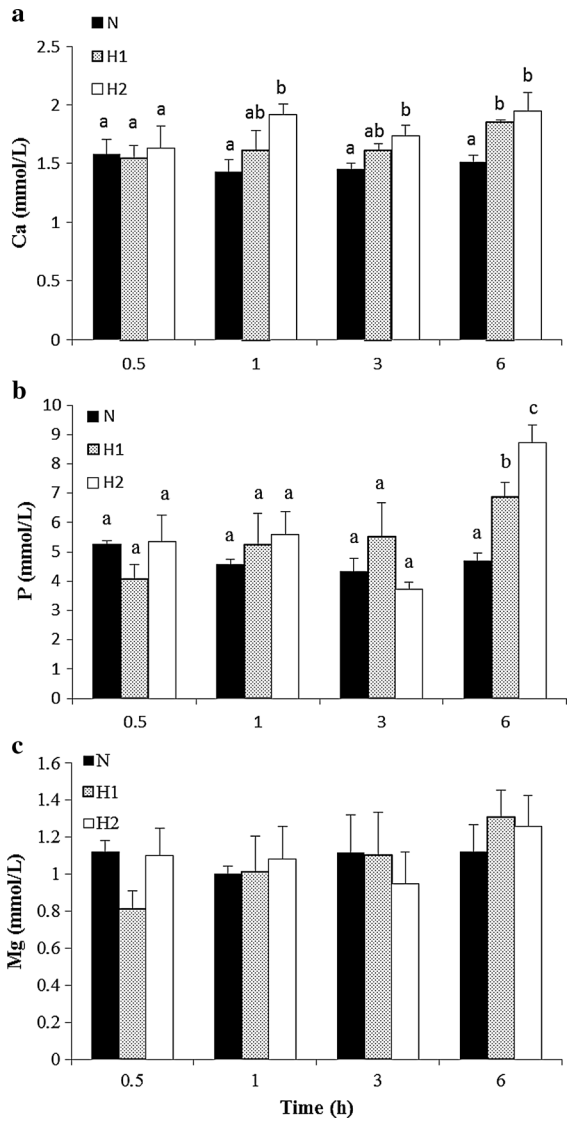


Fig. 9 Levels of serum electrolytes (calcium, inorganic phosphore and magnesium) of Amur sturgeon in different dissolved oxygen. Values are expressed as mean ± standard error of mean. *Different letters* in the same time point indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test). *Ca* calcium, *P* inorganic phosphore, *Mg* magnesium

proteins and genes in the stress process, such as the molecular chaperones *HSP70* and *HSP90* (Palmisano et al. 2000; Joly et al. 2010). As important aquaculture-related stress factors, both stocking density and hypoxia can activate this system.

HSP70 is an important protection factor in maintenance of homeostasis associated with stress conditions and immune reactions (Basu et al. 2002). Thus,

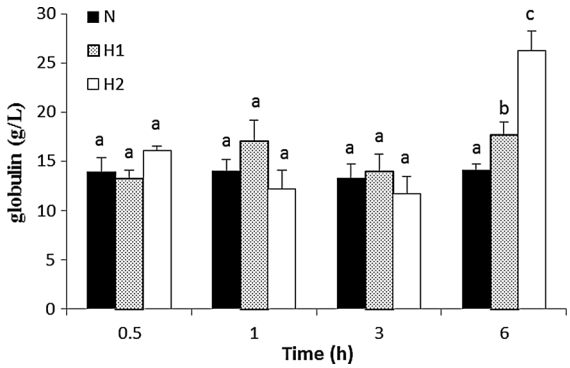


Fig. 10 Levels of serum globulin of Amur sturgeon in different dissolved oxygen. Values are expressed as mean ± standard error of mean ± standard error of mean. *Different letters* indicate significant differences ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple test)

we checked the expression levels of the *HSP70* upon different stocking density in liver, spleen and kidney. In present study, we observed a positive relationship between *HSP70* transcript and stocking density in liver, spleen and kidney suggesting that *HSP70* is an unspecific biomarker to the special species. Previous researches also observed over expression of *HSP70* in crowding environment (Gornati et al. 2004a; Caipang et al. 2008; Aksakal et al. 2011). Specifically, *HSP70* induction by stocking density was inhibited after 60 days of crowding. It is possible that Amur sturgeon developed a physiological adjustment to the long term of this environment.

HSP70 has also been identified to induce by hypoxia (Ton et al. 2002). In mammalian cardiac tissue, *HSP70* increases to almost three times baseline levels in response to ischemia (Marber et al. 1995). In fish, induced *HSP70* levels were also found under hypoxia stress (Lückstädt et al. 2004; Mu et al. 2013). However, in rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*), there were no elevated *HSP70* levels in response to hypoxia (Airaksinen et al. 1998; Gamperl et al. 1998). In present study, the mRNA expressions of the *HSP70* gene after hypoxia treatment were up-regulated in all of the liver, spleen and kidney tissues. We supposed that the up-regulated mRNA expression of as*HSP70* under hypoxia stress indicated that the as*HSP70* gene was inducible and involved in the immune response of Amur sturgeon.

HSP90 acts as a buffering system against the effects of random genetic variation and environmental change to ensure normal development (Picard 2002). In the current study, the levels of *HSP90* mRNA were significantly affected by stocking density and its duration. Comparing to as*HSP70*, as*HSP90* in liver and spleen did not showed inhibition after 60 days of crowding. The differences of these two HSPs to crowding stress may be caused by different functions in the cellular stress response. It is speculated that *HSP70* is primarily responsible for the recovery of stressor-induced protein abnormalities, through its chaperon function, whereas *HSP90* is crucial for cell signaling (Neckers and Ivy 2003).

Compared with the progress made in the study of *HSP70*, fewer reports about *HSP90* under hypoxia condition have been found in aquatic animals. Huang et al. (2014) reported as*HSP90* expression was up-regulated significantly at 4, 24 and 96 h post-hypoxia in the gills of *Haliotis diversicolor*. Li et al. (2009) found that *HSP90* was induced at 2 h and depressed at 8 h during hypoxia stress. Similar to the result of *HSP70*, as*HSP90* mRNA expression levels were up-regulated significantly in all liver, spleen and kidney after hypoxia stress (H1 and H2). We deduced that both as*HSP70* and as*HSP90* genes were inducible and played key roles in the immune response of Amur sturgeon under hypoxia stress.

Analysis of biochemical parameters could help to identify the general health status of animals and provide early warning signal in stressed organism (Agrahari et al. 2007). Plasma electrolytes are indicators of the secondary stress response in fish, providing an indirect indication of altered cortisol levels (Biswas et al. 2006; Roque et al. 2010). Previous study indicated that phosphorus and calcium levels were sensitive to fish stocking density (Hrubec et al. 2000). In the present study, phosphorus and calcium levels in high stocking density were much higher compared to fish in low stocking density. Similar differences in blood values were observed in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) raised in high stocking density (Hrubec et al. 1996). In hypoxia-stressed fish of this study, calcium and phosphorus increased rapidly, whereas magnesium concentration was not affected. It is reported that high globulin content in serum indicates a strong immune-competent ability (Li et al. 2012). In our study, serum globulin was significantly up-regulated in high stocking density and hypoxia condition (6 h). Globulin concentrations in fish from high stocking density and

hypoxia condition indicated an increase of immune function of Amur sturgeon.

In conclusion, the full-length sequences of *HSP70* and *HSP90* from the special chondr-ganoid scale fish, *Acipenser schrenckii*, were identified and characterized for the first time. The assessment of as*HSP70* and as*HSP90* mRNA levels under crowding and hypoxia stress indicated that the transcriptions of as*HSP70* and as*HSP90* were sensitive to crowding and hypoxia, so we deduced that as*HSP70* and as*HSP90* gene might good indicators of stressful situations for Amur sturgeon. Linking with the serum globulin and electrolytes levels, we suggest that both chronic (crowding) and acute (hypoxia) stressors could cause considerable stress for Amur sturgeon. These results of this study may provide useful information for further basic and application research in the immune function of Chondrostei under various stress conditions.

Acknowledgments This research was supported by Special Fund for Agro-scientific Research in the Public Interest (201003055).

References

Agrahari S, Pandey KC, Gopal K (2007) Biochemical alteration induced by monocrotophos in the blood plasma of fish *Channa punctatus* (Bloch). Pestic Biochem Physiol 88:268–272

Airaksinen A, Rabergh CMI, Sistonen L, Nikinmaa M (1998) Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. J Exp Biol 201:2543–2551

Aksakal E, Ekinci D, Erdoğan O, Beydemir S, Alım Z, Ceyhun SB (2011) Increasing stocking density causes inhibition of metabolic–antioxidant enzymes and elevates mRNA levels of heat shock protein 70 in rainbow trout. Livest Sci 141:69–75

Alfredo M, Frederic B, Ferenc M (2000) Cloning and expression analysis of an inducible *HSP70* gene from tilapia fish. FEBS Lett 474:5–10

Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. Gene 295:173–183

Bemis WE, Findeis EK, Grande L (1997) An overview of Acipenseriformes. Environ Biol Fishes 48:25–71

Biswas AK, Seoka M, Takii K, Maita M, Kumai H (2006) Stress response of red sea bream *Pagrus major to* acute handling and chronic photoperiod manipulation. Aquaculture 252:566–572

Bukau B, Horwich AL (1998) The *HSP70* and Hsp60 chaperone machines. Cell Pres 92:351–366

Caipang CMA, Brinchmann MF, Berg I, Iversen M, Eliassen R, Kiron V (2008) Changes in selected stress and immune–related genes in Atlantic cod, *Gadus morhua*, following overcrowding. Aquacult Res 39:1533–1540

Dang W, Hu Y, Zhang M, Sun L (2010) Identification and molecular analysis of a stress–inducible *HSP70* from *Sciaenops ocellatus*. Fish Shellfish Immunol 29:600–607

Deane EE, Woo NYS (2010) Growth hormone attenuates branchial *HSP70* expression in silver sea bream. Fish Physiol Biochem 36:135–140

Demand J, Lüders J, Höhfeld J (1998) The carboxy–terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. Mol Cell Biol 18(4):2023–2028

Douxfils J, Deprez M, Mandiki SN, Milla S, Henrotte E, Mathieu C, Silvestre F, Vandecan M, Rougeot C, Mélard C, Dieu M, Raes M, Kestemont P (2012) Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication–clues to physiological acclimation and humoral immune modulations. Fish Shellfish Immunol 33(5):1112–1122

Ellis T, North B, Scott AP, Bromage NR, Porter M, Gadd D (2002) The relationships between stocking density and welfare in farmed rainbow trout. J Fish Biol 61:493–531

Feder ME, Hofmann GE (1999) Heat–shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Gamperl AK, Vijayan MM, Pereira C, Farrell AP (1998) β-Receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. Am J Physiol 274:428–436

Gething MJ, Sambrook J (1992) Protein folding in the cell. Nature 355:33–45

Gornati R, Papis E, Rimoldi S, Terova G, Saroglia M, Bernardini G (2004a) Rearing density influences the expression of stress–related genes in sea bass (*Dicentrarchus labrax*, L). Gene 341:111–118

Gornati R, Terova G, Vigetti D, Prati M, Saroglia M, Bernardini G (2004b) Effects of population density on seabass (*Dicentrarchus labrax* L.) gene expression. Aquaculture 230:229–239

Gupta RS (1995) Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants and fungi species. Mol Biol Evol 12(6):1063–1073

Hrubec TC, Smith SA, Robertson JL, Feldman B, Veit HP, Libey G, Tinker MK (1996) Blood biochemical reference intervals for sunshine bass (*Morone chrysops* × *Morone saxatilis*) in three culture systems. Am J Vet Res 57:624–627

Hrubec TC, Cardinale JL, Smith SA (2000) Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis hybrid*). Vet Clin Pathol 29:7–12

Huang Y, Cai X, Zou Z, Wang S, Wang G, Wang Y, Zhang Z (2014) Molecular cloning, characterization and expression analysis of three heat shock responsive genes from *Haliotis diversicolor*. Fish Shellfish Immunol 36(2):590–599

Imorou T, Emile D, Fiogbe BK, Patrick K (2007) Rearing of African catfish (*Clarias gariepinus*) and vundu catfish (*Heterobranchus longifilis*) in traditional fish ponds (whedos): effect of stocking density on growth, production and body composition. Aquaculture 262:165–172

Joly AL, Wettstein G, Mignot G, Ghiringhelli F, Garrido C (2010) Dual role of heat shock proteins as regulators of apoptosis and innate immunity. J Innate Immun 2:238–247

Kiang JG, Tsokos GC (1998) Heat Shock Protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol Therapeut 80:183–201

Li F, Luan W, Zhang C, Zhang J, Wang B, Xie Y, Li S, Xiang J (2009) Cloning of cytoplasmic heat shock protein 90 (FcHSP90) from *Fenneropenaeus chinensis* and its expression response to heat shock and hypoxia. Cell Stress Chaperon 14(2):161–172

Li XF, Liu WB, Lu KL, Xu WN, Wang Y (2012) Dietary carbohydrate/lipid ratios affect stress, oxidative status and non–specific immune responses of fingerling blunt snout bream, *Megalobrama amblycephala*. Fish Shellfish Immunol 33:316–323

Lindquist S, Craig EA (1988) The heat–shock proteins. Annu Rev Genet 22:631–677

Livak KJ, Schmittgen TD (2002) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Lückstädt C, Schill RO, Focken U, Köhler HR, Becker K (2004) Stress protein HSP70 response of Nile Tilapia *Oreochromis niloticus* (Linnaeus, 1758) to induced hypoxia and recovery. Verh Ges Ichthyol Bd 4:137–141

Ludwig A (2008) Identification of Acipenseriform species in trade. J Appl Ichthyol 24:2–19

Ma KX, Chen GW, Liu DZ (2012) cDNA cloning of heat shock protein 90 gene and protein expression pattern in response to heavy metal exposure and thermal stress in planarian *Dugesia japonica*. Mol Biol Rep 39:7203–7210

Marber MS, Mestril R, Chi SH, Sayen R, Yellon DM, Dillmann WH (1995) Overexpression of the rat inducible 70-kD stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. J Clin Invest 95:1446–1456

Martinovic D, Villeneuve DL, Kahl MD, Blake LS, Brodin JD, Ankley GT (2009) Hypoxia alters gene expression in the gonads of zebrafish (*Danio rerio*). Aquat Toxicol 95:258–272

Ming JH, Xie J, Xu P, Liu WB, Ge XP, Liu B, He YJ, Cheng YF, Zhou QL, Pan LK (2010) Molecular cloning and expression of two *HSP70* genes in the Wuchang bream (*Megalobrama amblycephala* Yih). Fish Shellfish Immunol 28(3):407–418

Montero D, Izquierdo MS, Tort L, Robaina L, Vergara JM (1999) High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol Biochem 20:53–60

Mu W, Wen H, Li J, He F (2013) Cloning and expression analysis of a HSP70 gene from Korean rockfish (*Sebastes schlegeli*). Fish Shellfish Immunol 35(4):1111–1121

Mulero I, Garciae-Ayala A, Meseguer J, Mulero V (2007) Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: a mini review. Aquaculture 268:244–250

Neckers L, Ivy SP (2003) Heat shock protein 90. Curr Opin Oncol 15:419–424

Ni M, Wen H, Li J, Chi M, Bu Y, Ren Y, Zhang M, Song Z, Ding H (2013) The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. Fish Shellfish Immunol 36(2):325–335

North BP, Turnbull JF, Ellis T (2006) The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 255:466–479

Palmisano AN, Winton JR, Dickhoff WW (2000) Tissue-specific induction of *HSP90* mRNA and plasma cortisol response in chinook salmon following heat shock, seawater challenge, and handling challenge. Mar Biotechnol 2:329–338

Picard D (2002) Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 59:1640–1648

Pockley AG (2003) Heat shock proteins as regulators of the immune response. Lancet 362:469–476

Pratt WB (1997) The role of the *HSP90* based chaperone system in signal transduction by nuclear receptors and receptor signaling via map kinase. Annu Rev Pharmacol Toxicol 37:297–326

Rehulka J (2000) Influence of astaxanthin on growth rate condition and some blood indexes of rainbow trout *Oncorhynchus mykiss*. Aquaculture 190:27–47

Roque A, Yildiz HY, Carazo I, Duncan N (2010) Physiological stress responses of sea bass (*Dicentrarchus labrax*) to hydrogen peroxide (H_2O_2) exposure. Aquaculture 304:104–107

Saitou N, Nei M (1987) The neighbor–joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Salas-Leiton E, Anguis V, Martín-Antonio B, Crespo D, Planas JV, Infante C, Cañavate JP, Manchado M (2010) Effects of stocking density and feed ration on growth and gene expression in the Senegalese sole (*Solea senegalensis*): potential effects on the immune response. Fish Shellfish Immunol 28:296–302

Schram E, Van der Heul JW, Kamstra A, Verdegem MCJ (2006) Stocking density-dependent growth of Dover sole (*Solea solea*). Aquaculture 252:339–347

Schreck CB, Olla BL, Davis MW (1997) Behavioral responses to stress. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) Fish stress and health in aquaculture society for experimental biology seminar series, vol 62. Cambridge University Press, Cambridge, pp 145–170

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6:1025–1037

Su XR, Du LL, Li YY, Li Y, Zhou J, Li TW (2010) Cloning and expression of *HSP70* gene of sipuncula *Phascolosoma esculenta*. Fish Shellfish Immunol 28:461–466

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Ton C, Stamatiou D, Dzau VJ, Liew CC (2002) Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development. Biochem Biophys Res Commun 296:1134–1142

Tsan M, Gao B (2004) Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 286:739–744

Wallin RP, Lundqvist A, More SH, Von Bonin A, Kiessling R, Ljunggren HG (2002) Heat-shock proteins as activators of the innate immune system. Trends Immunol 23:130–135

Weigl E, Kopecek P, Raska M, Hradilova S (1999) Heat shock proteins in immune reactions. Folia Microbiol 44:561–566

Wu RS (2002) Hypoxia: from molecular responses to ecosystem responses. Mar Pollut Bull 45:35–45

Yang DG, Zhu YJ, Luo YP, Zhao JH, Chen JW (2011) Effect of stocking density on growth performance of juvenile Amur Sturgeon (*Acipenser schrenckii*). J Appl Ichthyol 27:541–544

Yue X, Liu B, Sun L, Tang B (2011) Cloning and characterization of a *HSP70* gene from Asiatic hard clam *Meretrix meretrix* which is involved in the immune response against bacterial infection. Fish Shellfish Immunol 30:791–799

Zhuang P, Kynard B, Zhang L, Zhang T, Zhang Z, Li D (2002) Overview of biology and aquaculture of Amur sturgeon (*Acipenser schrenckii*) in China. J Appl Ichthyol 18:659–664