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The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress

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ABSTRACT

Stocking density and hypoxia are considered priority issues in aquaculture research. In this study, two experiments were carried out in order to investigate the effects of chronic stress (stocking density) and acute stress (hypoxia) on the immune physiology responses (hematology, serum cortisol, glucose, total protein and the mRNA expression of *CYP 1A*) of juvenile Amur sturgeon (*Acipenser schrenckii*). In the chronic stress study, three triplicate groups of Amur sturgeon (42.0 ± 2.3 g) were reared in nine square concrete ponds (4.4 × 4.4 × 0.45 m^3) at three stocking densities (3.7, 6.9 and 9.0 kg/m^3) for 50 days. In the acute stress study, three triplicate groups: normal group (7 mg/l), hypoxia group 1 (5 mg/l) and hypoxia group 2 (3 mg/l) were used in nine 100 L indoor tanks. Sampling was performed at the end of the stocking density experiment (50 days) and at 0, 0.5, 1.5, 3 and 6 h after hypoxia stress. The results showed that increased stocking density reduced the morphological indexes (hepatosomatic index, spleen-somatic index and kidney-somatic index), while total protein and hemoglobin increased significantly in the stressed group. In response to hypoxia, the levels of cortisol, glucose and hematological parameters elevated significantly after this stress. As for spleen-somatic index, there was a decline after hypoxia though H1 group returned to the normal level at 3 h and 6 h after hypoxia stress. Additionally, In order to better understand the immune response of Amur sturgeon to chronic and acute stressors, we cloned the complete coding sequence of Amur sturgeon *CYP 1A* for the first time and investigated its tissue-specific expression and stress-induced expression. *CYP 1A* mRNA in liver showed over expressions both in crowding condition and in hypoxia stress. The same trend was also found in spleen and kidney which may provide evidence that *CYP 1A* could serve as a good indicator of immune response in Amur sturgeon. In addition, the result suggested a typical immune response both in high stocking density and hypoxia stress. But the chronically stressed fish might have an adaptation capability to survive under a stable crowding condition without a change in some immune parameters (cortisol, glucose, WBCs and RBCs).

1. Introduction

Sturgeons are notable because they are chonrdr-ganoid scale fish, with some characters between chondrichthyes and osteichthyes [1] and all living representatives of sturgeons are listed in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) [2,3]. At present, sturgeons have been raised in many countries for its considerable value in economy and ecology [4,5]. Amur sturgeon, *Acipenser schrenckii*, distributed throughout the Amur River (Heilongjiang River in Chinese) basin and in the Ussuri and Sungari River, has becoming one of the popular cultured species in China and accounting for 50% of the total sturgeon production in China [6]. Li reported the effect of stocking density on serum concentrations of thyroid hormones on this species [7]. Yang studied the growth performances of juvenile Amur sturgeon in different stocking density [8]. But little is known about the effect of acute and chronic stressors on the immune response of this species.

Stocking density and low dissolved oxygen are two major stressors in aquaculture. Stocking density is considered one of the most important variables in intensive aquaculture because it directly influences physiology, welfare and behavior of farmed fish [9–13]. Many former studies revealed that high density may impair the welfare of some fish species [11,14,15]. In contrast, null effects of

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high densities upon production were reported in wedge sole (*Dicologlossa cuneata*) and winter flounder (*Pseudopleuronectes americanus*) [16,17]. The pattern of these responses appears to be species specific. Respiration, whether by terrestrial or aquatic animals requires oxygen. For terrestrial animals oxygen in the environment is rarely limiting. But in the aquatic environment, oxygen is one relevant factor to consider. The ecological and physiological effects of hypoxia have been studied extensively in fishes [18,19]. In addition, low oxygen (in the present paper defined as hypoxia) has been shown to induce stress responses in fish [20–22]. These two adverse environmental factors might disturb the balance and harmony between fish and the environment, causing stress response in fish [23].

Spleen and kidney are major lymphoid organs of fishes [24,25]. The spleen functions as major secondary immune organ, as found in mammalian species, with abundant IgM^+ mature B cells [26–28]. It also plays a major role in the clearance of blood-borne antigens and immune complexes [29,30]. The spleno-somatic index (SSI) of fish is widely used as a simple measurable immune parameter with a potential role in immune response [31]. The entire kidney (including anterior kidney, mesonephric kidney and posterior kidney) is also a vital immune organ in fish. The head kidney/anterior kidney has the highest concentration of developing B lymphoid cells [26,28,32] and assumes hemopoietic functions [33,34]. In contrast, posterior kidney possesses both renal and immune function [27,28,35].

When subjected to a stressor, fishes employ a host of physiological adjustments to overcome the challenge [36]. One of the primary responses is mediated by the hypothalamus–pituitary–interrenal (HPI) axis, and is characterized by increased circulating levels of cortisol [37]. In fish, an increase in plasma cortisol has been widely employed as a quantitative measure of stress [38–40]. The changes of serum total protein and glucose are the secondary response to stress environment. Serum total protein content reflects the nutritional and metabolic status of fish, and indirectly reflects the level of non-specific immunity [41]. Serum glucose is also considered reliable markers of stress in animals such as brown trout, *Salmo trutta* and rainbow trout, *Oncorhynchus mykiss* [38,39].

The changes in levels of fish blood parameters will give an insight of fish's health status [42]. White blood cells in fish are considered to have immune functional role to play. Ellis found that, white blood cell count can be used to detect certain diseases and injury in fish body, as rearing environment can affect a number of circulating leukocytes in fish [43]. Rafatnezhad found that hemoglobin and red blood cell count may be influenced by stocking density [44].

The cytochromes P450 constitute a multigene family of enzymes involved in the oxidation of many endogenous and xenobiotics substrates [45]. Member of the cytochrome P450 gene family 1 (*CYP 1A*) is a useful biomarker in assessing contamination of the aquatic environment [46,47]. *CYP 1A* catalyze the activation of numerous hydrocarbon carcinogens and are induced by polynuclear and planar halogenated aromatic hydrocarbons and various natural compounds [48]. *CYP 1A* genes in non-mammalian taxa may be particularly important in evaluating the ecological or physiological pressures [49]. Gornati observed an increasing mRNA expression of *CYP 1A* in liver confirming that *CYP 1A* is an unspecific biomarker of stress response [50].

Due to the importance of stocking densities and hypoxia in commercial fish production, it is crucial to establish the physiological responses and the molecular mechanisms under these stressors in order to warrant an optimal welfare of Amur sturgeon. In the present study we cloned and characterized the complete coding sequence of Amur sturgeon *CYP 1A* gene for the first time. In addition, in order to establish the potential biological action of Amur sturgeon *CYP 1A*, the tissue-specific *CYP 1A* expression and the expression of *CYP 1A* in different tissues including spleen, kidney and liver in fish with different stress were detected. To better understand the immune response to acute and chronic stress we also determined the hematological indexes, serum cortisol and some serum biochemical parameters (glucose and total protein) of Amur sturgeon.

2. Methods and materials

2.1. Fish maintenance

The experiment was taken place in a commercial farm in Shandong province of China. Prior to the start of the experiment, fish were initially acclimated to experimental conditions in a square cement pool ($10 \times 10 \times 1$ m^3) for two weeks. During both the adaptation and experimental period, pelleted feed (Ningbo Tech-Bank, containing 42% of crude protein) was provided three times (8:00, 16:00, 23:00) daily at a feeding rate of 2% fish body weight per day.

2.2. Treatments and protocols

2.2.1. 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 = 9.0 kg/m^3, respectively). The experiment was conducted in 9 square concrete ponds ($4.4 \times 4.4 \times 0.45$ m^3) for a period of 50 days. At the end of the experimental period, 36 fish (4 from each tank) were captured, quickly anesthetized with tricaine methane sulfonate (MS-222) at a concentration of 200 mg/l, weighed and sampled within 20 min.

2.2.2. 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 1 group (H1, 3 mg/l) and hypoxia 2 group (H2, 1 mg/l). Nitrogen gas bubbling was used to quickly reduce the DO concentrations. Fish were euthanized, weighed (wet weight, liver weight, kidney weight and spleen weight) and tissue sampled prior to hypoxia stress (baseline levels in both groups) and at 0.5, 1, 3 and 6 h post–stress.

2.3. Blood and tissue sampling

Blood samples were taken gently from the caudal vein by 1–ml plastic syringe. Two different aliquots of blood were used for different analyses. The first aliquot was transferred to a plastic tube coated with EDTA-K2 as anticoagulant and was used for hematological indexes determination. A second aliquot was transferred to a plastic tube without EDTA-K_2, clot at 4 °C, centrifuged at 3000 g for 10 min, and then stored at −20 °C for subsequent analysis. Tissues were immediately frozen with liquid nitrogen and stored at −80 °C until mRNA analysis could be completed.

Biometric measurements including hepatosomatic index (HSI) = liver weight (g)/body weight (g) × 100, spleen-somatic index (SSI) = fresh spleen weight (g)/body weight (g) × 100 and kidney-somatic index (KSI) = kidney weight (g)/body weight (g) × 100 were also recorded from each fish.

2.4. Radioimmunoassay

Serum levels of cortisol in the serum of Amur sturgeon were measured using Iodine [I^{125}] Radioimmunoassay Kits (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino-US joint-venture

enterprise). The binding rate is highly specific with an extremely low cross reactivity to other naturally occurring steroids, which was less than 0.014% to most circulating steroids. The coefficients of intra-assay and inter-assay variation were 7.3% and 11.6%, respectively. Any samples with coefficient of variation higher than 10% were not included in the analyses. The assay sensitivity reached to 0.21 μg/dl by the kit protocol.

2.5. Biochemical and hematological assays

Serum biochemical parameters including glucose and total protein were determined with an automated biochemistry analyzer (Mindray BS180, China) according to Rehulka's method [51]. Hematological indexes including the numbers of white blood cells (WBCs), red blood cells (RBCs), neutrophilic granulocytes (NEUT) and hemoglobin values were measured using an automatic blood analyzer (Mindray, B1800, China).

2.6. Isolation and PCR amplification of CYP 1A cDNA

Total RNA was extracted from Amur sturgeon liver using RNAiso reagent (Takara, Japan) following the manufacturer's protocols. Then first-strand cDNA was synthesized respectively with 1 μg total RNA from each sample using random primers and Reverse Transcriptase M-MLV (Takara, Japan) in a 20 μl reaction. In order to isolate the *CYP 1A* cDNA from the Amur sturgeon, a 498 bp cDNA fragment of *CYP 1A* was first obtained from PCR amplification using specific primers (*CYP 1A*F1 and *P450R1*, Table 1). PCR reaction was carried out using PCR program as follows: 5 min denaturing step at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 64 °C and 40 s at 72 °C, followed by additional 10 min at 72 °C for extension. PCR product was then electrophoresed on a 1.5% agarose gel. The target cDNA fragments were purified using TIANgel midi Purification Kit (TIAGEN, China) then ligated into pGEM-T Vector (Promega, USA) and transformed into DH5α competent cells. Finally, recombinant plasmids were isolated and sequenced using the ABI3730XL sequencer to give at least 3-fold coverage.

3′ and 5′ ends RACE were performed using SMART™ RACE cDNA Amplification Kit (Clontech, USA). Gene specific primers (*CYP 1A* 5′ and *CYP 1A* 3′ listed in Table 1) were designed based on Amur sturgeon *CYP 1A* cDNA fragment. RACE was carried out according to the manufacturer's protocol. All DNAs subsequent to PCRs were gel purified and cloned, then sequenced as described above. BLASTN searches were used to verify gene identity.

2.7. Phylogenetic analysis and sequence analysis

To examine the similarity of Amur sturgeon *CYP 1A* to those of other species, eighteen *CYP 1A* amino acid sequences (Table 2) from various animals were retrieved from National Center for Biotechnology Information. Multi-sequences with deduced amino acid sequences for *CYP 1A* of Amur sturgeon were aligned using Clustal X (version 1.83) [52]. MEGA 4.0 software package [53] was used to construct and analyze phylogenetic tree using the Neighbor-Joining method with 1000 bootstrap trials [54]. Protein sequence analysis was performed with the ExPASy Molecular Biology Server (http://www.expasy.org/) scanning all known PROSITE motifs. Percent identities of proteins motifs between Amur sturgeon and other species were calculated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Table 1
Primer sequences for cloning and mRNA expression analysis.

Primer	Nucleotide sequence(5′ to 3′)	Application
*CYP 1A*F1	CTGAGGGACGAGTTTGGGCG	*CYP 1A* amplification
*CYP 1A*R1	GGTGAAGGGGAGGAAGGAGG	*CYP 1A* amplification
CYP 1A 5′	GAAGGAGCGGTAGTGGTCGG	5′ RACE
CYP 1A 3′	ATTGTGACCGACCACTACCGCT	3′ RACE
CYP 1A ex1	CAAGCAAGGGGAGGACTATG	RT-PCR and Q-PCR
CYP 1A ex2	CGGTGGAGAAAGAGCGAAG	RT-PCR and Q-PCR
*18S*F	GCCACACGAGATGGAGCA	Q-PCR
*18S*R	CCTGTCGGCGAAGGGTAG	Q-PCR

Table 2
Amino acid identities between *CYP 1A* in Amur sturgeon and *CYP 1A* in fish and other vertebrates.

Number	Species	Abbreviations	Accession number	Identities (%)
1	*Acipenser ruthenus*	*CYP 1A* 1	AEN19340.2	95
2	*Anguilla japonica*	*CYP 1A*	BAA88241.1	70
3	*Salmo salar*	*CYP 1A*	AAM00254.1	69
4	*Danio rerio*	*CYP 1A* 1	AAL54873.2	67
5	*Oncorhynchus mykiss*	*CYP 1A* 1	AAB40626.1	67
6	*Gobiocypris rarus*	*CYP 1A*	ABV01348.1	67
7	*Dicentrarchus labrax*	*CYP 1A*	CAB63650.1	67
8	*Paralichthys olivaceus*	*CYP 1A*	ABO38813.1	66
9	*Pseudopleuronectes americanus*	*CYP 1A*	ADV36120.1	66
10	*Takifugu obscurus*	*CYP 1A*	ABV24057.1	66
11	*Pelteobagrus fulvidraco*	*CYP 1A*	ABU62828.1	65
12	*Oreochromis niloticus*	*CYP 1A*	ACJ60906.2	64
13	*Balaenoptera acutorostrata*	*CYP 1A*	BAE46562.1	60
14	*Xenopus laevis*	*CYP 1A*	BAA37079.1	59
15	*Mus musculus*	*CYP 1A* 1	CAA68277.1	58
16	*Gallus gallus*	*CYP 1A* 5	NP_990477.1	58
17	*Corvus macrorhynchos*	*CYP 1A* 5	BAE75841.1	57
18	*Homo sapiens*	*CYP 1A* 1	NP_000490.1	55

2.8. PCR analysis

The expression profiles of *CYP 1A* mRNA in different tissues were studies through semi quantitative RT-PCR with specific-primers (*CYP 1A* ex1 and *CYP 1A* ex2, Table 1), including heart, liver, spleen, kidney, brain, pituitary, gill, stomach, intestine, fin and muscle from four fish. The expression levels of spleen, kidney and liver *CYP 1A* in fish exposed to acute and chronic stress were carried out on a LightCycler® 480 System (Roche, Switzerland) through quantitative PCR using SYBR Green (Takara, Japan) with specific-primers (*CYP 1A* ex1 and *CYP 1A* ex2, Table 1). PCR amplification of *18S* ribosomal RNA was applied as internal control to normalize the concentration of templates for Q-PCR. PCR reaction was optimized as follows: 30 s denaturing step at 94 °C, 35 cycles (for *CYP 1A*) or 25 cycles (for *18S* ribosomal RNA) of 30 s at 94 °C, 30 s at 58 °C (for *CYP 1A*) or 60 °C (for *18S* ribosomal RNA) and 30 s at 72 °C. In order to confirm the amplification of a single product per primer set, a melt curve analysis was performed at the end of the PCR run. After the program finished, the Ct values of the *CYP 1A* and *18S* genes were obtained from each sample. The relative expression level of gene could be calculated by $2^{-\Delta\Delta CT}$ method [55].

2.9. Statistical analyses

A one-way ANOVA with Duncan's test was used to test the effects of the treatments (SPSS 13.0). All data are presented as means ± standard error of mean (SEM), and a probability level of less than 0.05 was considered significant.

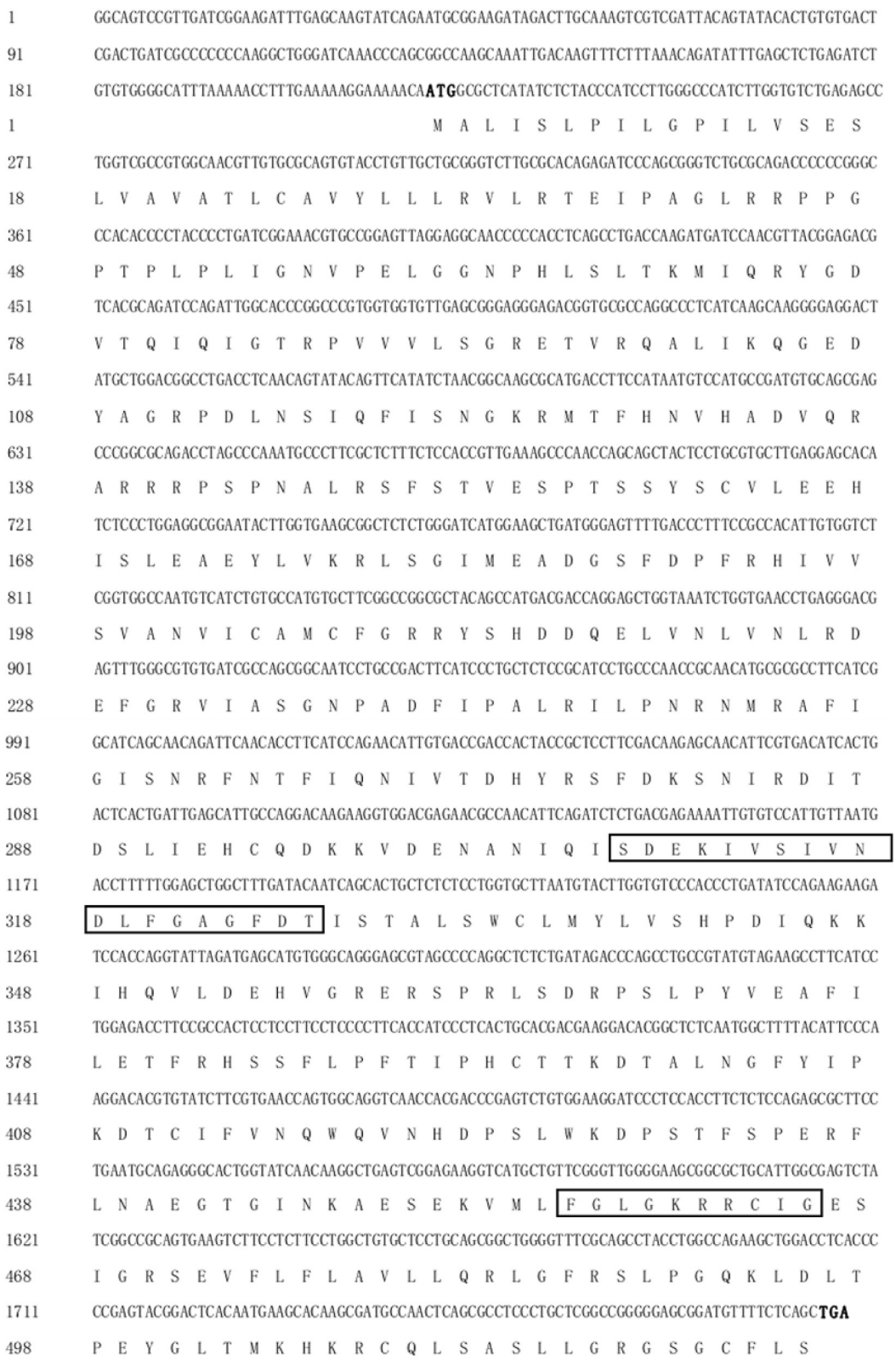

Fig. 1. The cDNA and deduced amino acid sequence of the *CYP 1A* gene in Amur sturgeon The initiation codon and termination codon were indicated in bold, the putative heme binding region was boxed, a highly conserved sequence among fish *CYP1A*s, 308-SDEKIVSIVNDLFGAGFDT-326 was also boxed, the nucleotides and amino acids were numbered along the left margin.

3. Results

3.1. Isolation and characterization of CYP 1A cDNA

The isolated cDNA of *CYP 1A* consisted of 1796 base pairs (bp), including the complete coding sequence (Fig. 1). The open reading frame (ORF) encoded a deduced protein of 525 amino acid residues with a predicted molecular mass of 58.79 kDa and theoretical isoelectric point of 8.57. The sequences have been submitted to GenBank with an accession number JX013935.2. From the results of BLAST in the National Center for Biotechnology Information, it was found that *CYP 1A* of Amur sturgeon exhibited high identity with those of other fishes. The deduced amino acid sequence of the sturgeon gene has a putative heme binding cysteine at position

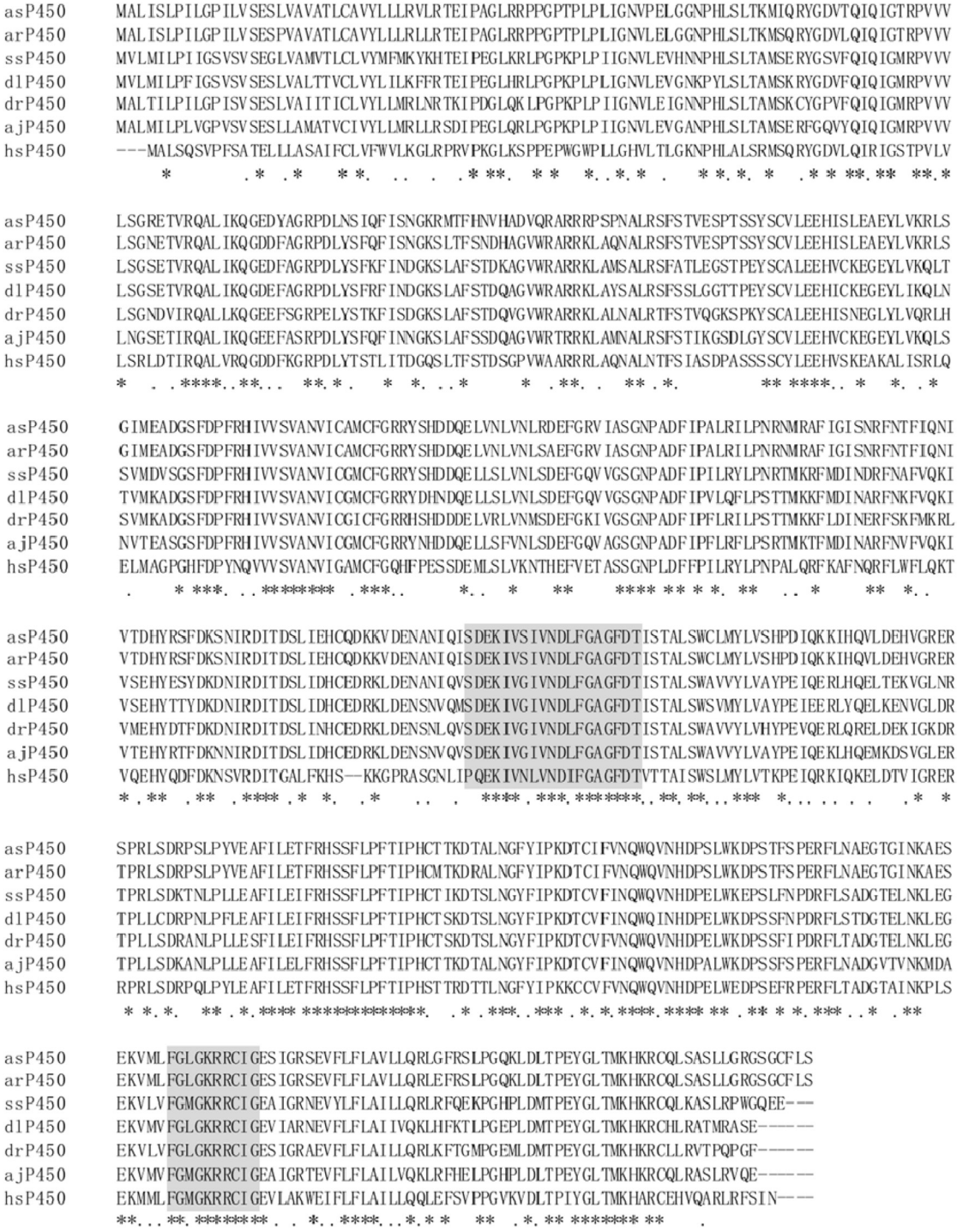

Fig. 2. Amino acid alignment of as*CYP 1A* with ar*CYP 1A*, ss*CYP 1A*, dl*CYP 1A*, dr*CYP 1A*, aj*CYP 1A* and hs*CYP 1A* (see materials and methods for sequence references and abbreviations). The symbols "(*)", "(:)", and "(.)" indicate identical residues, strongly conserved substitutions, and weakly conserved substitutions, respectively. as*CYP 1A* (*Acipenser schrenckii CYP 1A*), ar*CYP 1A* (*Acipenser ruthenus CYP 1A*), ss*CYP 1A* (*Salmo salar CYP 1A*), dl*CYP 1A* (*Dicentrarchus labrax CYP 1A*), dr*CYP 1A* (*Danio rerio CYP 1A*), aj*CYP 1A* (*Anguilla japonica CYP 1A*), hs*CYP 1A* (*Homo sapiens CYP 1A*).

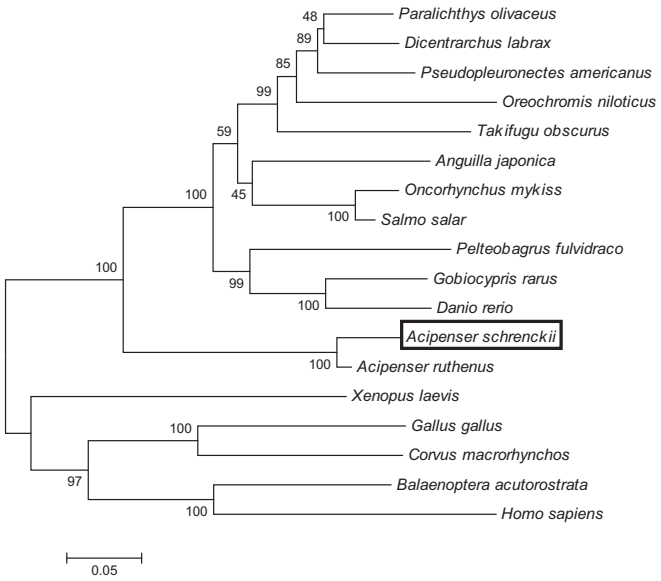

Fig. 3. Phylogenetic tree based on amino acid sequences for *CYP 1A* in vertebrates.

456, in a typical P450 signature sequence of FGLGKRRCIG. The sturgeon gene also has a sequence 308-SDEKIVSIVNDLFGAGFDT-326 that is very highly conserved among fish *CYP1A*s (Fig. 2).

3.2. Homology analysis

Phylogenetic analysis, based on deduced amino acids, was applied to determine the evolutionary position of Amur sturgeon *CYP 1A* (Fig. 3). As expected, Amur sturgeon *CYP 1A* was placed in the fish clade, closest related to Sterlet (*Acipenser ruthenus*) *CYP 1A*. The relationships displayed in the phylogenic tree were in agreement with the concept of traditional taxonomy, the sequence homology implicates that the *CYP 1A* are more similar to fish *CYP 1A* than the tetrapod *CYP 1A*. The results of ClustalW2, also showed that the deduced protein of *CYP 1A* in Amur sturgeon exhibited high identity with those of other fish. The comparison results of deduced amino acid sequences between Amur sturgeon *CYP 1A* and other similar *CYP 1A* was shown in Table 2. The amino acid sequences showed highest similarity (95%) to that in *A. ruthenus*. However, the homology was poorly conserved with terrestrial vertebrates: *Mus musculus* (58%), *Gallus gallus* (58%), *Corvus macrorhynchos* (55%) and *Homo sapiens* (55%).

3.3. Expression of CYP 1A in different tissues

The mRNA expression in different tissues was showed in Fig 4. *CYP 1A* mRNA was abundant in liver, and moderate in gill, kidney, heart and stomach. No *CYP 1A* mRNA was observed in adipose tissue, fin, muscle, pituitary, brain, intestines and spleen, but we could not exclude its presence. In addition, the levels of *18S* rRNA were used as an internal control and were found in all tissues studied at a similar intensity.

3.4. Effects of stocking density on Amur sturgeon

Table 3 showed the morphological indexes data in different stocking densities. At the end of the experiment, SSI varied inversely with regard to stocking density ($P < 0.05$). Similarly, the maximum HSI and KBI (3.39 ± 0.12 and 0.54 ± 0.03, respectively) were observed in LSD group ($P < 0.05$).

It is noticed that serum cortisol showed no significant difference among different stocking densities. Similarly, glucose was also not directly related to stocking density. However, TP in HSD group and MSD group were significantly higher than that in LSD group ($P < 0.05$) (Table 4).

Hematological indexes in different stocking densities including white blood cells, red blood cells and hemoglobin are showed in Table 5. The level of white blood cells as well as neutrophilic granulocytes appeared to be stable and there was no significant difference in different stocking densities. Red blood cells also showed no statistical difference among different stocking densities. However, hemoglobin revealed a significant increase in MSD group and HSD group.

At the end of the experiment, the liver *CYP 1A* mRNA expressions in HSD group and MSD group were significantly greater than that in LSD group ($P < 0.05$). Similar results were also found in spleen and kidney. But it's worth noting that crowding stress significantly induced *CYP 1A* mRNA expressions with spleen being more responsive (4.76–5.07 fold of control) than liver and kidney (Table 6).

3.5. Effects of hypoxia on Amur sturgeon

The morphological indexes data among different dissolved oxygen are compared in Fig. 5. Differences in spleno-somatic index were apparent. Spleno-somatic index in H1 and H2 group decreased significantly at 0.5 and 1.5 h after hypoxia stress. At 3 h and 6 h after hypoxia stress, the spleno-somatic index in H1 group returned to the normal group. However, spleno-somatic index in

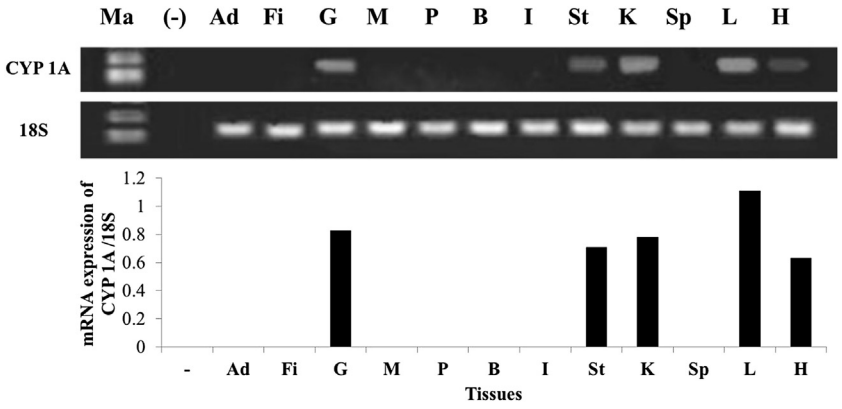

Fig. 4. RT-PCR expression analysis of *CYP 1A* in tissues of Amur sturgeon in normal condition PCR amplification of *18S* ribosomal RNA was used as an internal control for relative quantity of cDNA. Ad: adipose tissue; Fi: fin; G: gill; M: muscle; P: pituitary; B: brain; I: intestine; St: stomach; K: kidney; Sp: spleen; L: liver; H: heart; (−): mRNA not reversed transcribed; Ma: marker.

Table 3
Morphological indexes from Amur sturgeon reared in different stocking densities.

Parameter	LSD	MSD	HSD
SSI	0.27 ± 0.02b	0.21 ± 0.01a	0.20 ± 0.02a
KBI	0.54 ± 0.03b	0.43 ± 0.02a	0.45 ± 0.03a
HIS	3.39 ± 0.12b	2.54 ± 0.12a	2.46 ± 0.11a

Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test). LSD: low stocking density, MSD: medium stocking density, HSD: high stocking density, SSI: spleen-somatic index, KBI: kidney-somatic index, HSI: hepatosomatic index.

H2 group was significantly decreased 3 h and 6 h after hypoxia stress. Kidney-somatic index showed no significant difference either in H1 group or H2 group. There were no significant changes in hepatosomatic index in relation to the hypoxia stress, except for the 3 h after hypoxia stress which resulted significantly higher in the H2 group, compared to normal group and H1 group.

The levels of serum cortisol were significant increased after hypoxia stress except those at 6 h after hypoxia in H2 group, which showed no significant difference with the normal group (Fig. 6a). A significant increase of serum glucose levels occurred at 0.5 h, 1.5 h and 3 h after hypoxia stress in the H1 group and at 0.5 h and 1.5 h in H2 group. However, there was an obviously decline at 6 h after hypoxia stress in H1 group and 3 h after hypoxia stress in H2 group. No glucose concentration was detected at 6 h after hypoxia stress (Fig. 6b). Serum total protein exhibited no significant alteration except the final sampling point (6 h after hypoxia stress) which resulted significant increase with the decline of the dissolved oxygen content (Fig. 6c).

It is imperative to state that as not enough blood was sampled at 6 h after the hypoxia stress (Group H1 and H2) due to the bad condition of the fish, we failed to detect the hematological indexes at 6 h after this stress. The responses of white blood cells in different dissolved oxygen are showed in Fig. 7a, with significant increases at 1.5 h and 3 h after hypoxia stress in H1 group ($P < 0.05$). Similarly, white blood cells in H2 group showed significant increase at 0.5 h and 1.5 h after hypoxia stress. However, there is no significant difference 3 h after hypoxia stress in H2 group. The alterations of red blood cells were similar to those in white blood cells (Fig. 7b). Hemoglobin was significantly elevated during the hypoxia stress both in H1 and H2 group compared to the hemoglobin in normal group (Fig. 7c). Hypoxia significantly increased the numbers of neutrophilic granulocytes both in H1 and H2 group (Fig. 7d).

Fig. 8 showed the *CYP 1A* mRNA expression in different dissolved oxygen groups. The levels of *CYP 1A* mRNA expression in liver indicated a remarkable increase after hypoxia stress. However, there was a significant decrease at 6 h after hypoxia stress ($P < 0.05$). To our surprise, *CYP 1A* mRNA levels in H1 group were higher than those in H2 group (significant at 0.5 h and 6 h after hypoxia stress) (Fig. 8c). The changes of *CYP 1A* mRNA expression in kidney were similar to those in liver (Fig. 8a and b) with an up-regulation at 0.5 h and a down-regulation at 6 h after hypoxia stress. Remarkably, the increasing of *CYP 1A* mRNA expression in spleen was more sensitive (~30 fold at 3 h) than in liver (~9 fold at 3 h) and kidney (~5 fold at 3 h).

Table 4
Serum biochemical values (cortisol, glucose and total protein) in different stocking densities.

Parameter	LSD	MSD	LSD
Cortisol	0.29 ± 0.02	0.27 ± 0.04	0.26 ± 0.03
Glucose	1.32 ± 0.04	1.31 ± 0.19	1.31 ± 0.25
Total protein	15.48 ± 0.97a	25.85 ± 2.36b	23.03 ± 2.14b

Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test) LSD: low stocking density, MSD: medium stocking density, HSD: high stocking density.

Table 5
Hematological indexes from Amur sturgeon reared in different stocking densities.

Parameter	LSD	MSD	LSD
WBCs	200.04 ± 10.63	198.73 ± 11.10	183.95 ± 14.50
NEUT	92.43 ± 5.32	90.04 ± 7.83	91.04 ± 7.66
RBCs	0.17 ± 0.02	0.20 ± 0.03	0.22 ± 0.04
HGB	59.36 ± 1.15a	67.70 ± 2.95b	68.06 ± 3.75b

Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test). LSD: low stocking density, MSD: medium stocking density, HSD: high stocking density, WBCs: white blood cells, NEUT: neutrophilic granulocytes, RBCs: red blood cells, HGB: Hemoglobin.

4. Discussion

This study aimed to evaluate the effect of stocking density and hypoxia on the immuno-modulation in an endangered species, the Amur sturgeon. Barcellos found that acute and chronic stress lead to a decreased disease resistance in *Rhamdia quelen* Quoy and *Gaimard pimelodidae* [56]. Similar results were obtained by Salas-Leiton [57]. Fast reported the immune-related effects of Atlantic salmon (*Salmo salar*) subjected to short and long-term stress [22]. In addition, hypoxia has been shown to induce primary, secondary and tertiary stress responses in fish. Kvamme studied the effect of hypoxia on innate immune responses in Atlantic salmon [58]. But the appearance of immune parameters and defensive competence varies greatly between fish species, even between closely related species [59].

As found in mammalian species, the spleen as a secondary lymphatic and scavenging organ plays a vital role in hematopoiesis and antibody production processing [60]. In this study, spleno-somatic index decreased after hypoxia stress. The result showed a significant spleen contraction which might be related to hematopoiesis to improve oxygen carrying capacity in hypoxia condition. This hypothesis was further backed by rapid increases in red blood cells count observed in our study. Rapid increases in red blood cells count were also observed in hypoxia in juvenile Tambaqui (*Colossoma macropomum*) and silver trevally (*Pseudocaranx dantex*) [61,62]. Similarly, spleno-somatic index showed a decline with the increasing of the stocking density. Kidney is the main immunocompetent organ [63–65]. In addition, the kidney serves as an important immune–endocrine organ with key regulatory functions. Zwollo reported that head kidney has the highest concentration of developing B lymphoid cells [28]. In our research, stocking density negatively affected kidney somatic index. The result may suggest an immune response and/or physiological alteration exposed to this stressor. However, kidney somatic index showed no change in hypoxia condition.

Hepatosomatic index (HSI) has proved valuable as indicators of health status of fish cultured at different environment [66]. The

Table 6
CYP 1A mRNA expression in spleen, kidney and liver in different stocking densities.

Parameter	LSD	MSD	LSD
CYP 1A-S	1.03 ± 0.07a	4.76 ± 0.54a	5.07 ± 0.48a
CYP 1A-K	0.93 ± 0.11a	2.32 ± 0.35a	2.03 ± 0.31a
CYP 1A-L	1.07 ± 0.12a	1.78 ± 0.20b	1.93 ± 0.22b

Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test) LSD: low stocking density, MSD: medium stocking density, HSD: high stocking density, CYP 1A-S: *CYP 1A* mRNA expression in spleen, CYP 1A-K: *CYP 1A* mRNA expression in kidney, CYP 1A-L: *CYP 1A* mRNA expression in liver.

result of our study revealed that HSI in LSD group was significant higher than those in MSD and HSD groups indicating a high welfare status. The reduction of HSI by high stocking density has also been reported by previous studies [67,68]. With regard to hypoxia, HSI showed no significant difference except that at 3 h after hypoxia in H2 group. But we could not give a reasonable explanation for this phenomenon. Future studies are needed to focus on this mechanism.

Cortisol is an important stress hormone secreted from the hypothalamus–pituitary–interrenal axis (HPI) when fish is subjected to stress situation. The increase of cortisol in serum can be seen as the sensitive signal of fish stress [69]. It has been reported that serum glucose level might be up-regulated with the increasing of the level of cortisol [38,39]. The reason may be that cortisol can promote gluconeogenesis and glycogenolysis to meet the increasing demand of energy from fish under stress [69,70]. In our research, there were significant increase of serum cortisol and glucose levels during the period of hypoxia stress similar to observations in Siberian sturgeon (*Acipencer baeri*) and spotted wolffish (*Anarhichas minor*) [71,72]. This may indicated a mobilization of these metabolites to overcome the hypoxia disturbance. However, our results showed no significant differences among different stocking densities. It is generally believed that serum total protein reflects the level of non-specific immunity [41]. In our study, fish under crowding and hypoxia stress (6 h after hypoxia stress) showed a significant increase in the levels of the serum total protein. It is speculated that fish may increase specific proteins such as lysozyme or complement to enhance the immunity level to cope with stress [41,73].

White blood cells involve in defending the body against both infectious disease and foreign materials, playing an important role in the immune system [74]. An increase trend was observed after

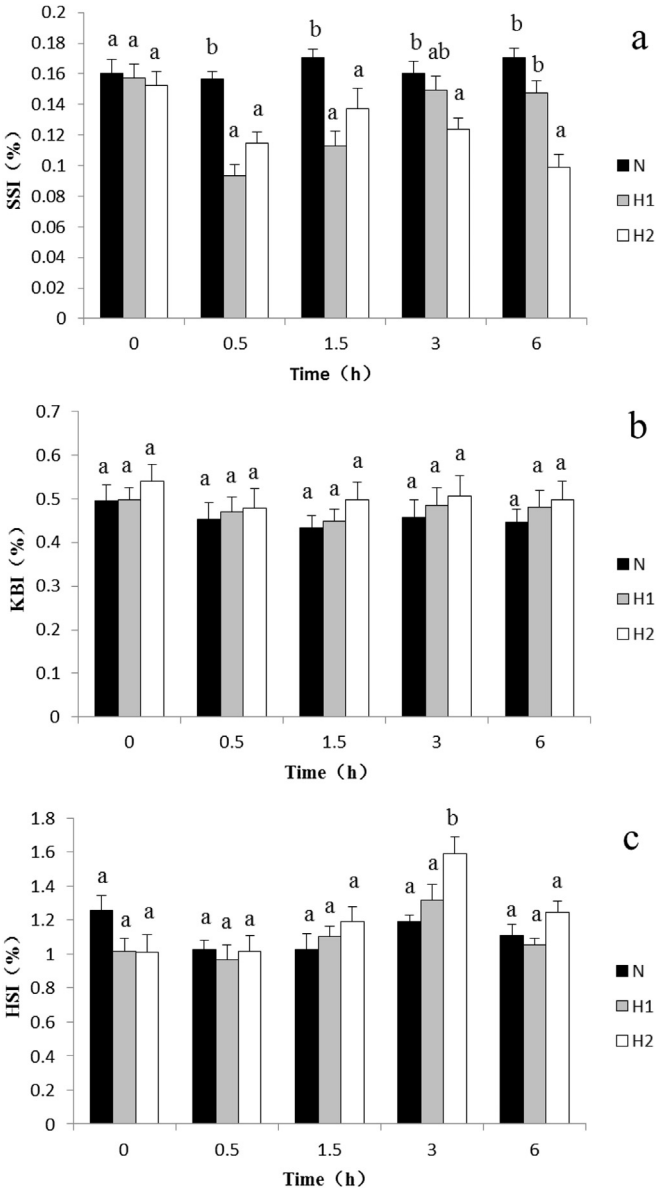

Fig. 5. Morphological indexes from Amur sturgeon exposed to different dissolved oxygen Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test). SSI: spleen-somatic index, KBI: kidney-somatic index, HSI: hepatosomatic index, N: normal group (6 mg/l), H1: hypoxia 1 group (3 mg/l), H2: hypoxia 2 group (1 mg/l).

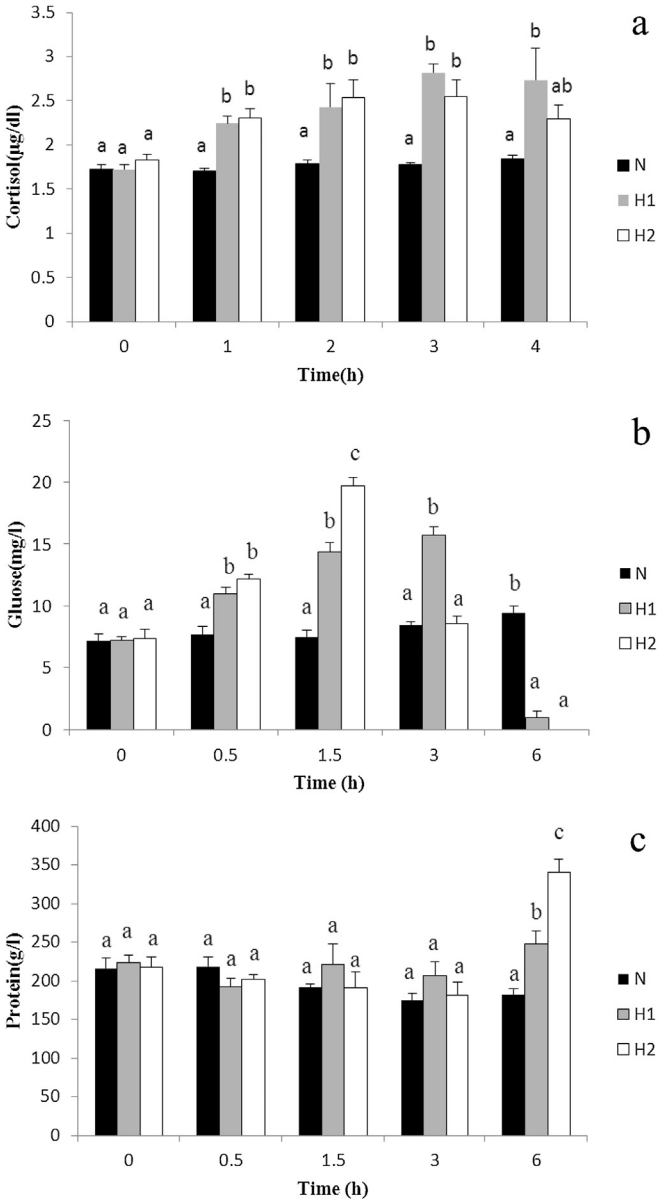

Fig. 6. Serum biochemical values (cortisol, glucose and total protein) from Amur sturgeon exposed to different dissolved oxygen Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test), N: normal group (6 mg/l), H1: hypoxia 1 group (3 mg/l), H2: hypoxia 2 group (1 mg/l).

hypoxia stress in this study which might be associated with an enhanced immune response. Red blood cells in our study also significantly elevated after hypoxia stress. The increase in the number of red blood cells may raise blood oxygen capacity and improves oxygen delivery to the tissues. This response has also been described in many studies [75,76]. However, in our study, neither white blood cells nor red blood cells showed significant alteration under different stocking densities. In our study, hemoglobin was significantly elevated during the hypoxia and crowding stress. Similarly, Qureshi reported that hemoglobin level in *Cyprinion watsoni* was significantly increased after hypoxia stress [77].

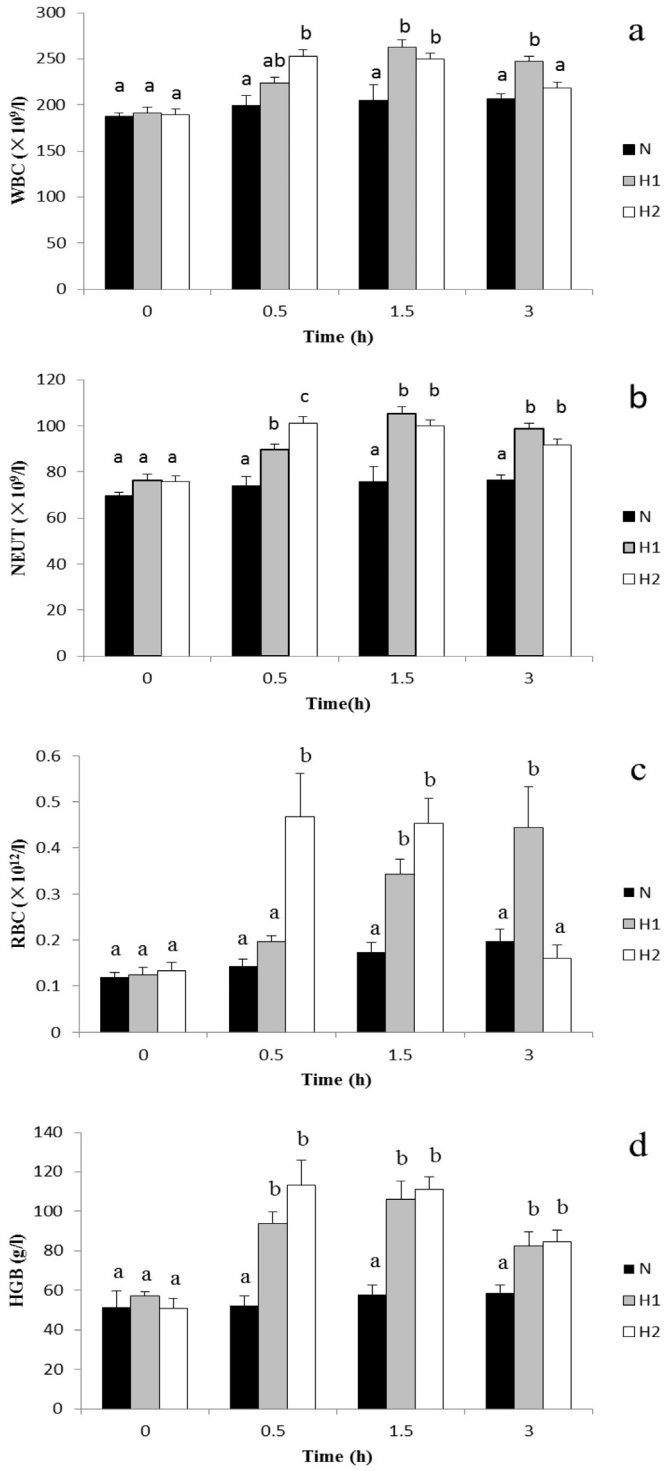

Fig. 7. Hematological indexes from Amur sturgeon in different dissolved oxygen Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test). WBCs: white blood cells, NEUT: neutrophilic granulocytes, RBCs: red blood cells, HGB: Hemoglobin, N: normal group (6 mg/l), H1: hypoxia 1 group (3 mg/l), H2: hypoxia 2 group (1 mg/l).

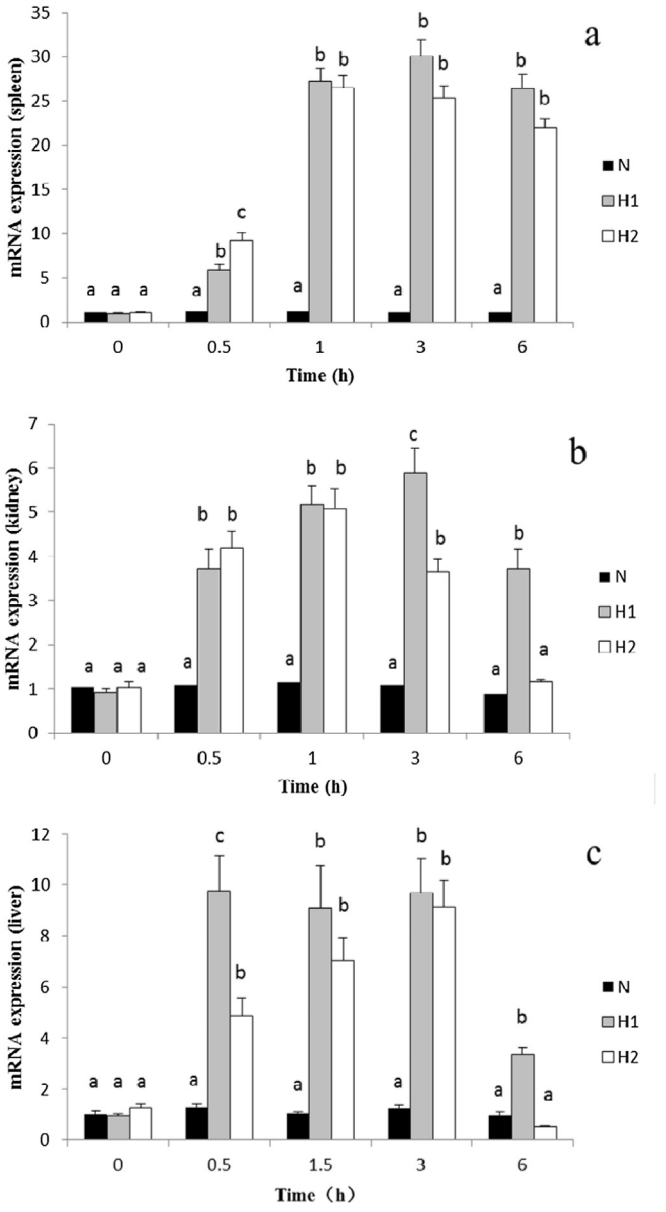

Fig. 8. *CYP 1A* mRNA expression of spleen (a), kidney (b) and liver (c) in different dissolved oxygen Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test), N: normal group (6 mg/l), H1: hypoxia 1 group (3 mg/l), H2: hypoxia 2 group (1 mg/l).

The cytochromes P450 gene superfamily consists of a large number of genes encoding P450 enzymes involved in the oxidation of many endogenous and xenobiotics substrates [44]. *CYP 1A* gene products catalyze the oxidation of environmental carcinogens and are therefore used as biomarker when assessing exposure to contaminants in environmental system [46,78]. In this study, we first cloned and characterized the complete CDS of *CYP 1A* in Amur sturgeon. Comparing the predicted amino acid sequence of *CYP 1A* with those of other species, we found all *CYP 1A* in fish shared higher identity, suggesting that *CYP 1A* was highly conservative during fish evolution.

Tissue distribution of *CYP 1A* mRNA was analyzed through semi quantitative RT-PCR. *CYP 1A* mRNA was strongly expressed in many tissues. The highest level of transcripts was found in liver. This result confirmed the expression pattern of *CYP 1A* reported in other fish species including zebrafish (*Danio rerio*), crucian carp (*Carassius carassius*) and Fugu obscurus (*Takifugu obscures*) [79–81]. The tissue-specific distribution of *CYP 1A* may indicate its physiological function for detoxification in fish.

In our study, we observed an increasing expression of *CYP 1A* in spleen, kidney and liver both in crowding environment and hypoxia stress suggesting that *CYP 1A* is an unspecific biomarker to the old species, Amur sturgeon. Gornati also observed an over expression of *CYP 1A* mRNA in crowding environment [50]. At the 100 μg/L BNF dose, hypoxia strongly increased *CYP 1A* expression in killifish embryos. However, hypoxia did not significantly influence *CYP 1A* expression in zebrafish embryos [82]. Rahman also reported a down-regulation of *CYP 1A* mRNA expression in Atlantic Croaker [83]. The differences seem to be related to fish species, fish size and the environment condition. It is worth noting that *CYP 1A* mRNA expression in hypoxia stress was significantly higher than that in crowding environment (in liver, 9 fold at 3 h after hypoxia stress and 1.78–1.93 fold in crowding stress, respectively). These results may suggested that the *CYP 1A* was more sensitive in acute stress (hypoxia).

In conclusion, the present study provides the first evidence that *CYP 1A* showed an up-regulation in some immune organs both in high stocking density and hypoxia stress which may provide an important implication that *CYP 1A* could serve as a good indicator of immune response in Amur Sturgeon. In addition, the integration of morphological indexes, hematological indexes, serum biochemical indexes and *CYP 1A* mRNA expression suggests a typical immune response both in high stocking density and hypoxia stress. However, the levels of some immune parameters (i.e. white blood cells, red blood cells and glucose) showed no significant changes in high stocking density. This may suggests that the chronically stressed fish might have an adaptation capability to survive under a stable crowding condition without a change in these immune parameters. To our knowledge, this is the first to study the immune response to acute and chronic stress in juvenile Amur sturgeon. It is imperative to conduct further studies on the influence of these stressors on other physiological aspect and provide a thorough understanding of the immune mechanisms of this species.

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References

[1] Wang D, Lu T, Liu H. Characterization and genetic diversity of the sturgeon *Acipenser schrenskii* Ig heavy chain. Immunobiology 2009;214(5):359–66.
[2] Bemis WE, Findeis EK, Grande L. An overview of Acipenseriformes. Environ Biol Fish 1997;48:25–71.
[3] Ludwig A. Identification of Acipenseriformes species in trade. J Appl Ichthyol 2008;24:2–19.
[4] Williot P, Sabeau L, Gessner J, Arlati G, Bronzi P, Gulyas T, et al. Sturgeon farming in Western Europe: recent developments and perspectives. Aquat Living Resour 2001;14:367–74.
[5] Wei Q, He J, Yang D, Zheng W, Li L. Status of sturgeon aquaculture and sturgeon trade in China: a review based on two recent nationwide surveys. J Appl Ichthyol 2004;20:321–32.
[6] Zhuang P, Kynard B, Zhang L, Zhang T, Zhang Z, Li D. Overview of biology and aquaculture of Amur sturgeon (*Acipenser schrenckii*) in China. J Appl Ichthyol 2002;18:659–64.
[7] Li D, Liu Z, Xie C. Effect of stocking density on growth and serum concentrations of thyroid hormones and cortisol in Amur sturgeon, *Acipenser schrenckii*. Fish Physiol Biochem 2012;38:511–20.
[8] Yang DG, Zhu YJ, Luo YP, Zhao JH, Chen JW. Effect of stocking density on growth performance of juvenile Amur Sturgeon (*Acipenser schrenckii*). J Appl Ichthyol 2011;27(2):541–4.
[9] Schreck CB, Olla BL, Davis MW. Behavioral responses to stress. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB, editors. Fish stress and health in aquaculture society for experimental biology seminar series, 62. Cambridge: Cambridge University Press; 1997. pp. 145–70.
[10] Montero D, Izquierdo MS, Tort L, Robaina L, Vergara JM. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol Biochem 1999;20:53–60.
[11] Ellis T, North B, Scott AP, Bromage NR, Porter M, Gadd D. The relationships between stocking density and welfare in farmed rainbow trout. J Fish Biol 2002;61:493–531.
[12] North BP, Turnbull JF, Ellis T. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2006;255:466–79.
[13] Schram E, van der Heul JW, Kamstra A, Verdegem MCJ. Stocking density–dependent growth of Dover sole (*Solea solea*). Aquaculture 2006;252:339–47.
[14] Lupatsch I, Santos GA, Schrama JW. Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. Aquaculture 2010;298:245–50.
[15] Yvette S, Wunderink SE, Silke H. Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): the involvement of cortisol, CRH and CRH–BP. Gen Comp Endocrinol 2011;171:203–10.
[16] Fairchild EA, Howell WH. Optimal stocking density for juvenile winter flounder *Pseudopleuronectes americanus*. J World Aquac Soc 2001;32:300–8.
[17] Herrera M, Vargas-Chacoff L, Hachero I, Ruíz-Jarabo I, Rodiles A, Navas JI. Physiological responses of juvenile wedge sole *Dicologoglossa cuneata* (Moreau) to high stocking density. Aquacult Res 2009;40:790–7.
[18] Pollock MS, Clarke LMJ, Dubé MG. The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environ Rev 2007;15:1–14.
[19] Douxfils J, Deprez M, Mandiki SNM, Milla S, Henrotte E, Mathieu C, et al. 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 2012;33(5):1112–22.
[20] Bernier NJ, Craig PM. CRF–related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. Am J Physiol Regul Integr Comp Physiol 2005;289:982–90.
[21] Welker TL, Lim C, Yildirim–Aksoy M, Klesius PH. Growth, immune function, and disease and stress resistance of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of bovine lactoferrin. Aquaculture 2007;262:156–62.
[22] Fast MD, Hosoya S, Johnson SC, Afonso LO. Cortisol response and immune–related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short– and long–term stress. Fish Shellfish Immunol 2008;24:194–204.
[23] Barton BA. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 2002;42(3):517–25.
[24] Press CMcL, Evensen O. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol 1999;9:309–18.
[25] Mulero I, Garcia–Ayala A, Meseguer J, Mulero V. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: a mini–review. Aquaculture 2007;268:244–50.
[26] Bromage ES, Kaattari IM, Zwollo P, Kaattari SL. Plasmablast and plasma cell production and distribution in trout immune tissues. J Immunol 2004;173:7317–23.
[27] Zwollo P, Cole S, Bromage E, Kaattari S. B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J Immunol 2005;174:6608–16.
[28] Zwollo P, Haines A, Rosato P, Gumulak–Smith J. Molecular and cellular analysis of B–cell populations in the rainbow trout using Pax5 and immunoglobulin markers. Dev Comp Immunol 2008;32:1482–96.
[29] Whyte SK. The innate immune response in finfish: a review of current knowledge. Fish Shellfish Immunol 2007;23:1127–51.
[30] Zabotkina EA. Comparative morphological characteristics of spleen of the perch *Perca fluviatilis* L from lakes with different water pH level. J Evol Biochem Physiol 2005;41:112–8.
[31] Lefebvre F, Mounaix B, Poizat G, Crivelli AJ. Impacts of the swim bladder nematode *Anguillicola crassus* on *Anguilla anguilla*: variations in liver and spleen masses. J Fish Biol 2004;64:435–47.
[32] Zwollo P, Mott K, Barr M. Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing B cell signatures. Dev Comp Immunol 2010;34:1291–9.
[33] Meseguer J, lópez–Ruiz A, García–Ayala A. Reticulo–endothelial stroma of the head–kidney from the seawater teleost gilthead seabream (*Sparus aurata* L): an ultrastructural and cytochemical study. Anat Rec 1995;241:303–9.
[34] Zapata A, Diez B, Cejalvo T, Gutierrez–de Frias C, Cortes A. Ontogeny of the immune system of fish. Fish Shellfish Immunol 2006;20:126–36.
[35] Zapata AG, Cooper EL. The immune system: comparative histophysiology. Chichester: John Wiley and Sons; 1990.
[36] Wendelaar Bonga SE. The stress response in fish. Physiol Rev 1997;77:591–625.
[37] Iwama GK, Afonso LOB, Vijayan MM. Stress in fishes. In: Evans DH, Claiborne JB, editors. The physiology of fishes. Boca Raton: CRC Press; 2005. pp. 320–42.

[38] Pickering AD, Pottinger TG, Christie P. Recovery of the brown trout, Salmo trutta L., from acute handling stress: a time–course study. J Fish Biol 1982;20:229–44.
[39] Van Raaij MTM, Pit DSS, Balm PHM, Steffens AB, Van den Thillart GEEJM. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Horm Behav 1996;30:85–92.
[40] Simontacchi C, Poltronieri C, Carraro C, Bertotto D, Xiccato G, Trocino A. Alternative stress indicators in sea bass *Dicentrarchus labrax*, L. J Fish Biol 2008;72:747–52.
[41] Ortuño J, Esteban MA, Meseguer J. Effects of short–term crowding stress on the gilthead seabream (*Sparus aurata* L) innate immune response. Fish Shellfish Immunol 2001;11:187–97.
[42] Harikrishnan R, Kim MC, Kim JS, Balasundaram C, Heo MS. Protective effect of herbal and probiotics enriched diet on haematological and immunity status of *Oplegnathus fasciatus* (Temminck & Schlegel) against *Edwardsiella tarda*. Fish Shellfish Immunol 2011;30:886–93.
[43] Ellis AE. Stress and the modulation of defence mechanisms in fish. In: Pickering AD, editor. Stress and fish. London: Academic Press; 1981. pp. 147–69.
[44] Rafatnezhad S, Falahatkar B, Gilani MHT. Effects of stocking density on haematological parameters, growth and fin erosion of great sturgeon (*Huso huso*) juveniles. Aquacult Res 2008;39:1506–13.
[45] Godard CAJ, Leaver MJ, Said MR, Dickerson RL, George S, Stegeman JJ. Identification of cytochrome P450 1B–like sequences in two teleost fish species (scup, *Stenotomus chrysopsand* plaice, *Pleuronectes platessa*) and in cetacean (striped dolphin, *Stenella coeruleoalba*). Mar Environ Res 2000;50:7–10.
[46] Williams DE, Lech JJ, Buhler DR. Xenobiotics and xenoestrogens in fish: modulation of cytochrome P450 and carcinogenesis. Mutat Res 1998;399:179–92.
[47] Mc Clain JS, Oris JT, Burton GA, Lattier D. Laboratory and field validation of multiple molecular biomarkers of contaminant exposure in rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 2003;22:361–70.
[48] Stegeman JJ, Hahn ME. Biochemistry and molecular biology of monooxygenases: current perspectives on multiplicity, functions and regulation of cytochrome P450 in aquatic species. In: Os–trander D, Malins D, editors. Biochemical, cellular and molecular biological perspectives in aquatic toxicology. Lewis Press; 1994. pp. 87–206.
[49] Miller HC, Mills GN, Bembo DG, Macdonald JA, Evans CW. Induction of cytochrome P4501A (CYP1A) in *Trematomus bernacchii* as an indicator of environmental pollution in Antarctica: assessment by quantitative RT-PCR. Aquat Toxicol 1998;44(3):183–93.
[50] Gornati R, Papis E, Rimoldi S, Terova G, Saroglia M, Bernardini G. Rearing density influences the expression of stress–related genes in sea bass (*Dicentrarchus labrax*, L). Gene 2004;341:111–8.
[51] Rehulka J. Influence of astaxanthin on growth rate condition and some blood indexes of rainbow trout *Oncorhynchus mykiss*. Aquaculture 2000;190:27–47.
[52] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[53] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.
[54] Saitou N, Nei M. The neighbor–joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.
[55] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real–time quantitative PCR and the $2^{-\Delta\Delta CT}$method. Methods 2001;25(4):402–8.
[56] Barcellos LJG, Luiz CK, Souza C, Rodrigues LB, Fioreze I, Rosmari MQ, et al. Hematological changes in jundiá (Rhamdia quelen Quoy and Gaimard pimelodidae) after acute and chronic stress caused by usual aquacultural management, with emphasis on immunosuppressive effects. Aquaculture 2004;237:229–36.
[57] Salas-Leiton E, Anguis V, Martín-Antonio B, Crespo D, Planas JV, Infante C, et al. 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 2010;28(2):296–302.
[58] Kvamme BO, Gadan K, Finne–Fridell F, Niklasson L, Sundh H, Sundell K, et al. Modulation of innate immune responses in Atlantic salmon by chronic hypoxia–induced stress. Fish Shellfish Immunol 2013;34(1):55–65.
[59] Schrøder MB, Villena AJ, Jørgensen T. Ontogeny of lymphoid organs and immunoglobulin producing cells in Atlantic cod (*Gadus morhua* L). Dev Comp Immunol 1998;22:507–17.
[60] Manning MJ, Nakanishi T. The specific immune system: cellular defences. In: Iwama G, Nakanishi T, editors. The fish immune system. New York: Academic Press; 1996. pp. 159–205.
[61] Affonso EG, Polez VLP, Correa CF, Mazon AF, Araujo MRR, Moraes G. Blood parameters and metabolites in the teleost fish *Colossoma macropomum* exposed to sulfide or hypoxia. Comp Biochem Physiol A 2002;133:375–82.
[62] Wells RMG, Baldwin J. Plasma lactate and glucose flushes following burst swimming in silver trevally (*Pseudocaranx dentex*: Carangidae) support the "releaser" hypothesis. Comp Biochem Physiol A 2006;143:347–52.
[63] Danneving BH, Lauve A, Press C, Landsverk T. Receptor–mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. Fish Shellfish Immunol 1994;4:3–18.
[64] Brattgjerd S, Evensen O. A sequential light microscopic and ultrastructural study on the uptake and handling of *Vibrio salmonicida* in the head kidney phagocytes of experimentally infected Atlantic salmon, *Salmo salar* L. Vet Pathol 1996;33:55–65.
[65] Kaattari S, Irwin MJ. Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertories. Dev Comp Immunol 1985;9:433–44.
[66] Grant ALM, Brown AR. A similarity hypothesis for shallow–cumulus transports. Q J Roy Meteor Soc 1999;125(558):1913–36.
[67] Leatherland JF, Cho CY. Effect of rearing density on thyroid and interrenal gland activity and plasma hepatic metabolite levels in rainbow trout, *Salmo gairdneri*, Richardson. J Fish Biol 1985;27:583–92.
[68] Vijayan MM, Ballantine JS, Leatherland JF. High stocking density alters the energy metabolism of brook charr, *Salvelinus fontinalis*. Aquaculture 1990;88:371–81.
[69] Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fisher 1999;9(3):211–68.
[70] Janssens PA, Waterman J. Hormonal regulation of gluconeogenesis and glycogenolysis in carp (*Cyprinus carpio*) liver pieces cultured in vitro. Comp Biochem Physiol A 1988;91(3):451–5.
[71] Lays N, Iversen MMT, Frantzen M, Jorgensen EH. Physiological stress responses in spotted wolfish (*Anarhichas minor*) subjected to acute disturbance and progressive hypoxia. Aquaculture 2009;295:126–33.
[72] Maxime V, Nonnotte G, Peyraud C, Williot P, Truchot JP. Circulatory and respiratory effects of an hypoxic stress in the Siberian sturgeon. Respir Physiol 1995;100:203–12.
[73] Ming J, Xie J, Xu P, Ge X, Liu W, Ye J. Effects of emodin and vitamin C on growth performance, biochemical parameters and two HSP70s mRNA expression of Wuchang bream (*Megalobrama amblycephala* Yih) under high temperature stress. Fish Shellfish Immunol 2012;32:651–61.
[74] Obach A, Quentel C, Laurencin FB. Effects of alpha-tocopherol acetate and dietary oxidized fish oil on the immune response of sea bass *Dicentrarchus labrax*. Dis Aquat Org 1993;15:175–85.
[75] Jensen FB, Weber RE. Internal hypoxia–hypercapnia in tench exposed to aluminium in acid water: effects on blood gas transport, acid–base status and electrolyte composition in arterial blood. J Exp Biol 1987;127:427–42.
[76] Soldatov AA. The effect of hypoxia on red blood cells of flounder: a morphologic and autoradiographic study. J Fish Biol 1996;48:321–8.
[77] Qureshi IZ, Hafeez MA, Shaikh SA. Hematological response of the fish, *Cyprinus watsoni*, to hypoxia, anemia and hyperthermia. Pak J Zool 1995;27:219–27.
[78] Williams TD, Lee JS, Sheader DL, Chipman JK. The cytochrome P450 1A gene (*CYP1A*) from European flounder (*Platichthys flesus*), analysis of regulatory regions and development of a dual luciferase reporter gene system. Mar Environ Res 2000;50:1–6.
[79] Jönsson ME, Jenny MJ, Woodin BR, Hahn ME, Stegeman JJ. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebrafish exposed to 3,3',4,4',5–pentachlorobiphenyl or 2,3,7,8–tetrachlorodibenzo–p–dioxin. Toxicol Sci 2007;100(1):180–93.
[80] Fu GH, Yang XL, Zhang HX, Yu WJ, Hu K. Effects of cytochrome P450 1A substrate (difloxacin) on enzyme gene expression and pharmacokinetics in crucian carp (hybridized Prussian carp). Environ Toxicol Phar 2011;31:307–13.
[81] Kima JH, Raisuddin S. Molecular cloning and β–naphthoflavone–induced expression of a cytochrome P450 1A (CYP1A) gene from an anadromous river pufferfish, Takifugu obscurus. Mar Pollut Bull 2008;57:433–40.
[82] McElroy A, Clark C, Duffy T, Cheng B, Gondek J, Fast M, et al. Interactions between hypoxia and sewage-derived contaminants on gene expression in fish embryos. Aquat Toxicol 2012;108:60–9.
[83] Rahman MS, Thomas P. Effects of hypoxia exposure on hepatic cytochrome P450 1A (CYP1A) expression in Atlantic croaker: molecular mechanisms of CYP1A down-regulation. PLoS ONE 2012;7(7):e40825.