Journal of Oceanology and Limnology
Vol. 36 No. 3, P. 956-972, 2018
https://doi.org/10.1007/s00343-018-7018-8

Stocking density affects the growth performance and metabolism of Amur sturgeon by regulating expression of genes in the GH/IGF axis*

REN Yuanyuan (任源远), WEN Haishen (温海深)**, LI Yun (李昀), LI Jifang (李吉方)

Key Laboratory of Mariculture (*Ocean University of China*), *Ministry of Education*, *Qingdao 266003*, *China*

Received Jan. 16, 2017; accepted in principle Mar. 9, 2017; accepted for publication Mar. 13, 2017

Abstract The effects of stocking density on the growth and metabolism of Amur sturgeon were assessed. Amur sturgeon were grown for 70 days at three different stocking densities (low stocking density, LSD: 5.5 kg/m^3; medium stocking density, MSD: 8.0 kg/m^3; and high stocking density, HSD: 11.0 kg/m^3), and the biometric index, muscle composition, and serum biochemical parameters were evaluated. In addition, pituitary, liver, and muscle samples were collected for gene cloning and expression analyses. After 70 days of growth, the fish maintained at HSD had significantly lower final body weight and specific growth rate, and a higher feed conversion ratio than those of the fish in the MSD and LSD groups. The HSD group had the lowest lipid and protein concentrations in serum and muscle. The serum cortisol concentration increased significantly in the HSD group, indicating that the stress-response system was activated in these fish. There was no change in the concentration of serum insulin-like growth factor 2 (IGF-2), while the concentrations of serum growth hormone (GH) and insulin-like growth factor 1 (IGF-1) decreased in the HSD group. The full-length cDNAs of *GH* and *IGF-2* genes (995-bp and 1 207-bp long, respectively), were cloned and analyzed. In the HSD group, the expressions of *GH* in the pituitary and growth hormone receptor (*GHR*) and *IGF-1* in the liver were down-regulated at the end of the 70-day experiment. In the HSD group, the transcript level of *IGF-2* significantly decreased in the liver, but did not change in muscle. Overall, our results indicated that a HSD negatively affects the growth performance and leads to changes in lipid and protein metabolism in Amur sturgeon. The down-regulated expression of genes related to the GH/IGF axis may be responsible for the poor growth performance of Amur sturgeon under crowding stress.

Keyword: Amur sturgeon; stocking density; growth; metabolism; growth hormone (GH)/insulin-like growth factor (IGF) axis

1 INTRODUCTION

The growth of fish is a complex physiological and metabolic process that is primarily controlled by the growth hormone (GH)/insulin-like growth factors (IGFs) axis (Moriyama et al., 2000; Beckman, 2011; de las Heras et al., 2015). Growth hormone and IGFs (including IGF-1 and IGF-2) are important hormones involved in many physiological processes and metabolic pathways related to somatic growth, development, metabolism, and reproduction (Reinecke et al., 2005; Salas-Leiton et al., 2010; de las Heras et al., 2015). The pituitary secretes GH, which circulates throughout the body and stimulates the synthesis and secretion of IGF-1 (Patel et al., 2005; Reindl and Sheridan, 2012). This process is mediated by binding of GH to the GH receptor (GHR), which is essential for the production and release of IGF-1 in the liver and other peripheral tissues (Kita et al., 2005; Tanamati et al., 2015). Then, IGF-1 regulates most of the somatotropic activities of GH such as cellular growth and differentiation (Mommsen, 2001; Otteson et al., 2002), DNA and protein synthesis (Duan, 1998), and lipid and carbohydrate metabolism (Moriyama et al., 2000; Reindl and Sheridan, 2012).

* Supported by the National Special Research Fund for Non-Profit Sector (Agriculture) (No. 201003055)
** Corresponding author: wenhaishen@ouc.edu.cn

Whereas much is known about the physiological functions of IGF-1, little is known about those of IGF-2. Several studies have reported the presence of IGF-2 in several teleost fish species such as *Pagrus auriga* (Ponce et al., 2008), *Dicentrarchus labrax* (Radaelli et al., 2008) and *Oreochromis niloticus* (Caelers et al., 2004). IGF-2 is initially translated as a preprohormone, consisting of a signal peptide, followed by B, C, A, D, and E domains in turn from the N-terminal (Jones and Clemmons, 1995; Ponce et al., 2008). It has been reported that IGF-2 may play a vital role in embryonic growth in mammals (DeChiara et al., 1990, 1991). In fish, *IGF-2* transcripts and/or the IGF-2 protein have been detected in larvae as well as in multiple juvenile and adult tissues (Caelers et al., 2004; Patruno et al., 2006; Ponce et al., 2008; Radaelli et al., 2008), suggesting that it plays important roles throughout the fish lifecycle.

The levels of circulating GH and IGFs and their mRNA transcripts in fish are considered to be markers of growth and physiological status (Dyer et al., 2004), which are directly or indirectly affected by various factors in aquaculture (e.g., temperature, salinity, photoperiod) (Gabillard et al., 2003; Deane and Woo, 2009; Mohammed-Geba et al., 2016), nutritional status (e.g., fasting, feeding, dietary composition) (Small and Peterson, 2005; Rolland et al., 2015; Tu et al., 2015), and other procedures related to fish culture (transport, manipulation, stocking density) (Rotlant et al., 2001; Wilkinson et al., 2006). For instance, *Oncorhynchus mykiss* had higher levels of *IGF-1* mRNA in muscle at 8°C than at 16°C (Gabillard et al., 2003). Prolonged starvation led to a significant decrease in circulating IGF-1 and IGF-2 in Atlantic salmon (Wilkinson et al., 2006) and reductions in hepatic *IGF-1* mRNA expression and pituitary *GH* mRNA expression in *Ictalurus punctatus* (Small and Peterson, 2005). Furthermore, dietary protein composition was found to be closely correlated with circulating GH and IGF-1 levels or the levels of their mRNAs in *Carassius auratus* (Tu et al., 2015), *O. niloticus* (Qiang et al., 2012), and rainbow trout (Rolland et al., 2015). Handling and confinement resulted in reduced levels of circulating GH in *Sparus aurata* (Rotlant et al., 2001), GH, IGF-1, and IGF-2 in *Salmo salar*, and IGF-1 and IGF-2 in rainbow trout (Wilkinson et al., 2006). Thus, it has been speculated that the GH/IGF axis plays extensive roles in fish under different environmental conditions and physiological status. However, few studies have analyzed the effect of stocking density and crowding stress on the levels of these hormones in plasma and on the transcript levels of their encoding genes.

Stocking density is a critical husbandry factor in intensive aquaculture. High stocking densities can cause chronic stress and affect the growth, welfare, and productivity of farmed fish (Herrera et al., 2012; Menezes et al., 2015; Ni et al., 2016). Several studies have indicated that inappropriate stocking densities negatively affect the growth performance of fish species including *S. salar* (Adams et al., 2007), *Arapaima gigas* (de Oliveira et al., 2012), *Scophthalmus rhombus* (Herrera et al., 2012), *Brycon insignis* (Tolussi et al., 2010), and *Argyrosomus regius* (Millán-Cubillo et al., 2016). The growth performance and the expression of GH in *Rhamdia quelen* significantly decreased with increased stocking density (Menezes et al., 2015), suggesting that crowding stress was closely related to *GH* expression. Furthermore, high stocking densities have been shown to negatively affect different metabolic pathways related to carbohydrate, lipid, and protein metabolism by activating stress responses in fish (Costas et al., 2008; Laiz-Carrión et al., 2012). For instance, Vargas-Chacoff et al. (2014) reported that *Eleginops maclovinus* maintained at a high stocking density had low levels of plasma glucose, triglyceride, and lactate. Inappropriate stocking density was also shown to alter the metabolism of triglycerides in *Salvelinus fontinalis* (Vijayan et al., 1990). However, low stocking densities can lead to higher production costs and lower profitability for the industry due to the inappropriate use of space (Millán-Cubillo et al., 2014). For these reasons, research on stocking density is attracting more attention. It is important to monitor the physiological responses and to explore the molecular mechanisms related to adaptation to crowding stress to ensure optimal health and welfare of farmed fish (Salas-Leiton et al., 2010).

Amur sturgeon (*Acipenser schrenckii* Brandt) is a riverine-resident species distributed in the Amur River. It has important economic value in the production of caviar and flesh; therefore, it has become an important breeding sturgeon species in China (Zhuang et al., 2002; Li et al., 2012). Previous studies have reported that stocking density affects the growth, physiology, and immunity of sturgeons (Jodun et al., 2002; Ni et al., 2014, 2016). However, there have been no reports on changes in the GH/IGF axis and how such changes modulate fish growth under crowding stress, or on *IGF-2* expression in sturgeon. The aim of this study was to elucidate the

effects of stocking density on the growth performance of Amur sturgeon and to explore the roles of GH, GHR, and IGFs in regulating growth under crowding stress. We cloned *GH* and *IGF-2* sequences from Amur sturgeon, measured the levels of GH, IGF-1, and IGF-2 in serum, and monitored changes in the transcript levels of *GH* in the pituitary and of *GHR*, *IGF-1*, *IGF-2* in the liver and muscle of fish grown under different stocking densities.

2 MATERIAL AND METHOD

2.1 Experimental procedures

Amur sturgeons (body weight, 225.46±32.28 g; length, 31.32±2.74 cm) were acquired from the Shandong Xunlong Fish Culture Farm (eastern China), where the trials were conducted. Before the experiments, the fish were maintained for 2 weeks in concrete ponds with continuously running water. Then, 2 700 fish were randomly selected and transferred to nine square concrete ponds (4.4 m× 4.4 m×0.45 m) at initial densities of 5.5 (low stocking density, LSD), 8.0 (middle stocking density, MSD) and 11.0 kg/m^3 (high stocking density, HSD) each with three replicates. The experimental ponds were supplied with continuous water flow (900 L/h) to maintain the water quality. The physicochemical parameters of the water in the ponds were maintained at satisfactory levels throughout the experiments: water temperature varied from 13.3°C to 17.2°C, dissolved oxygen (DO) from 7.2 to 9.6 mg/L, and pH from 7.8 to 8.3. During the 70-day experimental period, fish were fed three times a day with dry commercial feed (Ningbo Tech-Bank, Yuyao, China) at 1.5% (w/w) of total fish biomass. Body weight was measured (50 fish in each pond) and fish were sampled (6 per pond) on days 10, 30, 50, and 70. All fish were anaesthetized with 200 mg/L tricaine methane sulphonate (MS-222, Sigma, St. Louis, MO, USA) and sampled within 15 min. Blood samples were collected from the caudal vein, and the serum was obtained by centrifugation at 12 000×*g* for 10 min. Heart, liver, kidney, intestine, stomach, brain, pituitary, fin, gill, spleen, gonad, and muscle tissues were collected from fish from each group, immediately frozen in liquid nitrogen, and stored at -80°C until total RNA extraction.

2.2 Measurement of growth performance

At the beginning and end of the trial, the following biometric parameters were obtained: initial body length (IBL) (cm), initial body weight (IBW) (g), final body length (FBL) (cm), and final body weight (FBW) (g). Specific growth rate (SGR), daily weight gain (DWG), feed conversion rate (FCR), and condition factor (K) were calculated as follows: SGR (%)=100×(lnFBW–lnIBW)/time (d); DWG (g/(fish·d))=(FBW–IBW)/IBW/days×100; FCR=[weight of feed (g)/weight gain (g)]; K=weight/length^3×100.

2.3 Muscle composition analyses

Muscle samples of Amur sturgeon at different stocking densities (four fish per group) were analyzed to determine dry matter, crude lipid, crude protein, and ash (AOAC, 1995). Dry matter was determined by drying the muscle samples to constant weight at 105°C. Crude lipid was quantified by the Soxhlet ether extraction method using the Soxtec System HT6 (Tecator, Höganäs, Sweden). Crude protein was examined by the Kjeldahl method using an Auto Kjeldahl System (FOSS KT260, Hillerød, Denmark) and estimated by multiplying the nitrogen content by a factor of 6.25. Ash was measured using the combustion method in a muffle furnace at 550°C for 12 h.

2.4 Serum analyse

Blood samples (six fish per group) were collected from the caudal vein of fish and centrifuged to obtain serum for biochemical analyses. Serum samples were analyzed to determine the concentrations of glucose (GLU, mmol/L), total cholesterol (TC, mmol/L), triacylglycerol (TG, mmol/L), lactate (mmol/L), and total protein (TP, g/L) with commercial reagent kits according to their recommended protocols using an BS180 Automated Biochemistry Analyzer (ShenZhen Mindry Bio-Medical Electronicsco., LTD, Guangzhou, China).

The serum levels of cortisol and IGF-1 were assayed using Iodine [I^{125}] Radioimmunoassay (RIA) Kits (Tianjin Nine Tripods Medical & Bioengineering Co. Ltd., Tianjin, China) following the manufacturer's instructions. The assay sensitivity was 0.21 mg/dl. The inter-assay variation was 7.3% and the intra-assay variation was 11.6%. The GH and IGF-2 concentrations in fish serum were determined by enzyme-linked immunosorbent assay using a commercial kit (Nanjing Jiancheng Bioengineering Co. Ltd., Nanjing, China). Absorbance was measured spectrophotometrically at 450 nm. All samples were measured twice and the results shown are average values.

Table 1 Primer sequences used for gene cloning and mRNA expression analyses of *GH*, *GHR*, *IGF-1*, and *IGF-2*

Primer name	Sequence (5′→3′)	Description
GH-F	TCTGAGACCATCCCTGCTCCCACT	Amplification of cDNA fragment
GH-R	TACAACTTCCCATTGCTATGCCTTT	Amplification of cDNA fragment
IGF-2-F	GGTGGACGCCCTGCACTTAGTCTGTG	Amplification of cDNA fragment
IGF-2-R	GGAAGATAGCCTGCTCCTGGACCTACATAT	Amplification of cDNA fragment
GH 5′R	GTGAAAACACGGCTCAGGGAC	5′-RACE
GH 3′R	TCAACCTAAGAAACGATGATGC	3′-RACE
IGF-2 5′R1	TGGCTTTCAGGATAGATGGGAC	5′-RACE
IGF-2 3′R1	GGGGTCCCATCTATCCTGAA	3′-RACE
GH-e-F	TGGAGGAAGGCATTGTG	RT-PCR and qPCR primer
GH-e-R	AGCATCATCGTTTCTTAGGT	RT-PCR and qPCR primer
GHR-e-R	TGTGGGTGGAGTTCATAGAGC	RT-PCR and qPCR primer
GHR-e-R	GCAGTTGCCATTCAGGTTC	RT-PCR and qPCR primer
IGF-1-e-F	TCTTCAGTTTGTGTGTGGGG	RT-PCR and qPCR primer
IGF-1-e-R	GTGAGGATTTGGCTGGCTT	RT-PCR and qPCR primer
IGF-2-e-F	ACGAGGGGTCCCATCTATC	RT-PCR and qPCR primer
IGF-2-e-R	TGTAACGGGCTGTTTGCTT	RT-PCR and qPCR primer
18S-e-F	GCCACACGAGATGGAGCA	Reference primer
18S-e-R	CCTGTCGGCGAAGGGTAG	Reference primer

2.5 Molecular cloning of *GH* and *IGF-2* cDNAs

Total RNA was extracted from tissue samples using RNAiso reagent (TaKaRa, Otsu, Japan) following the manufacturer's protocol. The concentration and purity of RNAs were assessed using a Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing), and RNA integrity was confirmed by 1.5% agarose gel electrophoresis. Before first-strand cDNA was synthesized, gDNA was removed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Then, for each sample, first-strand cDNA was synthesized with 1 μg total RNA using random primers and Reverse Transcriptase M-MLV (TaKaRa) in a 20-μL reaction system.

To isolate the *GH* and *IGF-2* cDNAs, a pair of degenerate primers was designed for each gene (Table 1) based sequences of these genes from other species. Then, cDNA fragments of *GH* and *IGF-2* of Amur sturgeon were obtained by PCR amplification. The PCR reaction was performed in a final volume of 25 μL containing 1 μL cDNA, 2 μL 10 mmol/L dNTP mix, 2.5 μL reaction buffer, 0.5 μL each primer solution (Table 1), 0.2 μL Taq polymerase (TaKaRa), and 18.3 μL nuclease-free water. The PCR products were analyzed on a 1.5% w/v agarose gel and visualized using ethidium bromide staining. Putative gene fragments were cloned into the PEASY-T1 vector (Tiangen, Beijing, China), transformed into *Escherichia coli* (Trans5α, Transgen, Beijing, China) and sequenced with an ABI3730XL sequencer (Applied Biosystems, Foster City, CA, USA).

Based on the obtained *GH* and *IGF-2* cDNA fragments, gene-specific primers were designed to amplify the full-length cDNAs by PCR (Table 1). A SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA USA) was used for the 3′- and 5′-RACE reactions. The PCR products corresponding to the predicted length were isolated, purified, cloned into vectors, and sequenced as described above.

2.6 Sequence and phylogenetic analyses of *GH* and *IGF-2*

To examine the similarity of Amur sturgeon *GH* and *IGF-2* to those of other species, multiple sequence alignments of predicted amino acid sequences of GH and IGF-2 were produced using ClustalX version 1.83. Signal peptide regions, potential N-glycosylation sites, and other functional sites were predicted using SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/), ExPASy Molecular Biology Server (http://www.expasy.org/), and Gene Runner version 3.02 (Hasting Software, Hasting, USA). Phylogenetic analyses based on *GH* and *IGF-2* amino acid sequences were conducted using the neighbor-joining

Table 2 Growth parameters of Amur sturgeon after 70 days of growth at different stocking densities

Item	Experiment treatments		
	LSD	MSD	HSD
Initial body weight (IBW, g)	229.17±9.40	220.83±3.50	227.50±4.13
Final body weight (FBW, g)	407.22±7.52^{a}	383.33±5.00^{b}	364.45±6.31^{c}
Initial body length (IBL, cm)	31.70±1.47	30.98±1.16	31.29±1.47
Final body length (FBL, cm)	39.75±1.47^{a}	38.89±1.56^{ab}	38.20±0.88^{b}
Specific growth rate (SGR, %/d)	0.82±0.03^{a}	0.79±0.02^{a}	0.67±0.02^{b}
Daily weight gain (DWG, g/(n·d))	2.54±0.12^{a}	2.32±0.07^{a}	1.96±0.09^{b}
Feed conversion ratio (FCR)	1.05±0.05^{a}	1.15±0.03^{a}	1.38±0.07^{b}
Condition factor (*K*, g/cm^{3})	0.70±0.04^{a}	0.67±0.03^{a}	0.61±0.02^{b}

LSD: low stocking density; MSD: middle stocking density; HSD: high stocking density. SGR: specific growth rate (%)=100×(lnFBW–lnIBW)/time (d); DWG: daily weight gain (g/(fish·d))=(FBW–IBW)/IBW/days×100; FCR: feed conversion ratio=*F*/*n*(FBW–IBW), *n*=number of fish; condition factor (*K*)=weight/length^{3}×100; data are means±SEM. Values not sharing a common letter are significantly different (P<0.05).

method with MEGA 6.0 software using 1 000 bootstrap replicates.

2.7 Tissue distribution patterns of *GH* and *IGF-2* transcripts

The expression patterns of *GH* and *IGF-2* mRNAs in various tissues of Amur sturgeon were examined by RT-PCR using 18S ribosomal RNA (*18S*) as the internal control. Table 1 lists the primers used for these analyses. Total RNAs were extracted from heart, liver, kidney, spleen, stomach, intestine, gonad adipose tissue, fin, gill, muscle, brain, and pituitary tissues using RNAiso reagent (TaKaRa). The PCR cycling conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 5 s, 60°C for 35 s, and 72°C for 30 s; and 72°C for 10 min. The PCR products were checked on a 1.5% agarose gel, pre-stained with ethidium bromide, and visualized using the ChampGel 5000 gel imaging and analysis system (Beijing Sage Creation Science Co. Ltd., Beijing, China).

2.8 Quantitative real-time PCR analyses of genes related to GH/IGF axis

The transcript levels of *GH*, *GHR*, *IGF-1*, and *IGF-2* in various tissues of fish were determined by quantitative real-time PCR (qRT-PCR). The primers were designed based on the cloned sequences or those in Genbank using Primer5 software (Premier Biosoft International) (Table 1). All the cDNA products were diluted to 250 ng/μL. Each 20-μL qRT-PCR reaction mixture consisted of 2 μL template cDNA, 10 μL SYBR®FAST qPCR Master Mix (2×), 0.4 μL each primer, 0.4 μL ROX, and 6.8 μL nuclease-free water. The PCR amplifications were performed using the StepOne Plus Real-Time PCR system (Applied Biosystems) under the following conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, annealing at 55°C (for *GH*, *GHR*) or 60°C (for *IGF-1*, *IGF-2*) for 15 s, and final extension at 72°C for 2 min. Serially diluted cDNA samples from the control were used to calibrate the results. The relative mRNA levels of genes were normalized by the comparative $2^{-\Delta\Delta Ct}$ method using 18S as the internal control.

2.9 Statistical analysis

All data were determined by SPSS 17.0 and are expressed as mean values±standard error. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests were used to analyze the experimental data. Significant differences were accepted when P<0.05.

3 RESULT

3.1 Effects of stocking density on growth performance

The body weight of fish in all three treatments increased during the 70-day experiment (Fig.1a). At days 10 and 30, the average body weight of sturgeon did not differ significantly among the three stocking densities. However, at day 70, the FBW was highest in the LSD group, followed by the MSD and then the HSD group. A similar trend was observed for SGR. Fish in the HSD group had significantly lower SGR than those of fish in the LSD and MSD groups after 50 and 70 days of growth.

Table 2 summarizes the growth parameters of sturgeons after 70 days of culture. The initial stocking density significantly affected growth performance. The FBW, FBL, SGR, DWG, and K were significantly lower in the HSD group than in the LSD group at day 70. However, the FCR was significantly higher in the HSD group than in the LSD and MSD groups at day 70.

3.2 Serum biochemistry

The serum glucose concentration was positively related to stocking density (LSD < MSD < HSD), with maximum values in the HSD group (Table 3). The concentrations of cholesterol, triglycerides, and total protein in serum showed the opposite trend, with significantly lower levels in the HSD group than in the LSD group after 70 days of culture.

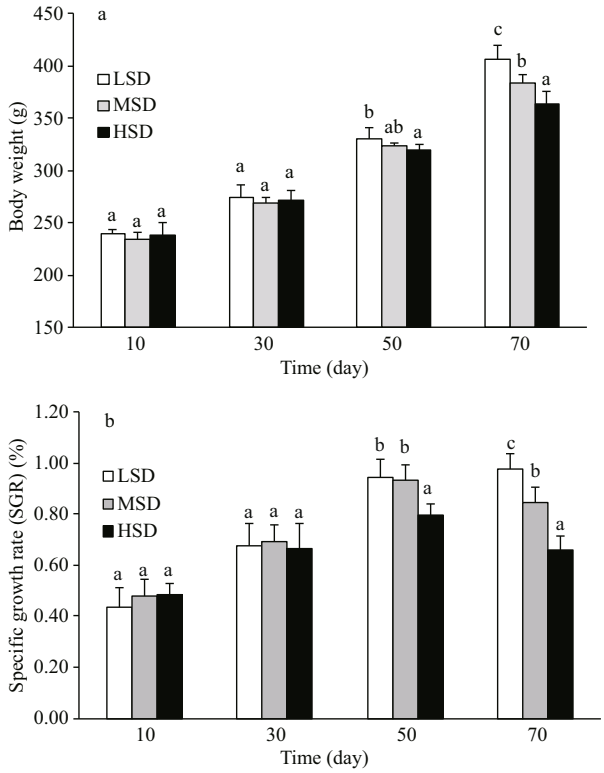

Fig.1 Changes of body weight (a) and specific growth rate (b) in Amur sturgeon cultured at different stocking density during 70 days

Data are presented as mean±SEM for each density tested (n=50). Values not sharing a common letter are significantly different. (P<0.05).

The lactate concentration did not differ significantly among the three groups. The serum cortisol level increased during the first 50 days, and then decreased until day 70. The serum cortisol concentration did not differ significantly among the three stocking densities in the first 30 days. At days 50 and 70, the serum cortisol concentration was markedly higher in the HSD group than in the LSD and MSD groups (Fig.2).

3.3 Muscle composition of Amur sturgeon

The moisture content of Amur sturgeon' muscle at all three stocking densities decreased during the experimental period (Fig.3a). At day 70, the moisture content was significantly higher in the HSD group than in the LSD and MSD groups. The ash content did not differ significantly among the three stocking densities during the experiment (Fig.3b). At days 10, 30, and 50, the crude lipid content (Fig.3c) and crude protein content (Fig.3d) in muscle did not differ significantly among the three groups. At day 70, however, the crude lipid and protein content in muscle were significantly lower in the HSD group than in the LSD and MSD groups.

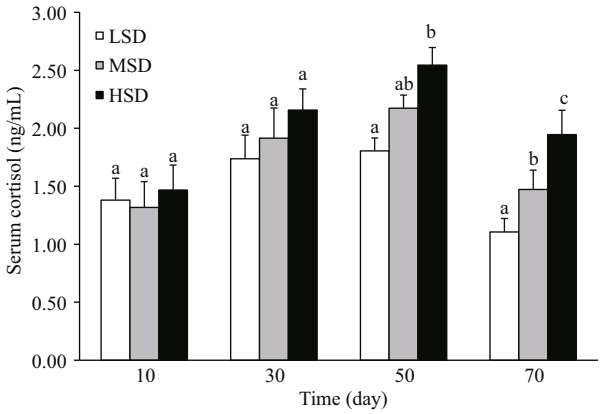

Fig.2 Serum cortisol concentrations in Amur sturgeon grown at different stocking densities for 70 days

Values are means±SEM. Different letters above bars indicate significant differences at each time point (P<0.05).

3.4 Molecular characterization and phylogenetic analysis *GH* and *IGF-2*

The full length cDNAs of *GH* and *IGF-2* were obtained from Amur sturgeon by assembling the core fragment, 5′-, and 3′-end sequences. The complete nucleotide sequence of *GH* (GenBank accession number: KC460212) was 995 bp long, and contained a 645-bp open reading frame (ORF) encoding a protein of 214 amino acids, a 52-bp 5′-untranslated terminal region (UTR), and a 298-bp 3′-UTR region with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail. The sequence of *GH* contained four characteristic cysteine residues (Cys76, Cys187, Cys204, and Cys212). The cDNA of *IGF-2* was 1 207 bp long (Genbank accession number: KC484697) and contained a 651-bp ORF encoding a putative protein of 216 amino acids, as well as a 131-bp 5′-UTR and a 425-bp 3′-UTR. The signal peptide of *IGF-2* consisted of 47 amino acids. The six cysteine residues (Cys64, Cys76, Cys103, Cys104, Cys108, and Cys117) in *IGF-2* were located at conserved positions. The *IGF-2* of Amur sturgeon contained B, C, A, D, and E domains consisting of 32, 11, 21, 6, and 94 amino acids, respectively.

To further understand the structural similarities at the protein level, the predicted amino acid sequences of GH and IGF-2 of Amur sturgeon were compared with those from other vertebrates using ClustalX (Fig.4). Multiple sequence alignments of GH revealed high sequence identity to GHs from other species. The predicted Amur sturgeon GH showed the highest homology with that of Siberian Sturgeon (*Acipenser baerii*) (97%) (Fig.4a). The deduced amino acid sequence of Amur sturgeon IGF-2 showed similarities

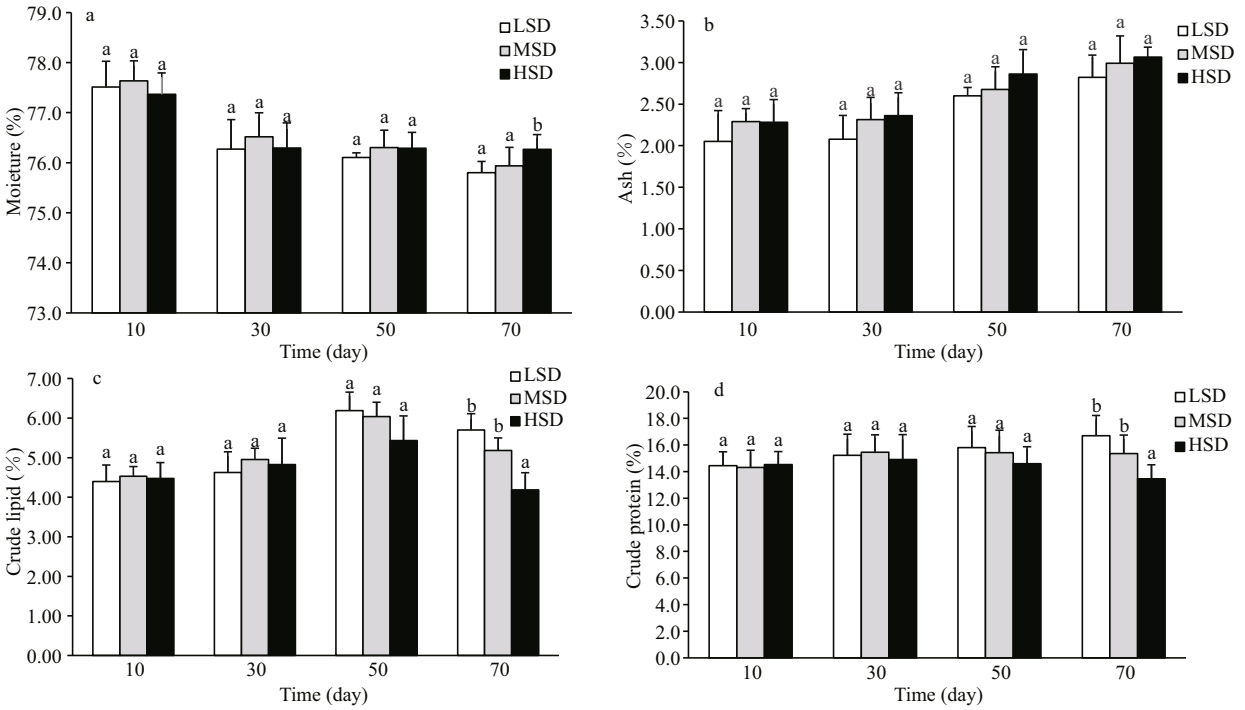

Fig.3 Changes in parameters of body composition (moisture, ash, crude fat and crude protein) of Amur sturgeon at different stocking density

Data are presented as mean±SD (n=8). Different superscript letters within same density indicate significant differences (P<0.05).

a

```
Amur sturgeon              MASGLLLCPVLLVILLVSP--KESGAYPMIPLSSLFTNAVLRAQYLHQLAADIYKDFERTYVPDEQRHS-SKNSPSAFCYSETIPAP
Siberian sturgeon          MASALLLCPVLLVILLVSP--EESGAYPMIPLSSLFTNAVLRAQYLHQLAADIYKDFERTYVPDEQRHS-SKNSPSAFCYSETIPAP
Chicken                    MAPGSWFSPLLIAVVTLGLPQEAAATFPAMPLSNLFANAVLRAQHLHLLAAETYKEFERTYIPEDQRYT-NKNSQAAFCYSETIPAP
Western clawed frog        MATG-FCSSFGLLVVLLLKNVADVGAFPSVPLFSLFTNAVSRAQYIHMLAADTYRDYERTYITDEQRHS-NKNSHVVSCYSETIPYP
Human                      MATGSRTSLLLAFGLLCLPWLQEGSAFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSL-CFSESIPTP
Mouse                      MAADSQTPWLLTFSLLCLLWPQEAGAFPAMPLSSLFANAVLRAQHLHQLAADTYKEFERA-YIPEGQRYS-IQNAQAAFCFSETIPAP
Gilthead seabream          -----MDRVVLMLSVMSLG--VSSQPITDG--QRLFSIAVSRVQHLHLLAQRLFSDFESSLQTEEQPQL-NKIFLQDFCNCDYIISP
Atlantic salmon            -----MGQVFLLMPVLLVSCFLSQGAAMEN--QRLFNIAVNRVQHLHLLAQKMFNDFEGTLLSDERRQL-NKIFLLDFCNSDSIVSP
Grass carp                 -----MARALVLLSVVLVSLLVNQGTASEN--QRLFNNAVIRVQHLHQLAAKMINDFEDNLLPEERRQL-SKIFPLSFCNCDSIEAP
Channel catfish            -----MARALVLLSVVVASLLFSQGATFES--QRLFNNAVIRVQHLHQLAAKMMDDFEEALLPEERKQL-SKIFPLSFCNSDSIEAP
                                 .    :          .         **  *: *.: :* **     ::*    .. :     :      * .: *  *

Amur sturgeon              TGKDEAQQRSDVELLQFSLALIQSWISPLQSLSRVFTNSLVFSTSDR-VFEKLKDLEEGIVALMRDLGEGG---FGSSTLLKLTYDK
Siberian sturgeon          TGKDEAQQRSDVELLQFSLALIQSWISPLQSLSRVFTNSLVFSTSDR-VFEKLKDLEEGIVALMRDLGEGG---FGSSTLLKLTYDK
Chicken                    TGKDDAQQKSDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDR-VFEKLKDLEEGIQALMRELEDRS---PRGPQLLRPTYDK
Western clawed frog        TDKDNTHQKSDLELLRFSLNLIQSWLNPVQALNKVFSNNLVFGSSD--VYERLKYLEEGIQALMQELEDGS---FRSFPFLRPPYER
Human                      SNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRLEDGS---PRTGQIFKQTYSK
Mouse                      TGKEEAQQRTDMELLRFSLLLIQSWLGPVQFLSRIFTNSLMFGTSDR-VYEKLKDLEEGIQALMQELEDGS---PRIGQILKQTYDK
Gilthead seabream          IDKHETQRSSVLKLLSISYRLVESWEFPSRSLS--GGS----APRNQ-ISPKLSELKTGIHLLIRANEDGAEIFPDRSALQLAPYGN
Atlantic salmon            IDKQETQKSSVLKLLHISFRLIESWEYPSQTLA--ISNSLMVRNSNQ-ISEKLSDLKVGINLLIKGSQDGVLSLDDNDSQHLPPYGN
Grass carp                 TGKDETQKSSMLKLLRISFRLIESWEFPSQTLSGQVSNSLTVGNPNQ-ITEKLADLKVGISVLIKGCLDGQPNMDDNDSLPL-PFED
Channel catfish            AGKDEAQKSSVLKLLHTSYRLIESWEFPSRNL----------GNPNH-ISEKLADLKMGIGVLIEGCVDGQTGLDENDSLAP-PFED
                            .:.:::: : ::**  *  *::**  * : *           :  :   *  *: **  *:    :              .: :

Amur sturgeon              FDVNLRNDDALFKNYGLLSCFKKDMHKVEAYLKVVKCRRFVESNCTL
Siberian sturgeon          FDVNLRNDDGLFKNYGLLSCFKKDMHKVETYLKVMKCRRFVGSNCTL
Chicken                    FDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVMKCRRFGESNCTI
Western clawed frog        FDINLRSDDALVKVYGLLSCFKKDMHKVETYLKVMKCRRFVESNCTI
Human                      FDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCR-SVEGSCGF
Mouse                      FDANMRSDDALLKNYGLLSCFKKDLHKAETYLRVMKCRRFAESSCAF
Gilthead seabream          YYQSLGTDESLRRTYELLACFKKDMHKVETYLTVAKCRLSPEANCTL
Atlantic salmon            YYQNLGGDGNIRRNYELLACFKKDMHKVETYLTVAKCRKSLEANCTL
Grass carp                 FYLTMG-ESSLRESFRLLACFKKDMHKVETYLRVANCRRSLDSNCTL
Channel catfish            FYQTLS-EGNLRKSFRLLSCFKKDMHKVETYLSVAKCRRSLDSNCTL
                                 :  : .  : ** **:**:.*.*::* : :**    ..* :
```

To be continued

Fig.4 Continued

b

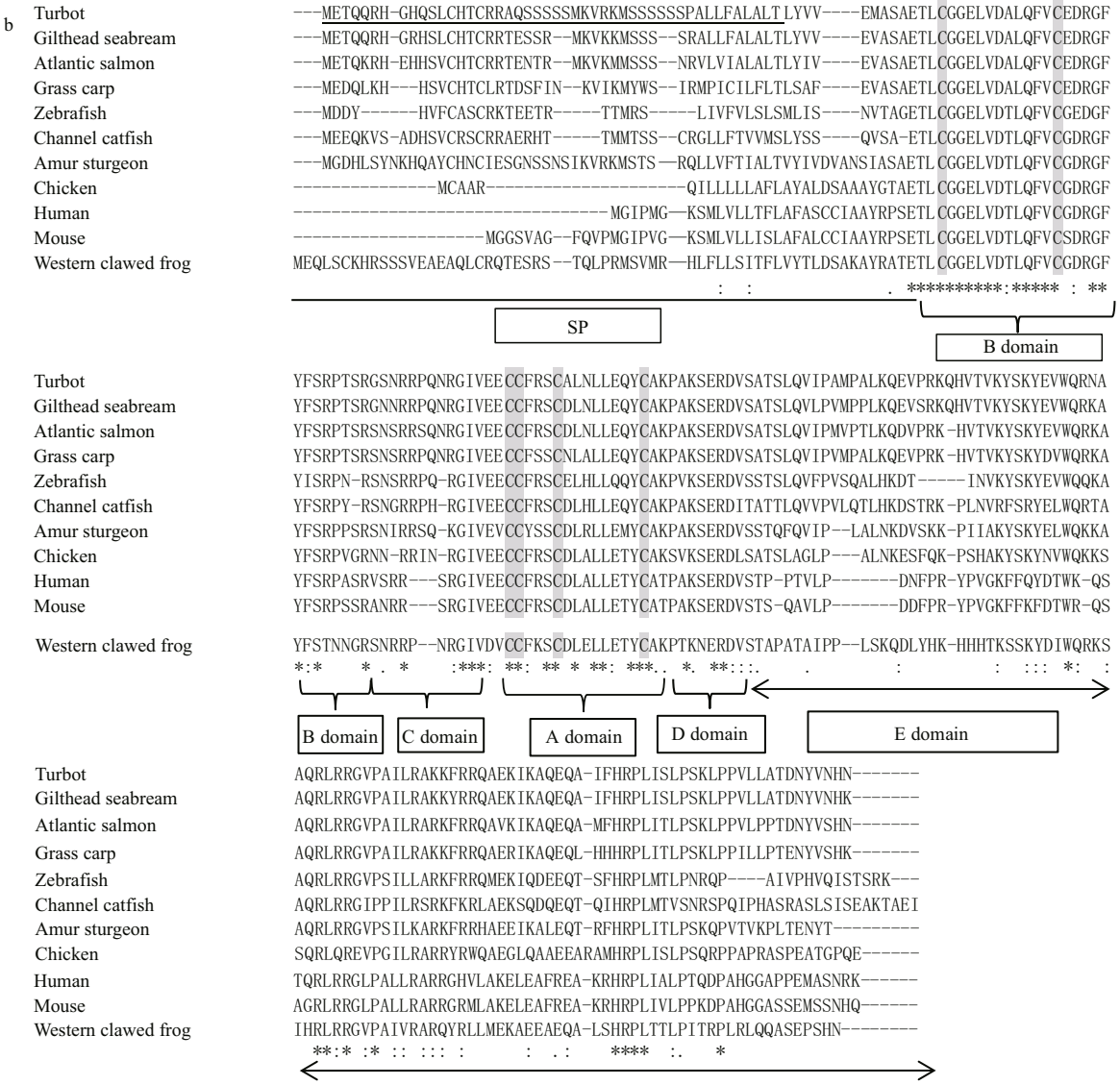

Fig.4 Multiple sequence alignments of Amur sturgeon *GH* (a) and *IGF-2* (b) amino acid sequences with those of other species

Signal peptides are underlined. Conserved cysteines are shaded. The asterisk indicates the stop codon. Predicted glycosation site are indicated in solid box. The GenBank Accession Numbers used are as follows: Amur sturgeon *GH*: AGI96360; Siberian sturgeon *GH*: ACJ60679; Chicken *GH*: NP_989690; Western clawed frog *GH*: NP_001083848; Human *GH*: AAA98618; Mouse *GH*: AAH61157; Gilthead seabream *GH*: AAB19750; Atlantic salmon *GH*: AAA49558; Grass carp *GH*: ABV74334; Channel catfish *GH*: AAC60745; Amur sturgeon *IGF-2*: AGJ72849; Turbot *IGF-2*: AEJ89913; Gilthead seabream *IGF-2*: AAY46224; Atlantic salmon *IGF-2*: ABY88873; Grass carp *IGF-2*: ABK55615; Zebrafish IG2: NP_571508; Channel catfish *IGF-2*: NP_001187875; Chicken *IGF-2*: NP_001025513; Human *IGF-2*: ABD93451; Mouse *IGF-2*:AAH53489; Western clawed frog *IGF-2*: AAI56000.

ranging from 50% (to mouse IGF-2) to 66% (to Atlantic Salmon IGF-2) (Fig.4b). The A and B domains were more conserved than other domains in IGF-2.

A phylogenetic analysis was conducted to study the evolutionary relationships between Amur sturgeon *GH* and *IGF-2* and those of other vertebrates (Fig.5). The tree showed that Amur sturgeon *GH* was closely related to that of Siberian Sturgeon, which belongs to the same family (Acipenseriformes). Notably, sturgeon *GHs* did not form a clade with other fish subgroups, but grouped into a clade with *GHs* from mammals, birds, and amphibians (Fig.5a). Amur sturgeon *IGF-2* grouped with fish *IGF-2s* and showed close relationships with *IGF-2s* of *S. salar* and *Ctenopharyngodon idella* (Fig.5b). The phylogenetic relationships based on the *IGF-2* amino acid sequences were consistent with the traditional classification.

3.5 Tissue distribution of *GH* and *IGF-2* transcripts

The levels of *GH* and *IGF-2* mRNA were

determined in different tissues of Amur sturgeon (Fig.6). Transcripts of *GH* were detected only in the pituitary (Fig.6a), whereas transcripts of *IGF-2* were detected in all examined tissues of Amur sturgeon. The highest transcript level of *IGF-2* was in the kidney, followed by gonad adipose tissue, the liver, gill, and stomach, with the lowest transcript levels in the brain and heart (Fig.6b).

3.6 Serum GH, IGF-1 and IGF-2

The effects of stocking density on serum GH, IGF-1, and IGF-2 concentrations in serum are shown in Fig.7. During the first 50 days, serum GH was not affected by stocking density. However, by day 70, serum GH concentrations were lower in the HSD group than in the other groups (Fig.7a). A similar trend was observed for IGF-1, with no significant differences among the three groups during the first 30 days. At days 50 and 70, the IGF-1 concentration was lower in the HSD group and higher in the LSD group, showing an inverse correlation with stocking density (Fig.7b). The serum IGF-2 concentration did not differ significantly among the three groups (Fig.7c).

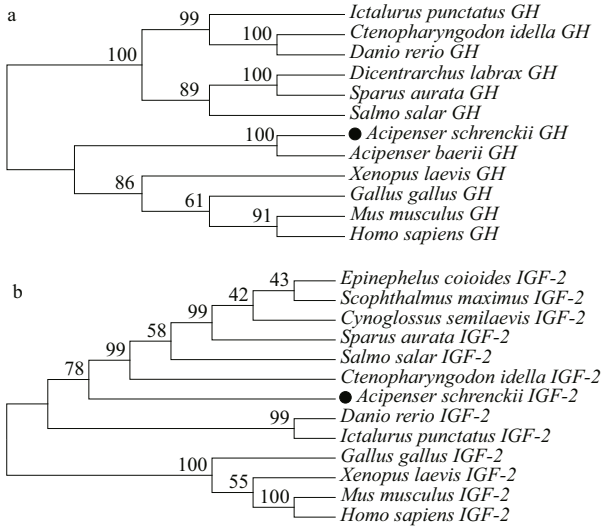

Fig.5 Phylogenetic analysis of *GH* (a) and *IGF-2* (b)

Phylogenetic trees were constructed using MEGA 6.0 software with the neighbor-joining method.

3.7 Transcript levels of *GH*, *GHR*, *IGF-1* and *IGF-2* in various tissues

To further evaluate variations at the molecular level, the transcript levels of *GH*, *GHR*, *IGF-1*, and *IGF-2* in various tissues of Amur sturgeon were examined by quantitative real-time PCR (Fig.8). The transcript level *GH* in the pituitary decreased with increasing stocking density (Fig.8a), and was approximately two-fold higher in the LSD group than in the HSD group. The expression of hepatic *GHR* showed a similar profile, with lower *GHR* mRNA levels in the HSD group (Fig.8b). In addition,

Table 3 Concentrations of metabolites in serum of Amur sturgeon after 70 days of growth at different stocking densities

Parameters	Stocking density		
	LSD	MSD	HSD
Glucose (mmol/L)	1.86±0.19	2.00±0.36	2.05±0.18
Total protein (g/L)	26.50±1.63^{a}	26.22±2.62^{ab}	23.89±1.67^{b}
Cholesterol (mmoL/L)	2.77±0.58^{a}	2.59±0.69^{ab}	2.02±0.45^{b}
Triglyceride (mmoL/L)	7.06±0.64^{a}	6.60±0.71^{ab}	6.04±0.43^{b}
Lactate (mmoL/L)	1.27±0.24	1.18±0.36	1.37±0.19

Values are means±SEM. Values followed by different letters are significantly different (P<0.05).

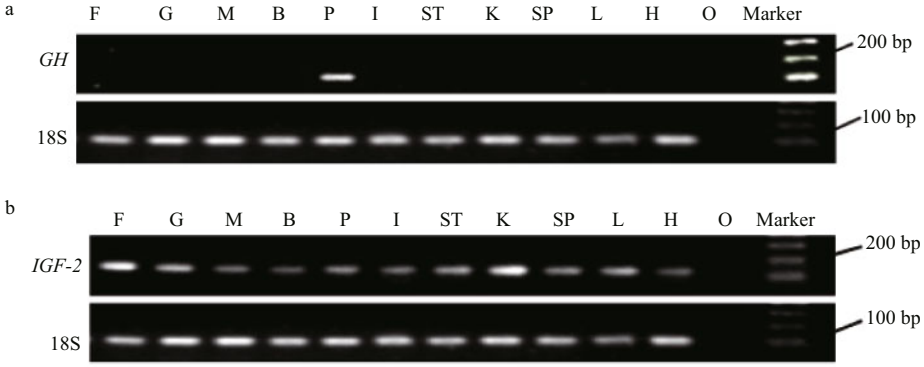

Fig.6 Expression patterns of *GH* (a) and *IGF-2* (b) in different tissues of Amur sturgeon detected by RT-PCR

Integrity of RNAs from each tissue was ensured by uniform amplification of 18S transcripts (lower panel). F: fin; G: gill; M: muscle; B: brain; P: pituitary; I: intestine; ST: stomach; K: kidney; SP: spleen; L: liver; H: heart; O: negative control (water as template); marker: molecular weight standard.

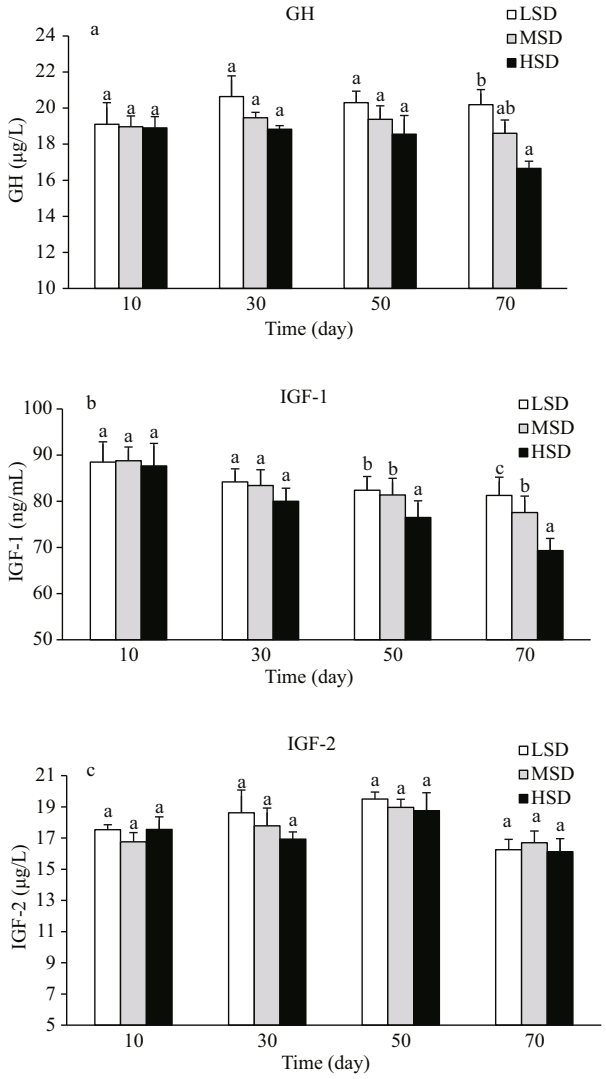

Fig.7 Concentrations of GH (a), IGF-1 (b) and IGF-2 (c) in serum of Amur sturgeon grown at three different stocking densities during a 70-day experiment

Values are means±SEM. Different letters indicate significant differences among experimental groups at each time point (P<0.05).

increased stocking density markedly down-regulated the expression of *IGF-1* in the liver and muscle (LSD>MSD>HSD) (Fig.8c). The expression level of *IGF-2* in the liver was significantly lower in the HSD group than in the LSD and MSD group. However, the transcript levels of *IGF-2* mRNA in muscle did not differ significantly among the three groups (Fig.8d).

4 DISCUSSION

The initial stocking density is an important factor in intensive aquaculture. An inappropriate stocking density negatively affects the growth and metabolism of fish at different stages of development, leading to reduced yields and profitability (Ambrosio et al., 2008; de las Heras et al., 2015). Therefore, it is important to determine the optimum stocking densities at different stages of the life cycle for the healthy and sustainable development of farmed fish (Rowland et al., 2006). In this study, the effect of stocking density on sturgeon growth could be separated into two different phases. In the first phase (days 10 and 30), growth parameters including SGR, DWG, body weight, and body length were similar among all three experimental groups. In the second phase (from day 50 onwards), Amur sturgeon showed density-dependent growth performance; that is, the values of these parameters were lower in the HSD group than in the other two groups. These results are consistent with those previously reported for *S. salar* (Hosfeld et al., 2009), *O. niloticus* (Telli et al., 2014), and *Chelon labrosus* (de las Heras et al., 2015), indicating that the influence of stocking density on fish growth is dynamic and increases over time. The highest FCR was in the HSD group. In our trial, food consumption was not a limiting factor during the experiment, because fish were fed three times per day and proportionally to their body mass. Thus, we speculate that less feeding energy was used for growth of Amur sturgeon at a high stocking density. Similar trends have been reported for *O. niloticus* grown at different stocking densities (Telli et al., 2014).

A high stocking density can cause chronic crowding stress and disturb metabolism in intensive fish rearing (Sangiao-Alvarellos et al., 2005; de las Heras et al., 2015; Yarahmadi et al., 2016). Previous studies have shown that short- and long-term crowding stress can elevate plasma cortisol levels (primary stress response) and consequently trigger metabolic reorganization (secondary stress response) and reduce the growth rate (tertiary stress response) of fish (Mommsen et al., 2001; Ellis et al., 2002). Ruane et al. (2002) subjected *C. carpio* grown at high- and low-densities to a confinement treatment, and found that plasma cortisol levels were higher in the fish from the high-density treatment. Iguchi et al. (2003) and Lupatsch et al. (2010) also found that a higher stocking density resulted in higher cortisol levels in *Plecoglossus altivelis* and *Danio rerio*, respectively. In our study, cortisol concentrations increased with increasing stocking density, suggesting that a high stocking density induced a chronic stress response in Amur sturgeon. Notably, serum cortisol levels decreased in all treatments from day 50 to day 70. This was probably because of acclimation to the crowding environment, as indicated by the return of

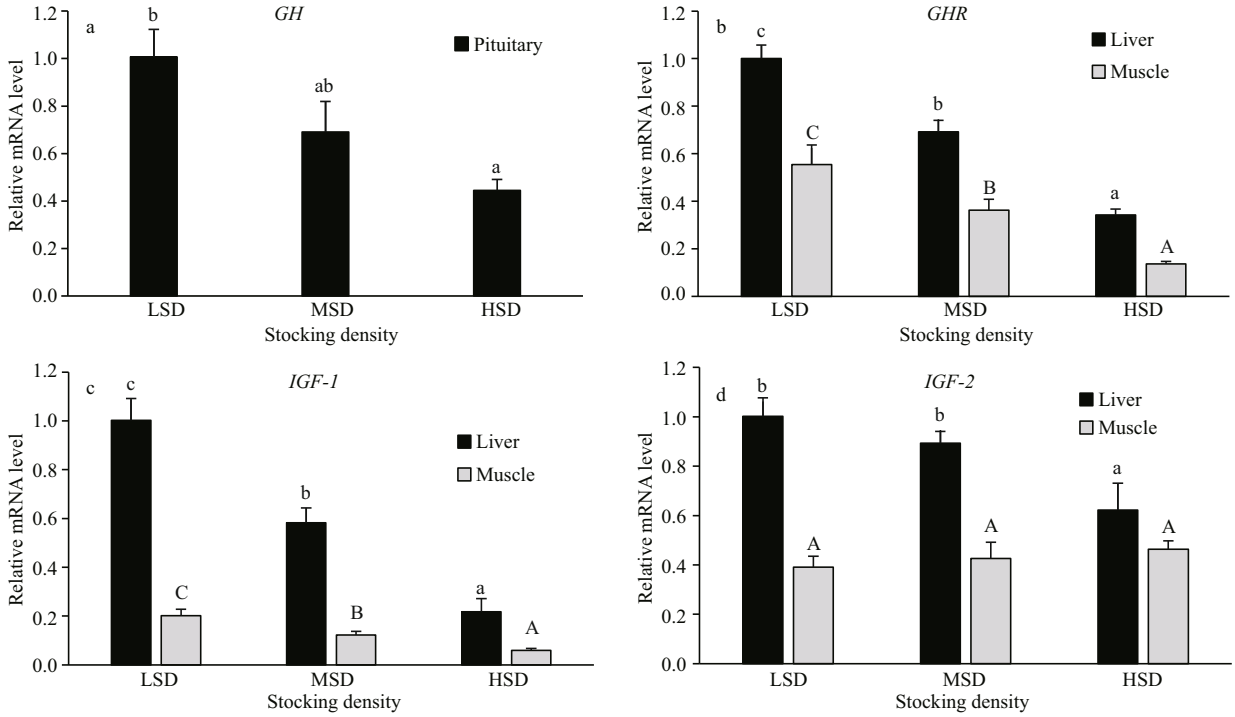

Fig.8 Transcript levels of *GH* (a), *GHR* (b), *IGF-1* (c) and *IGF-2* (d) in tissues of Amur sturgeon after 70 days of growth

Gene transcript levels were determined by relative quantitative real-time PCR, with 18S rRNA as internal control. Values are means±SEM (n=12). Different letters above bars indicate significant difference (P<0.05, one-way ANOVA, Duncan's test).

cortisol concentrations to relatively normal levels. Further studies are required to verify this hypothesis.

Stress caused by a high stocking density can enhance the consumption of energy reserves and the reallocation of metabolic energy. These changes can interfere with other physiological processes such as growth and immunity (Laiz-Carrión et al., 2009). Iguchi et al. (2003) reported that the concentrations of plasma glucose and lactate, which are metabolic indicators of the secondary stress response, increased under stress conditions. However, the serum glucose and lactate concentrations did not differ significantly among the three stocking densities in the present study. These changes do not appear to be consistent among farmed fish, but instead appear to be species-specific. Vargas-Chacoff et al. (2014) reported lower plasma glucose and lactate levels in *E. maclovinus* at higher stocking densities. The differences among species may be due to their different sensitivities and responses to crowding stress. In addition, compared with fish in the LSD and MSD groups, those in the HSD group showed significantly lower serum triglyceride and protein levels, indicating that crowding enhanced energy demands and mobilization. Similar results were reported for *E. maclovinus* (Vargas-Chacoff et al., 2014), in which high stocking density significantly affected triglyceride levels. In addition, intense social interactions at a high stocking density might contribute to increased metabolic demands and retarded growth (McKenzie et al., 2012; Liu et al., 2015).

Body composition and the condition factor roughly indicate the growth profiles and body energy stores of fish (Goede and Barton, 1990). Previous studies have demonstrated that stocking density may affect the body composition of various fish species (Piccolo et al., 2008; Ni et al., 2016). For example, moisture, ash, and crude protein contents in muscle of *Solea solea* were not affected by stocking density, while the crude lipid content was lower in fish at a high stocking density (Piccolo et al., 2008). In this study, muscle composition showed similar variations among the three groups in the first 30 days. However, the crude lipid and protein content in muscle was significantly lower in the HSD group than in the other two groups after 70 days of growth. Also, at the end of the 70-day experiment, the condition factor was significantly lower in the HSD group than in the LSD group. Similar trends were observed in sturgeon kept at a high stocking density (Rafatnezhad et al., 2008; Ni et al., 2016). A high stocking density can activate a series of defense mechanisms, diverting energy from growth to other stress-response and energy-consuming processes (Lupatsch et al., 2010). The decreased contents of some muscle components and the reduced condition

factor in fish in the HSD group in this study appeared to be related to more energy consumption caused by crowding stress, leading to reduced energy available for growth. In general, a high stocking density affected the metabolic pathways related to lipid and protein metabolism and resulted in the mobilization of energy to meet the increased energy requirements imposed by stress. Further, Amur sturgeon in the LSD group showed not only superior growth performance, but also better nutrient composition.

The significant variation in the growth performance of Amur sturgeon among the different stocking densities prompted our interest in the expression of genes related to the GH/IGF axis, which plays a vital role in the neuroendocrine regulation of vertebrate growth (Patel et al., 2005; Beckman, 2011; de las Heras et al., 2015). First, we cloned and analyzed *GH* and *IGF-2* sequences from Amur sturgeon, and detected their expression patterns in different tissues. The complete coding region of *GH* encoded a putative protein of 214 amino acids with four cysteine residues. These cysteine residues participate in the formation of two disulfide bonds, which are probably related to tertiary folding and the biological activity of GH (Chang et al., 1992; Deng et al., 2014). The deduced amino acid sequence of *GH* showed relatively high sequence identity to those of other teleosts, suggesting that its structure and function were evolutionarily conserved. Interestingly, a phylogenetic analysis of the *GH* sequence showed that species in the Acipenseriformes group formed a single cluster separate from other teleosts, and closer to amphibians and endotherms, indicating their special status in the evolutionary tree of vertebrates. The *IGF-2* cDNA from Amur sturgeon encoded a putative protein of 216 amino acids. This is the first report of an *IGF-2* cDNA sequence from a member of the Acipenseridae. The putative IGF-2 sequence included six cysteine residues. Most of the cysteine residues in IGF proteins are involved in binding to receptors or IGF-binding proteins (Duval et al., 2002). In the phylogenetic analysis of *IGF-2*, the Amur sturgeon sequence was most similar to those from other fish species and less similar to those from vertebrates, consistent with the phylogenetic distances among these species. The RT-PCR analyses demonstrated that *GH* was exclusively expressed in the pituitary of Amur sturgeon. This finding is consistent with those of Li et al. (2005), who reported that the expression of *GH* mRNA was dominantly detected in the pituitary of *E. coioides*. This result is not unexpected, because the pituitary is known to be the main site of GH production and secretion. In contrast, *IGF-2* was expressed ubiquitously in all analyzed tissues, indicating that this hormone may exert important physiological functions in bony fish. As in *O. niloticus* (Caelers et al., 2004), *D. labrax* (Terova et al., 2007), and *Umbrina cirrosa* (Patruno et al., 2006), there were high levels of *IGF-2* mRNAs in the liver, adipose tissue, and muscle of Amur sturgeon, indicating a possible role of IGR-2 in metabolism. Transcripts of *IGF-2* were also detected in the kidney and gill in our study. These results are consistent with those reported for *Solea senegalensis* (Funes et al., 2006) and *P. auriga* (Ponce et al., 2008), suggesting that IGF-2 may also play roles in excretion and osmoregulation.

As in other vertebrates, the growth hormone/insulin-like growth factor (GH/IGF) axis (including *GH*, *GHR*, *IGF-1*, *IGF-2*) participates in regulating many growth-promoting processes in fish (Reindl and Sheridan, 2012). This axis and its components are influenced by environmental conditions and nutrition (Duan, 1998; Hanson et al., 2014; Menezes et al., 2015; Tu et al., 2015). In fish, *GH* is closely related to somatic growth and developmental processes. For example, *GH* transcription was higher in fast-growing females than in males in *Cynoglossus semilaevis* (Ma et al., 2012) and *Anguilla anguilla* (Degani et al., 2003). Furthermore, the transcript levels of *GH* decreased with increased stocking density and fasting conditions in *R. quelen* (Menezes et al., 2015). Similar trends were observed in our study; that is, there were lower levels of *GH* expression and serum GH in the HSD group than in the LSD or MSD groups at the end of the 70-day experiment. These changes may be responsible for the reduced growth of Amur sturgeon in the HSD group. In fish, GH is associated with and affected by cortisol (de las Heras et al., 2015). In the current study, the increased cortisol level in the HSD group may account for the changes in circulating GH and *GH* transcript levels.

The biological actions of growth hormones are mediated by the transmembrane growth hormone receptor (*GHR*) (Reindl and Sheridan, 2012). In mammals, the binding of growth hormone to its receptor causes rapid activation of some signal transduction pathways such as the tyrosine kinase JAK2 (Han et al., 1996), mitogen-activated protein kinases, and signal transducer and activator of transcription pathways (Carter-Su et al., 1996). These pathways then regulate cell growth and differentiation, and stress adaptation to the environment. Environmental

factors such as temperature, photoperiod, salinity, and nutritional status have been shown to affect *GHR* expression patterns in numerous tissues of fish (Reinecke, 2005; Poppinga et al., 2007; Peterson and Waldbieser, 2009). In this study, the transcript levels of *GHR* significantly decreased in the liver and muscle of fish in the HSD group, indicating that chronic stress down-regulated *GHR* expression. Growth hormone and cortisol have been reported to be involved in regulating *GHR* expression in various fish species (Jiao et al., 2006; Very and Sheridan, 2007). At present, it is unclear whether crowding stress regulates *GHR* by increasing cortisol concentrations or by affecting GH secretion. Further studies are necessary to investigate the specific mechanism by which chronic stress alters *GHR* transcription. In addition, considering the relationship among *GHR*, *GH*, and *IGF*s, reduced expression of hepatic *GHR* in Amur sturgeon would attenuate GH sensitivity, possibly leading to reduced IGF production. The reduced transcript levels of *IGF-1* and *IGF-2* in the HSD group in this study are consistent with this hypothesis.

In fish, IGFs are the primary mediators of the growth-promoting effects of GH. The IGFs regulate many biological processes such as cell proliferation and differentiation, protein synthesis, and tissue maintenance (Patel et al., 2005; Allard and Duan, 2011). After the reduction in the transcript levels of *GH*, we observed a significant decrease in the transcript levels of *IGF-1* in the liver and muscle of fish at higher stocking densities after 70 days of culture, consistent with the changes in serum IGF-1 concentrations. A positive correlation between somatic growth and plasma IGF-1 has been reported in vertebrates (Patel et al., 2005), and circulating IGF-1 is regarded as a biomarker of growth performance in fish (Picha et al., 2014). In this study, the reduction in growth performance in the HSD group was accompanied by changes in IGF-1 serum levels. In addition, the transcript levels of *IGF-2* in the liver were lower in fish in the HSD group. This finding is consistent with those previously reported for *S. senegalensis* cultured at different stocking densities (Salas-Leiton et al., 2010). However, the levels of circulating IGF-2 and *IGF-2* mRNA in muscle did not differ significantly among the three experimental groups. We observed that *IGF-1* and *IGF-2* were differentially regulated by crowding stress, indicating that IGF-1 and IGF-2 play different roles in the muscle in response to crowding stress. The suppression of IGF synthesis and gene expression by cortisol has been observed in fish and other vertebrates (Delany et al., 2001; Peterson and Small, 2005; Leung et al., 2008). A cortisol treatment was shown to decrease *IGF-1* gene expression in hepatocytes isolated from sea bream (Leung et al., 2008), and to reduce plasma IGF-1 and *IGF-1* mRNA levels in the liver in *Oreochromis mossambicus* (Kajimura et al., 2003). In this study, the significant reduction in *IGF-1* and *IGF-2* transcript levels in Amur sturgeon in the HSD group may be related to increased serum cortisol levels.

5 CONCLUSION

To better understand the functions of GH and IGF-2 in growth and other physiological processes, we analyzed the cDNA sequences and expression patterns of *GH* and *IGF-2* in different tissues of Amur sturgeon cultured at different stocking densities. The results showed that increasing stocking density negatively affected the growth performance of Amur sturgeon. High stocking density during a 70-day experiment caused chronic crowding stress, and affected the growth and metabolism of Amur sturgeon, as indicated by changes in serum lipid and protein contents. The expression of many genes in the GH/IGF axis and circulating levels of GH and IGF-1 were down-regulated in fish cultured at a high stocking density, suggesting that crowding stress influenced growth performance via regulation of the GH/IGF axis. Further studies are required to explore the specific mechanisms and signaling pathways involved in these changes.

6 ACKNOWLEDGEMENT

The authors thank the Shandong Xunlong Fisheries Farm for providing the fish and the experimental site.

References

Adams C E, Turnbull J F, Bell A, Bron J E, Huntingford F A. 2007. Multiple determinants of welfare in farmed fish: stocking density, disturbance, and aggression in Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, **64**(2): 336-344.

Allard J B, Duan C. 2011. Comparative Endocrinology of Aging and Longevity Regulation. *Frontiers in Endocrinology*, **2**(75): 1-23.

Ambrosio P P, Costa C, Sánchez P, Flos R. 2008. Stocking density and its influence on shape of *Senegalese sole* adults. *Aquaculture International*, **16**(4): 333-343.

Association of Official Analytical Chemists (AOAC). 1995. Official Methods of Analysis of Official Analytical Chemists International. 16th edn. Association of Official Analytical Chemists, Arlington, VA, USA.

Beckman B R. 2011. Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. *General and Comparative Endocrinology*, **170**(2): 233-252.

Caelers A, Berishvili G, Meli M L, Eppler E, Reinecke M. 2004. Establishment of a real-time RT-PCR for the determination of absolute amounts of IGF-I and IGF-II gene expression in liver and extrahepatic sites of the tilapia. *General and Comparative Endocrinology*, **137**(2): 196-204.

Carter-Su C, Schwartz J, Smit L J. 1996. Molecular mechanism of growth hormone action. *Annual Review of Physiology*, **58**(1): 187-207.

Chang Y S, Liu C S, Huang F L, Lo T B. 1992. The primary structures of growth hormones of three cyprinid species: bighead carp, silver carp, and grass carp. *General and Comparative Endocrinology*, **87**(3): 385-393.

Costas B, Aragão C, Mancera J M, Dinis M T, Conceição L E C. 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. *Aquaculture Research*, **39**(1): 1-9.

de las Heras V, Martos-Sitcha J A, Yúfera M, Mancera J M, Martínez-Rodríguez G. 2015. Influence of stocking density on growth, metabolism and stress of thick- lipped grey mullet (*Chelon labrosus*) juveniles. *Aquaculture*, **448**: 29-37.

de Oliveira E G, Pinheiro A B, de Oliveira V Q, Da Silva Júnior M, de Moraes M G, Rocha Í R C B, de Sousa R R, Costa F H F. 2012. Effects of stocking density on the performance of juvenile pirarucu (*Arapaima gigas*) in cages. *Aquaculture*, **370-371**: 96-101.

Deane E E, Woo N Y S. 2009. Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. *Reviews in Fish Biology and Fisheries*, **19**(1): 97-120.

DeChiara T M, Efstratiadis A, Robertsen E J. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*, **345**(6270): 78-80.

DeChiara T M, Robertson E J, Efstratiadis A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. Cell, **64**(4): 849-859.

Degani G, Tzchori I, Yom-Din S, Goldberg D, Jackson K. 2003. Growth differences and growth hormone expression in male and female European eels [*Anguilla anguilla* (L.)]. *General and Comparative Endocrinology*, **134**(1): 88-93.

Delany A M, Durant D, Canalis E. 2001. Glucocorticoid suppression of IGF I transcription in osteoblasts. *Molecular Endocrinology*, **15**(10): 1 781-1 789.

Deng S P, Wu B, Zhu C H, Li G L. 2014. Molecular cloning and dimorphic expression of growth hormone (*gh*) in female and male spotted scat *Scatophagus argus*. *Fisheries Science*, **80**(4): 715-723.

Duan C M. 1998. Nutritional and developmental regulation of insulin-like growth factors in fish. *The Journal of Nutrition*, **128**(2): 306S-314S.

Duval H, Rousseau K, Eliès G, Le Bail P Y, Dufour S, Boeuf G, Boujard D. 2002. Cloning, characterization, and comparative activity of turbot IGF-I and IGF-II. *General and Comparative Endocrinology*, **126**(3): 269-278.

Dyer A R, Barlow C G, Bransden M P, Carter C G, Glencross B D, Richardson N, Thomas P M, Williams K C, Carragher J F. 2004. Correlation of plasma IGF-I concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture*, **236**(1-4): 583-592.

Ellis T, North B, Scott A P, Bromage N R, Porter M, Gadd D. 2002. The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology*, **61**(3): 493-531.

Funes V, Asensio E, Ponce M, Infante C, Cañavate J P, Manchado M. 2006. Insulin-like growth factors I and II in the sole *Solea senegalensis*: cDNA cloning and quantitation of gene expression in tissues and during larval development. *General and Comparative Endocrinology*, **149**(2): 166-172.

Gabillard J C, Weil C, Rescan P Y, Navarro I, Gutiérrez J, Le Bail P Y. 2003. Effects of environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology*, **133**(2): 233-242.

Goede R W, Barton B A. 1990. Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. *In*: Adams S M ed. Biological Indicator of Stress in Fish. American Fisheries Society Symposium, Bethesda, USA. **8**: 93-108.

Han Y L, Leaman D W, Watling D, Rogers N C, Groner B, Kerr I M, Wood W I, Stark G R. 1996. Participation of jak and stat proteins in growth hormone-induced signaling. *Journal of Biological Chemistry*, **271**(10): 5 947-5 952.

Hanson A M, Kittilson J D, Martin L E, Sheridan M A. 2014. Environmental estrogens inhibit growth of rainbow trout (*Oncorhynchus mykiss*) by modulating the growth hormone-insulin-like growth factor system. *General and Comparative Endocrinology*, **196**: 130-138.

Herrera M, Ruíz-Jarabo I, Hachero I, Vargas-Chacoff L, Amo A, Mancera J M. 2012. Stocking density affects growth and metabolic parameters in the brill (*Scophthalmus rhombus*). *Aquaculture International*, **20**(6): 1 041-1 052.

Hosfeld C D, Hammer J, Handeland S O, Fivelstad S, Stefansson S O. 2009. Effects of fish density on growth and smoltification in intensive production of Atlantic salmon (*Salmo salar* L.). *Aquaculture*, **294**(3-4): 236-241.

Iguchi K, Ogawa K, Nagae M, Ito F. 2003. The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). *Aquaculture*, **220**(1-4): 515-523.

Jiao B W, Huang X G, Chan C B, Zhang L, Wang D S, Cheng C H K. 2006. The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. *Journal of Molecular Endocrinology*, **36**(1): 23-40.

Jodun W A, Millard M J, Mohler J. 2002. The effect of rearing density on growth, survival, and feed conversion of juvenile atlantic sturgeon. *North American Journal of Aquaculture*, **64**(1): 10-15.

Jones J I, Clemmons D R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews*, **16**(1): 3-34.

Kajimura S, Hirano T, Visitacion N, Moriyama S, Aida K, Grau E G. 2003. Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. *Journal of Endocrinology*, **178**(1): 91-99.

Kita K, Nagao K, Okumura J. 2005. Nutritional and tissue specificity of IGF-I and IGFBP-2 gene expression in growing chickens-a review. *Asian-Australasian Journal of Animal Sciences*, **18**(5): 747-754.

Laiz-Carrión R, Fuentes J, Redruello B, Guzmán J M, Martín del Río M P, Power D, Mancera J M. 2009. Expression of pituitary prolactin, growth hormone and somatolactin is modified in response to different stressors (salinity, crowding and food-deprivation) in gilthead sea bream *Sparus auratus*. *General and Comparative Endocrinology*, **162**(3): 293-300.

Laiz-Carrión R, Viana I, Cejas J R, Ruiz-Jarabo I, Jerez S, Martos J A, Eduardo A B, Mancera J M. 2012. Influence of food deprivation and high stocking density on energetic metabolism and stress response in red porgy, *Pagrus pagrus* L. *Aquaculture International*, **20**(3): 585-599.

Leung L Y, Kwong A K Y, Man A K Y, Woo N Y S. 2008. Direct actions of cortisol, thyroxine and growth hormone on IGF-I mRNA expression in sea bream hepatocytes. *Comparative Biochemistry and Physiology Part A*: *Molecular & Integrative Physiology*, **151**(4): 705-710.

Li D P, Liu Z D, Xie C X. 2012. Effect of stocking density on growth and serum concentrations of thyroid hormones and cortisol in Amur sturgeon, *Acipenser schrenckii*. *Fish Physiology and Biochemistry*, **38**(2): 511-520.

Li W S, Chen D, Wong A O L, Lin H R. 2005. Molecular cloning, tissue distribution, and ontogeny of mRNA expression of growth hormone in orange-spotted grouper (*Epinephelus coioides*). *General and Comparative Endocrinology*, **144**(1): 78-89.

Liu B L, Liu Y, Wang X P. 2015. The effect of stocking density on growth and seven physiological parameters with assessment of their potential as stress response indicators for the Atlantic salmon (*Salmo salar*). *Marine and Freshwater Behaviour and Physiology*, **48**(3): 177-192.

Lupatsch I, Santos G A, Schrama J W, Verreth J A J. 2010. Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. *Aquaculture*, **298**(3-4): 245-250.

Ma Q, Liu S F, Zhuang Z M, Lin L, Sun Z Z, Liu C L, Ma H, Su Y Q, Tang Q S. 2012. Genomic structure, polymorphism and expression analysis of the growth hormone (GH) gene in female and male half-smooth tongue sole (*Cynoglossus semilaevis*). *Gene*, **493**(1): 92-104.

McKenzie D J, Höglund E, Dupont-Prinet A, Larsen B K, Skov P V, Pedersen P B, Jokumsen A. 2012. Effects of stocking density and sustained aerobic exercise on growth, energetics and welfare of rainbow trout. *Aquaculture*, **338-341**: 216-222.

Menezes C, Ruiz-Jarabo I, Martos-Sitcha J A, Toni C, Salbego J, Becker A, Loro V, Martínez-Rodríguez G, Mancera J M, Baldisserotto B. 2015. The influence of stocking density and food deprivation in silver catfish (*Rhamdia quelen*): a metabolic and endocrine approach. *Aquaculture*, **435**: 257-264.

Millán-Cubillo A F, Martos-Sitcha J A, Ruiz-Jarabo I, Cárdenas S, Mancera J M. 2016. Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (*Argyrosomus regius*, Asso 1801). *Aquaculture*, **451**: 87-92.

Mohammed-Geba K, Yúfera M, Martínez-Rodríguez G, Mancera J M. 2016. Molecular endocrine changes of Gh/Igf1 axis in gilthead sea bream (*Sparus aurata* L.) exposed to different environmental salinities during larvae to post-larvae stages. *Fish Physiology and Biochemistry*, **42**(4): 1 177-1 186.

Mommsen T P. 2001. Paradigms of growth in fish. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*, **129**(2-3): 207-219.

Moriyama S, Ayson F G, Kawauchi H. 2000. Growth regulation by insulin-like growth factor-I in fish. *Bioscience*, *Biotechnology*, *and Biochemistry*, **64**(8): 1 553-1 562.

Ni M, Wen H S, Li J F, Chi M L, Bu Y, Ren Y Y, Zhang M, Song Z F, Ding H M. 2014. The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. *Fish & Shellfish Immunology*, **36**(2): 325-335.

Ni M, Wen H S, Li J F, Chi M L, Bu Y, Ren Y Y, Zhang M, Song Z F, Ding H M. 2016. Effects of stocking density on mortality, growth and physiology of juvenile Amur sturgeon (*Acipenser schrenckii*). *Aquaculture Research*, **47**(5): 1 596-1 604.

Otteson D C, Cirenza P F, Hitchcock P F. 2002. Persistent neurogenesis in the teleost retina: evidence for regulation by the growth-hormone/insulin-like growth factor-I axis. *Mechanisms of Development*, **117**(1-2): 137-149.

Patel K, Macharia R, Amthor H. 2005. Molecular mechanisms involving IGF-1 and myostatin to induce muscle hypertrophy as a therapeutic strategy for duchenne muscular dystrophy. *Acta Myologica*: *Myopathies and Cardiomyopathies*: *Official Journal of the Mediterranean Society of Myology*, **24**(3): 230-241.

Patruno M, Maccatrozzo L, Funkenstein B, Radaelli G. 2006. Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*, **144**(2): 137-151.

Peterson B C, Small B C. 2005. Effects of exogenous cortisol on the GH/IGF-I/IGFBP network in channel catfish. *Domestic Animal Endocrinology*, **28**(4): 391-404.

Peterson B C, Waldbieser G C. 2009. Effects of fasting on IGF-I, IGF-II, and IGF-binding protein mRNA

concentrations in channel catfish (*Ictalurus punctatus*). *Domestic Animal Endocrinology*, **37**(2): 74-83.

Piccolo G, Marono S, Bovera F, Tudisco R, Caricato G, Nizza A. 2008. Effect of stocking density and protein/fat ratio of the diet on the growth of Dover sole (*Solea solea*). *Aquaculture Research*, **39**(16): 1 697-1 704.

Picha M E, Biga P R, Galt N, McGinty A S, Gross K, Hedgpeth V S, Siopes T D, Borski R J. 2014. Overcompensation of circulating and local insulin-like growth factor-1 during catch-up growth in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) following temperature and feeding manipulations. *Aquaculture*, **428-429**: 174-183.

Ponce M, Infante C, Funes V, Manchado M. 2008. Molecular characterization and gene expression analysis of insulin-like growth factors I and II in the redbanded seabream, *Pagrus auriga*: transcriptional regulation by growth hormone. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*, **150**(4): 418-426.

Poppinga J, Kittilson J, Mccormick S D, Sheridan M A. 2007. Effects of somatostatin on the growth hormone-insulin-like growth factor axis and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **273**(2-3): 312-319.

Qiang J, Yang H, Wang H, Kpundeh M D, Xu P. 2012. Growth and IGF-I response of juvenile Nile tilapia (*Oreochromis niloticus*) to changes in water temperature and dietary protein level. *Journal of Thermal Biology*, **37**(8): 686-695.

Radaelli G, Poltronieri C, Bertotto D, Funkenstein B, Simontacchi C. 2008. Cellular localization of insulin-like growth factor-II protein in the sea bass (*Dicentrarchus labrax*) from hatching to adult. *Histology and Histopathology*, **23**(5): 523-530.

Rafatnezhad S, Falahatkar B, Tolouei Gilani M H. 2008. Effects of stocking density on haematological parameters, growth and fin erosion of great sturgeon (*Huso huso*) juveniles. *Aquaculture Research*, **39**(14): 1 506-1 513.

Reindl K M, Sheridan M A. 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comparative Biochemistry and Physiology Part A*: *Molecular & Integrative Physiology*, **163**(3-4): 231-245.

Reinecke M, Björnsson B T, Dickhoff W W, McCormick S D, Navarro I, Power D M, Gutiérrez J. 2005. Growth hormone and insulin-like growth factors in fish: where we are and where to go. *General and Comparative Endocrinology*, **142**(1-2): 20-24.

Rolland M, Dalsgaard J, Holm J, Gómez-Requeni P, Skov P V. 2015. Dietary methionine level affects growth performance and hepatic gene expression of GH-IGF system and protein turnover regulators in rainbow trout (*Oncorhynchus mykiss*) fed plant protein-based diets. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*, **181**: 33-41.

Rotllant J, Balm P H, Pérez-Sánchez J, Wendelaar-Bonga S E, Tort L. 2001. Pituitary and Interrenal Function in Gilthead Sea Bream (*Sparus aurata* L. Teleostei) after Handling and Confinement Stress. *General & Comparative Endocrinology*, **121**(3): 333-342.

Rowland S J, Mifsud C, Nixon M, Boyd P. 2006. Effects of stocking density on the performance of the australian freshwater silver perch (*Bidyanus bidyanus*) in cages. *Aquaculture*, **253**(1-4): 301-308.

Ruane N M, Carballo E C, Komen J. 2002. Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). *Aquaculture Research*, **33**(10): 777-784.

Salas-Leiton E, Anguis V, Martín-Antonio B, Crespo D, Planas J V, Infante C, Cañavate J P, 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 Immunology*, **28**(2): 296-302.

Sangiao-Alvarellos S, Guzmán J M, Láiz-Carrión R, Míguez J M, Martín de Río M P, Mancera J M, Soengas J L. 2005. Interactive effects of high stocking density and food deprivation on carbohydrate metabolism in several tissues of gilthead sea bream *Sparus auratus*. *Journal of Experimental Zoology Part A*: *Comparative Experimental Biology*, **303**(9): 761-775.

Small B C, Peterson B C. 2005. Establishment of a time-resolved fluoroimmunoassay for measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*). *Domestic Animal Endocrinology*, **28**(2): 202-215.

Tanamati F, da Silva S C C, Del Pilar Rodriguez Rodriguez M, Schuroff G P, Nascimento C S D, Del Vesco A P, Gasparino E. 2015. GHR and IGF-I gene expression and production characteristics associated with GH gene polymorphism in Nile tilapia. *Aquaculture*, **435**: 195-199.

Telli G S. Ranzani-Paiva M J T, de Carla Dias D, Sussel F R, Ishikawa C M, Tachibana L. 2014. Dietary administration of *Bacillus subtilis* on hematology and non-specific immunity of Nile tilapia *Oreochromis niloticus* raised at different stocking densities. *Fish & Shellfish Immunology*, **39**(2): 305-311.

Terova G, Rimoldi S, Chini V, Gornati R, Bernardini G, Saroglia M. 2007. Cloning and expression analysis of insulin-like growth factor I and II in liver and muscle of sea bass (*Dicentrarchus labrax*, L.) during long-term fasting and refeeding. *Journal of Fish Biology*, **70**(Supplement B): 219-233.

Tolussi C E, Hilsdorf A W S, Caneppele D, Moreira R G. 2010. The effects of stocking density in physiological parameters and growth of the endangered teleost species piabanha, *Brycon insignis* (Steindachner, 1877). *Aquaculture*, **310**(1-2): 221-228.

Tu Y Q, Xie S Q, Han D, Yang Y X, Jin J Y, Liu H K, Zhu X M. 2015. Growth performance, digestive enzyme, transaminase and GH-IGF-I axis gene responsiveness to different dietary protein levels in broodstock

allogenogynetic gibel carp (C*arassius auratus gibelio*) CAS III. *Aquaculture*, **446**: 290-297.

Vargas-Chacoff L, Martínez D, Oyarzún R, Nualart D, Olavarría V, Yáñez A, Bertrán C, Ruiz-Jarabo I, Mancera J M. 2014. Combined effects of high stocking density and *Piscirickettsia salmonis* treatment on the immune system, metabolism and osmoregulatory responses of the Sub-Antarctic Notothenioid fish *Eleginops maclovinu*s. *Fish & Shellfish Immunology*, **40**(2): 424-434.

Very N M, Sheridan M A. 2007. Somatostatin regulates hepatic growth hormone sensitivity by internalizing growth hormone receptors and by decreasing transcription of growth hormone receptor mRNAs. *American Journal of Physiology-Regulatory*, *Integrative and Comparative Physiology*, **292**(5): R1 956-R1 962.

Vijayan M M, Ballantyne J S, Leatherland J F. 1990. High stocking density alters the energy metabolism of brook charr, *Salvelinus fontinalis*. *Aquaculture*, **88**(3): 371-381.

Wilkinson R J, Porter M, Woolcott H, Longland R, Carragher J F. 2006. Effects of aquaculture related stressors and nutritional restriction on circulating growth factors (GH, IGF-I and IGF-II) in Atlantic salmon and rainbow trout. *Comparative Biochemistry and Physiology Part A*: *Molecular & Integrative Physiology*, **145**(2): 214-224.

Yarahmadi P, Miandare H K, Fayaz S, Caipang C M A. 2016. Increased stocking density causes changes in expression of selected stress- and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology*, **48**: 43-53.

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. *Journal of Applied Ichthyology*, **18**(4-6): 659-664.