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Effects of stocking density on lipid deposition and expression of lipid-related genes in Amur sturgeon (*Acipenser schrenckii*)

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Abstract To investigate the correlation between lipid deposition variation and stocking density in Amur sturgeon (*Acipenser schrenckii)* and the possible physiological mechanism, fish were conducted in different stocking densities (LSD 5.5 kg/m^3, MSD 8.0 kg/m^3, and HSD 11.0 kg/m^3) for 70 days and then the growth index, lipid content, lipase activities, and the mRNA expressions of lipid-related genes were examined. Results showed that fish subjected to higher stocking density presented lower final body weights (FBW), specific growth ratio (SGR), and gonad adipose tissue index (GAI) ($P < 0.05$). Lower lipid content was observed in the liver, gonad adipose tissue and muscle in sturgeons held in HSD group ($P < 0.05$). The serum concentrations of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) decreased significantly with increasing stocking density, while no significant change was observed for low-density lipoprotein cholesterol (LDL-C). Furthermore, the cDNAs encoding lipoprotein lipase (LPL) and hepatic lipase (HL) were isolated in Amur sturgeon, respectively. The full-length LPL cDNA was composed of 1757 bp with an open reading frame of 501 amino acids, while the complete nucleotide sequences of HL covered 1747 bp encoding 499 amino acids. In the liver, the activities and mRNA levels of LPL were markedly lower in HSD group, which were consistent with the variation tendency of HL. Fish reared in HSD group also presented lower levels of activities and mRNA expression of LPL in the muscle and gonad. Moreover, the expressions of peroxisome proliferator-activated receptor α (PPARα) in both the liver and skeletal muscle were significantly upregulated in HSD group. Overall, the results indicated that high stocking density negatively affects growth performance and lipid deposition of Amur sturgeon to a certain extent. The downregulation of LPL and HL and the upregulation of PPARα may be responsible for the lower lipid distribution of Amur sturgeon in higher stocking density.

Keywords Amur sturgeon · Stocking density · Lipid deposition · Lipases · Gene expression

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Introduction

Stocking density is one of the most important parameters in intensive aquaculture since it may greatly affect growth, welfare, and productivity of farmed fish (Vijayan et al. 1990; Marco et al. 2008; Herrera et al. 2012; Menezes et al. 2015). Identifying the optimum stocking densities for each economic species and production phase are necessary to ensure fish's health and to maximize profitability (Rowland et al. 2006). Adverse effects on growth performance and productivity at high stocking densities were observed in pirarucu (*Arapaima gigas*) (De Oliveira et al. 2013), brill (*Scophthalmus rhombus*) (Herrera et al. 2012), and piabanha (*Brycon insignis*) (Tolussi et al. 2010). Low

stocking density also negatively affects growth performance and welfare in some species such as meager (*Argyrosomus regius*) (Millán-Cubillo et al. 2016) and Atlantic salmon (*Salmo salar*) (Adams et al. 2007). Further, an inappropriate stocking density may disturb some metabolism pathways in species-specific way, many of which were related to lipid metabolism. In *Scophthalmus rhombus*, triglycerides and total polar lipid were significantly enhanced in high stocking density (Herrera et al. 2012). *Brycon insignis* and *Piaractus mesopotamicus* reared at lower stocking density presented higher lipid deposition in the liver, with no difference in their hepatosomatic index values (Tolussi et al. 2010; Bittencourt et al. 2010). Moreover, for gilthead seabream (*Sparus aurata)*, high stocking density produced some effects on lipid metabolism to meet the increased energy demand, with a decrease in hepatasomatic index and alterations in liver fatty acid composition (Montero et al. 1999). Because of the importance of stocking densities for commercial fish production, it is crucial to evaluate the physiological changes and the molecular mechanisms of energy substances that respond to crowding in order to ensure an optimal welfare and health status.

As the predominant sources of metabolic energy, lipids play essential roles in whole life history of fish including growth, development, reproduction, and movement. Nowadays, with the rapid development of the intensive aquaculture industry, more and more farmed fish appears to have lipid-associated problems such as fatty liver disease or fatty acid deficiency, which seriously influence fish's health and production quality. Keeping the appropriate lipid composition and metabolism in their life cycle would be of great importance for fish culture. Lipids metabolism refers to the processes that involve digestion, absorption, synthesis, catabolism (oxidation), and deposition of lipids, which are mainly regulated by some key enzymes such as lipoprotein lipase (LPL) and hepatic lipase (HL), as well as transcription factors like peroxisome proliferator-activated receptor α (PPARα) (Oku et al. 2006; Pawlak et al. 2015). LPL and HL are two members of the lipase gene superfamily, which encode lipases that share high degrees of structural similarity with each other but play different roles in lipid metabolism (Wong and Schotz. 2002; Mukherjee 2003; Saera-Vila et al. 2005). LPL catalyzes the hydrolysis of plasma chylomicron and very low-density lipoproteins, while HL is critical for chylomicron-remnant and high-density lipoprotein metabolism (Wang et al. 1992; Santamarina-Fojo et al. 1994; Mukherjee 2003). PPARα, as a ligand-activated nuclear receptor, takes part in lipid catabolism by regulating the transcription of target genes encoding enzymes that are involved in peroxisomal and mitochondrialβ-oxidation of fatty acids (Corcoran et al. 2015), and its expression is enriched in tissues with high fatty acid oxidation rates such as the liver, heart, kidney, and skeletal muscle (Philippe et al. 2006).

It has been demonstrated that the activities of HL and LPL are regulated in response to the physiological status of fish, which is highly mediated by various aquaculture conditions and stressors. The effects of nutritional regulation (e.g., fasting and feeding, dietary composition) on LPL and/or HL have been extensively studied in several teleost species such as red sea bream (*Pagrus major*) (Oku et al. 2006;), rainbow trout (*Oncorhynchus mykiss*) (Albalat et al. 2006), Atlantic salmon (Morais et al. 2011)), darkbarbel catfish (*Pelteobagrus vachelli*) (Zheng et al. 2010), and gilthead sea bream (*Sparus aurata*) (Saera-Vila et al. 2005). The relationships between stress factors (e.g., temperature, stocking density) and lipid-related genes expression have also been characterized (Liang et al. 2003; Perez-Sanchez et al. 2013). After crowding exposure, the mRNA expressions of LPL and HL in gilthead sea bream were significantly upregulated at 3 and 24 h, respectively (Perez-Sanchez et al. 2013). It has also been reported that the activity of LPL may affect the hypotriglyceridemic effects of fibrates, thiazolindinediones, and fatty acids, which are known activators (and/or ligands) of the PPARs (Schoonjans et al. 1996). As results, the mRNA levels of above genes are speculated to be correlated with each other and be all closely related to lipid deposition and metabolism in fish.

Amur sturgeon (*Acipenser schrenckii* Brandt) is a valuable economic fish species distributed in the Amur River and has become one of the most popular breeding sturgeons in China (Zhuang et al. 2002; Li et al. 2012). Previous studies have shown that stocking density affected the growth and physiology in sturgeons (Jodun et al. 1993; Li et al. 2012), however, there has been no research to evaluate relationship between stocking densities and lipid variation under controlled culture conditions in Amur sturgeon, especially how chronic stress affects levels of genes regulating lipid deposition and metabolism. In the present study, we investigated the effects of stocking density on the lipids levels by measuring the lipid content and lipase activity in tissues.

Further, we reported the molecular cloning and expression of LPL and HL as well as the mRNA expression patterns of PPARα at three different stocking densities, which provide valuable evidences for the significance of LPL, HL, and PPARα in the regulation of lipid deposition and metabolism in Amur sturgeon.

Materials and methods

Fish and experimental conditions

Amur sturgeons (initial body weight = 225.5 ± 32.3 g) were obtained from a commercial farm (Shandong Xunlong Sci-Tech Co., Ltd) in which this research was conducted. Prior to experiment, fish were reared in concrete ponds for 2 weeks. After adaptation, 2700 fish were randomly selected and stocked into square concrete ponds (4.4 × 4.4 × 0.45 m^3) at initial experimental densities of 5.5, 8.0, and 11.0 kg/m^3 with three replicates. Amur sturgeons were maintained for 70 days and fed three times a day with dry commercial feed (Ningbo Tech-Bank, containing 42% of crude protein) at ration of 1.5% of a total fish biomass. The experimental ponds were supplied with deep-well water from a flow-through system equipped with mechanical filters. Water was renewed at approximately 15 L/min in every pond. The following physicochemical parameters of the water were always at satisfactory levels for fish culture throughout the experiments: water temperatures were maintained from 13.3 to17.2 °C, DO from 7.2 to 9.6 mg/L, and pH from 7.8 to 8.3. Then 54 fish (6 from each pond) were sampled at the 70th day. All fish were treated with 200 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO). Blood samples were collected from caudal vein and precipitated at 4 °C for 6 h. Then, serum was centrifuged at 16,000 r min^{-1} for 10 min and stored at −40 °C before analysis. Tissues including the brain, pituitary, fin, gills, heart, liver, kidney, intestine, stomach, spleen, gonad and muscle at each stocking densities from experimental Amur sturgeons were collected, immediately frozen with liquid nitrogen, and stored at −80 °C until extraction of total RNA.

Measurement of growth performance

Biometric samplings were performed at the beginning and end of the trial. Fish were anesthetized with MS-222 (200 mg/L) and measured for length, body weight, and tissues weight. Condition factor (CF), specific growth rate (SGR, % per day), hepatosomatic index (HSI), and gonad adipose tissue index (GAI) were calculated as follows: condition factor (K) = weight/length^3 × 100; specific growth ratio (SGR, %) = 100 × (lnFBW–lnIBW)/time (days); HSI: hepatosomatic index (%) = (liver weight/body weight) × 100; GAI: gonad adipose tissue index (%) = (gonad adipose tissue weight/body weight) × 100; where IBW and FBW are initial and final body weight (g) of fish.

Measurement of lipid content and lipase activity

Fat was reported to accumulate around gonad in Amur sturgeon. We defined this compound as gonad adipose tissue for the first time. The lipid content of the muscle, liver, and gonad adipose tissue of Amur sturgeon ($n = 4$) was analyzed according to Folch's method (Folch et al.1957). Serum parameters included total cholesterol (TC, mg/L), triacylglycerol (TG, mg/L), high density lipoprotein cholesterol (HDL-c, mmol/L) and low density lipoprotein cholesterol (LDL-c, mmol/L) were quantified with commercially available reagent kits using BS180 Automated Biochemistry Analyzer (ShenZhen Mindry Bio-Medical Electronicsco., LTD, Guangzhou, China).

Portions of the frozen liver, gonad adipose tissue and muscle samples from sturgeons (4 fish from each stocking density) were homogenized in nine volumes of homogenization buffer (0.01 mol/L Tris-HCl, 0.0001 l mol/L EDTA-2Na, 0.8% NaCl liquor and 0.01 mol/L sucrose) at pH 7.4. All analyses were conducted in triplicate. Homogenates were centrifuged at 20,000*g* at 4 °C for 20 min and the clear intermediate phase between the top layer and the pellet was used for LPL and HL activity assays. Both LPL and HL activities were determined using UV spectrophotometry with commercially available reagent kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. One unit of LPL or HL activity was defined as 1 μmol of free fatty acid released per gram of tissue protein per hour in the reaction system.

Molecular cloning of LPL and HL cDNAs

Total RNA of different tissues was extracted using RNAiso reagent (Takara, Japan) following the manufacturer's protocols. The concentration and purity of

extracted RNA were quantified using a UV spectrophotometer (ChampGel 5000, China) by $A260_{nm}/A280_{nm}$ ratios. A 1.5% agarose gel was used to detect RNA integrity. After RNA quality was determined, first-strand cDNA was synthesized from 1 μg of total RNA from each sample using random primers and reverse transcriptase M-MLV (Takara, Japan) in a 10-μL reactions.

Based on the sequences of reported Chinese sturgeon LPL (GenBank ID: FJ436088) and HL (GenBank ID: FJ436062) cDNA sequences, two pairs of primers (Table 1) were designed, respectively. PCR reaction was performed in a final volume of 25 μl containing 1 μl of cDNA from the liver following the Taq polymerase manufacturer's instructions (Takara, Japan). Touchdown PCR reaction was carried out using a Biometra TPersonal Thermal Cycler (Biometra, Germany) as follows: initial denaturing at 95 °C for 10 s, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 68 to 58 °C, decreasing 1 °C each cycle, and 30 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, finally ended with 10 min at 72 °C for extension. PCR products were analyzed on a 1.5% *w/v* agarose gel and visualized by ethidium bromide staining. Putative gene fragments were subcloned into the PEASY-T1 vector (Tiangen, China), transformed in *E. coli* (Trans5α, Transgen, China) and sequenced with an ABI3730XL sequencer (ABI, USA).

Based on the obtained fragments of LPL and HL cDNA, primers were designed for amplification of the cDNA ends of the two genes using the SMART™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's instructions. The resulting product was PCR amplified with a universal primer (UPM) and a specific primer (Table 1). After PCR, the amplified 5′ and 3′ cDNAs were purified, cloned, and sequenced.

Sequence characterization and phylogenetic analysis of LPL and HL cDNA

Sequencing data were analyzed using online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Multiple sequence alignment of the amino acid sequences of LPL and HL were produced using ClustalX version 1.81 (Thompson et al. 1997). Signal peptide regions and potential N-glycosylation sites were predicted using SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and Gene Runner version 3.02 (Hasting Software, Hasting, USA), respectively. The phylogenetic tree was constructed using the neighbor-joining methods (1000 replicates, bootstrap phylogeny

Table 1 Primers used in this study

Primers name	Sequence (5′ → 3′)	Description
LPL-F	GCCTGAACTTCCTGACGATGACA	Amplification of cDNA fragment
LPL-R	TCGGTTGACCTTGTTGTTTGTGAG	Amplification of cDNA fragment
HL-F	ATGGTCGGTGGATGGGATGCT	Amplification of cDNA fragment
HL-R	GAAAGGTGCTCCGTTCGGGTAGA	Amplification of cDNA fragment
LPL5′-R	CCGCTGTTCCTCCGCATACTCAA	5′-RACE
LPL3′-R	ACAAGGTCAACCGAATCACAGGC	3′-RACE
HL5′-R	CTCTGTAGAAAACTGGTGGGACTC	5′-RACE
HL3′-R	GCTACAACATCAAGAAGGTCCGA	3′-RACE
LPL-e-F	AAGGTGGTGTTCAGTGCC	RT-PCR and qPCR
LPL-e-R	CAGGTCTTTTGCGTTCGT	RT-PCR and qPCR
HL-e-F	TTATGTCCCAAAATCCAACTTCCG	RT-PCR and qPCR
HL-e-R	TCCAGCATCCCATCCACCGA	RT-PCR and qPCR
PPAR-e-F	GAACAAGGAGGCAGAGGTGAG	RT-PCR and qPCR
PPAR-e-R	TATGAAGCCATTCCCGTAAGC	RT-PCR and qPCR
18S-e-F	GCCACACGAGATGGAGCA	Reference primer
18S-e-R	CCTGTCGGCGAAGGGTAG	Reference primer

test) of MEGA4.1 software (Tamura et al. 2007) based on LPL and HL amino acid sequences.

Tissue distribution pattern of LPL and HL transcripts

The expression profiles of Amur sturgeon LPL and HL mRNA were determined by semi-quantitative RT-PCR using 18S as internal control. Total RNA were extracted from the liver, heart, intestine, stomach, kidney, spleen, gonad adipose tissue, fin, gill, muscle, brain, and pituitary using RNAiso reagent (Takara, Japan). A uniform quantity of DNA-free RNA was used to synthesize first-strand cDNA using reverse transcriptase M-MLV (Takara, Japan) following manufacturer's protocol. Semi-quantitative RT-PCR program was carried out 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 (primers were listed in Table 1). PCR products were checked on a 1.5% agarose gel and visualized by ethidium bromide staining on a Gel system (ChampGel 5000, China).

Quantitative real-time PCR of LPL, HL and PPARα transcripts

The expression levels of PPARα, LPL, and HL genes at different stocking densities were measured by the quantitative real-time PCR. Equal quantities of total RNA in the same tissue of 3 fish from the same stocking density were mixed together to synthesize the first strand cDNAs. PCR amplification was performed using the iCycler iQ Multicolor Real-Time PCR Detection System (Eppendorf, Hamburg, USA) and the iQ™ SYBR Green Supermix (Takara, Japan) following the manufacturer's instructions. The gene-specific primer pairs for LPL, HL, and PPARα are listed in Table 1. The real-time PCR conditions used the following conditions: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, annealing at 58 °C (for LPL) or 61 °C (for HL) of 20 s respectively, and extension at 72 °C for 20 s. The samples from normal control with serial dilutions of total cDNA were used as calibrators in this research. The relative mRNA levels were normalized by the comparative $2^{-\Delta\Delta CT}$ method using the 18S rRNA gene as internal controls (Livak and Schmittgen, 2001).

Statistical analysis

All data were expressed as mean values ± standard error of the mean. One-way analysis of variance (ANOVA) was used to analyze the relevant values and Duncan's multiple range tests was used for post hoc comparisons. Statistical significance was considered as $P < 0.05$.

Results

The effects of stocking density on growth performance

Our results indicated that stocking density had a significant effect on growth performance of Amur sturgeon after 70-day breeding (Table 2). The final body weight showed a decreasing tendency with increasing stocking densities. Fish farmed in HSD group showed the lowest final body weight (364.5 ± 6.3 g), while those in the lowest density showed the highest final body weight (407.2 ± 7.5 g) ($P < 0.05$). Similar tendency was observed for SGR and CF, with both SGR and CF decreased significantly in HSD group, which ranged from (0.8 ± 0.03)% to (0.7 ± 0.02)% and (0.7 ± 0.04)% to (0.6 ± 0.02)%, respectively (Table 2). HSI levels showed no considerable differences among the three stocking densities after 70 days. However, GAI in HSD group (0.1 ± 0.01) were significantly lower than

Table 2 Statistics for growth parameters of Amur sturgeon reared at different stocking densities after 70 days

Parameters	Stocking densities (kg/m^3)		
	LSD	MSD	HSD
IBW (g)	229.2 ± 9.4	220.8 ± 3.5	227.5 ± 4.1
FBW (g)	407.2 ± 7.5^a	383.3 ± 5.0^b	364.5 ± 6.3^c
SGR (%)	0.8 ± 0.03^a	0.8 ± 0.02^a	0.7 ± 0.02^b
CF	0.7 ± 0.04^a	0.7 ± 0.03^a	0.6 ± 0.02^b
HSI (%)	3.0 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
GAI (%)	0.2 ± 0.02^a	0.2 ± 0.03^a	0.1 ± 0.01^b

Values are the mean ± standard error of mean (SEM) ($n = 12$). Values not sharing a common letter are significantly different ($P < 0.05$)

IBW initial body weight, *FBW* final body weight, condition factor (*CF*) = weight/length^3 × 100, specific growth ratio (SGR, %) = 100 × (lnFBW−lnIBW)/time (days), *HSI* hepatosomatic index (%) = (liver weight/body weight) × 100, *GAI* gonad adipose tissue index (%) = (gonad adipose tissue weight/body weight) × 100

that of in LSD group (0.2 ± 0.02) and in MSD group (0.2 ± 0.03) ($P < 0.05$) (Table 2).

The effects of stocking density on lipid content

After 70-day breeding, the changes of lipid content of the liver, muscle, and gonad adipose tissue exhibited the similar tendency among tested fishes at different stocking densities (Table 3). Fish subjected to higher stocking density had lower total lipid content in the liver and muscle ($P < 0.05$). Meanwhile, lipid content of gonad adipose tissue was also negatively correlated with stocking density. After 70-day culture, the lipid content in gonad adipose tissue in HSD group was (51.8 ± 2.5) % wet weight, while in LSD group and MSD group that were (62.6 ± 4.7) and (57.5 ± 3.5) % wet weigh, respectively.

The statistical changes of four serum lipid parameters in Amur sturgeon at different stocking densities after 70-day breeding were listed in Table 4. In line with the values for lipid content described above, the serum concentrations of total cholesterol and triglyceride decreased with increasing stocking density, which showed significant difference between LSD and HSD groups ($P < 0.05$). However, these two indexes in MSD showed no considerable differences with other two stocking densities after 70 days. The high-density lipoprotein cholesterol (HDL-C) level at LSD and MSD group (0.3 ± 0.03 mmol/L and 0.3 ± 0.04 mmol/L) were significantly higher than that at HSD group (0.2 ± 0.02 mmol/L). Moreover, the changes in serum concentrations of low-density lipoprotein cholesterol (LDL-C) were not significantly among different stocking densities ($P > 0.05$) (Table 4).

Table 3 Lipid content (% wet weight) of different tissues in Amur sturgeon after 70-day breeding

Parameters	Stocking densities (kg/m^3)		
	LSD	MSD	HSD
Liver	16.9 ± 2.2^a	14.7 ± 1.9^a	10.1 ± 2.0^b
Muscle	5.4 ± 0.1^a	5.2 ± 0.2^a	4.2 ± 0.1^b
Gonad adipose tissue	62.6 ± 4.7^a	57.5 ± 3.5^{ab}	51.8 ± 2.5^b

Values are the mean ± standard error of mean (SEM) (n = 4). Values not sharing a common letter are significantly different ($P < 0.05$)

Table 4 Statistics for lipid parameters in serum of Amur sturgeon reared at different stocking densities after 70 days

Serum parameters	Stocking densities (kg/m^3)		
	LSD	MSD	HSD
Total cholesterol (mmol/L)	2.8 ± 0.6^a	2.6 ± 0.7^{ab}	2.0 ± 0.5^b
Triglyceride (mmol/L)	7.1 ± 0.4^a	6.6 ± 0.7^{ab}	6.0 ± 0.4^b
HDL-C (mmol/L)	0.3 ± 0.03^b	0.3 ± 0.04^b	0.2 ± 0.02^a
LDL-C (mmol/L)	1.0 ± 0.3	1.0 ± 0.3	0.9 ± 0.2

Values are the means ± SEM (n = 4). Values not sharing a common letter are significantly different ($P < 0.05$)

HDL-C high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol

The effects of stocking density on LPL and HL activity

The activities of LPL and HL in liver, muscle, and gonad adipose tissue were measured after 70-day culture (Fig. 1). LPL activity in gonad adipose tissue was significantly lower in HSD group (3.0 ± 0.2 U/mgprot) compared with that in LSD group (2.3 ± 0.2 U/mgprot) ($P < 0.05$), but showed no significant difference with MSD group (Fig. 1a). LPL and HL activity in the liver showed a similar change, which were inversely correlated with stocking density ($P < 0.05$). Moreover, LPL activity in muscle showed a decreasing trend after 70-day breeding, which decreased from 2.1 ± 0.3 to 1.2 ± 0.2 U/mgprot ($P < 0.05$) with the increasing density. The HL activity was significantly lower ($P < 0.05$) in the fish raised at HSD group than in the fish subjected to the other treatments (Fig. 1b).

Molecular characterization and phylogenetic analysis of LPL and HL

The full-length cDNAs of LPL and HL were obtained from the liver of Amur sturgeon by assembling the core fragment, 5′ and 3′ end sequences. The complete nucleotide sequences of LPL (Genbank accession number: JX948105) covered 1757 bp, containing a 1506-bp open reading frame (ORF) encoded a protein of 501 amino acids, a 139-bp 5′-untranslated terminal region (UTR), and a 112-bp 3′-UTR region with a canonical polyadenylation signal sequence AATAAA. The obtained full-length cDNA of HL is 1747 bp long

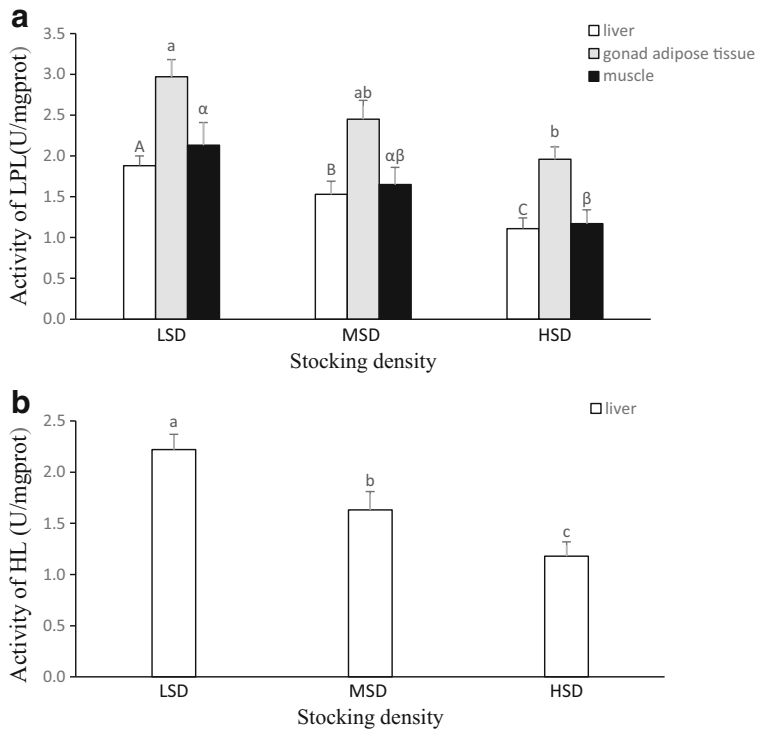

Fig. 1 Responses of HL (**a**) activity and LPL (**b**) activity in tissues of Amur sturgeon at different stocking density after 70-day breeding. Values are the means ± SEM ($n = 4$). Values not sharing a common letter are significantly different ($p < 0.05$)

(Genbank accession number: KC311364) with an ORF of 1500 bp encoding a putative protein of 499 amino acids, a 5′-UTR of 118 bp and a 3′ UTR of 139 bp. The deduced amino acid sequences of Amur sturgeon LPL and HL contained putative signal peptides, N-glycosylation sites and several other functional features, including conserved catalytic triad, putative lipid-binding region, and polypeptide "lid" (Fig. 2), all of which played important roles in lipid enzymatic function.

Phylogenetic analysis was conducted to study the evolutionary relationship of Amur sturgeon LPL and HL to those of other vertebrates (Fig. 3). Tree topology showed that the deduced amino acid sequences of LPL and HL clearly grouped into two clades, respectively. Both LPL and HL in the phylogenic tree were divided into two main groups. All fish species were in the same group forming a clade, distinguished from endotherms. Both genes of Amur sturgeon were first clustered with their respective counterparts form Chinese sturgeon (*Acipenser sinensis*), which belongs to the same Acipenseriformes family. The phylogenetic relationship based on LPL and HL amino acid sequences was in consistence with traditional classification.

Tissue distribution of LPL and HL transcripts

The tissue-specificities of Amur sturgeon LPL and HL were determined by semi-quantitative RT–PCR method using 18S as internal control (Fig. 4). The results showed that Amur sturgeon HL gene was mainly expressed in the liver, and lower expression levels were detected in the kidney, gonad adipose tissue, heart, muscle, brain and spleen. Expressions of HL in the gill, stomach, fin, stomach, intestine, and pituitary were not detectable (Fig. 4a). The expressions of LPL gene were detected in all examined tissues of Amur sturgeon. The highest expression level of LPL was found in gonad adipose tissue, followed by the muscle, heart, stomach, and liver, with the lowest expression in pituitary and brain (Fig. 4b). All the negative control exhibited no products.

Expression profile of LPL, HL, and PPARα at different stocking densities

The temporal expressions of LPL, HL, and PPARα in sturgeon at different stocking density were measured by quantitative real-time PCR (Fig. 5). After 70-day culture, the relative mRNA level of LPL in the liver of tested fish at HSD group were significantly lower than those of LSD

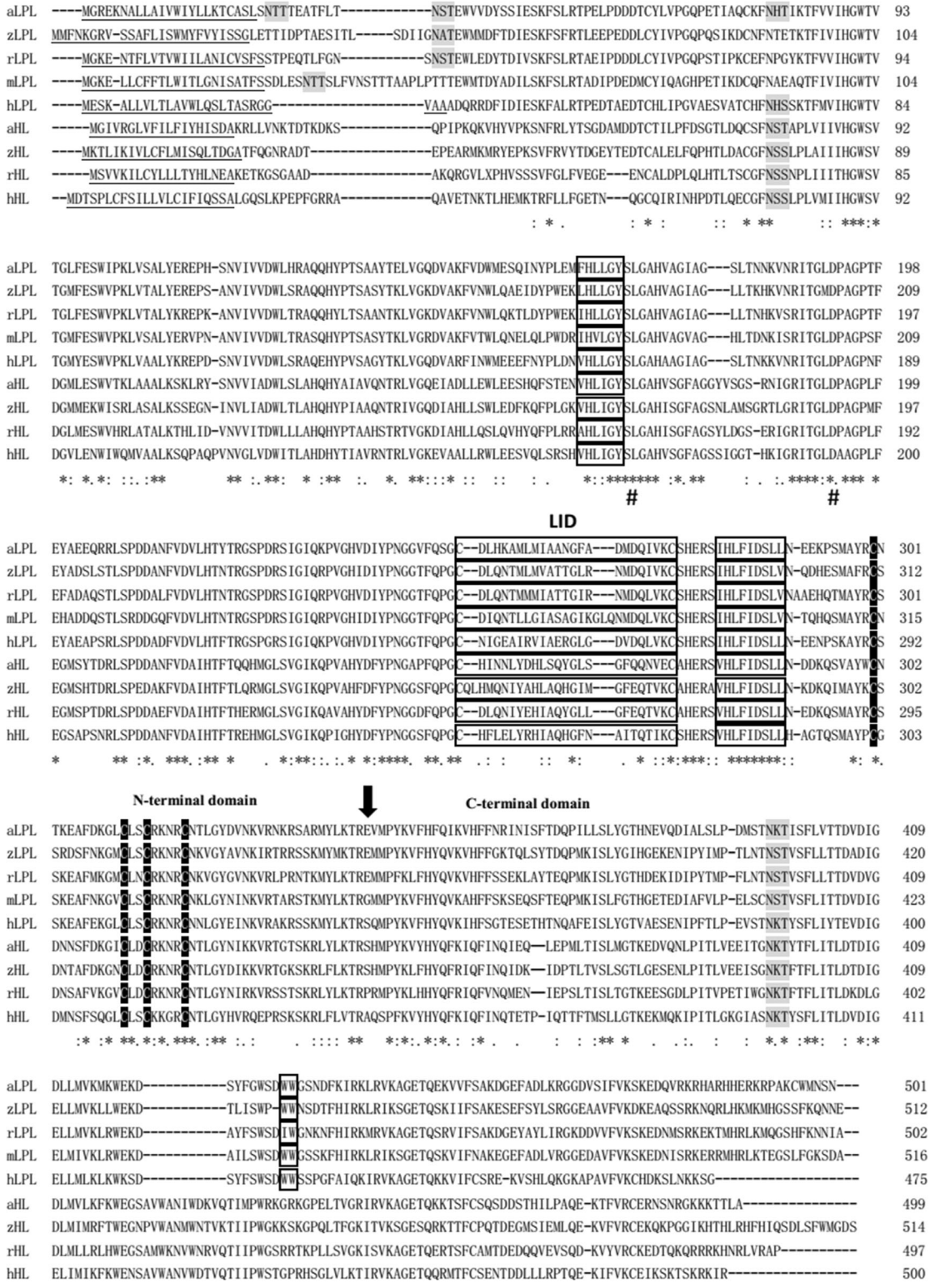
LID
N-terminal domain
C-terminal domain

Fig. 2 Multiple sequence alignment of LPL and HL amino acid sequences. Amur sturgeon LPL (aLPL, AGC24229) is aligned with LPL of zebra fish (zLPL, AAH64296;), medaka (mLPL, BAL61270), rainbow trout (rLPL, NP_001118076) and human (hLPL, CAG33335). Amur sturgeon HL (aHL, AGI04247) is aligned with HL of zebra fish (zHL, AAH53243), red seabream (rHL, ABY82366), and human (hHL, AAI36496). Putative signal peptides are *underlined*. Predicted N-linked glycosylation sites are gray shaded. Catalytic triad (*number sign*), identical residues (*asterisk*), conserved cysteine residue (*black shade*), putative lipid-binding region (*dotted box*), polypeptide "lid" (*solid box*), and N-terminal domain and C-terminal domain (*downwards arrow*) are indicated

group and MSD group ($P < 0.05$). The similar expression pattern of LPL was found in gonad adipose tissue and muscle, which decreased with the increasing density ($P < 0.05$) (Fig. 5a). Moreover, increased stocking density downregulated the expression of HL markedly in the liver ($P < 0.05$), which was about twofold higher in LSD group compared with that in HSD group (Fig. 5b). On the contrary, the expression patterns of PPARα in the liver and muscle increased significantly with the increasing density. After 70-day culture, the expression level of PPARα in fish liver of HSD group was significantly higher than those of LSD and MSD group ($P < 0.05$). The mRNA levels of PPARα in gonad adipose tissue

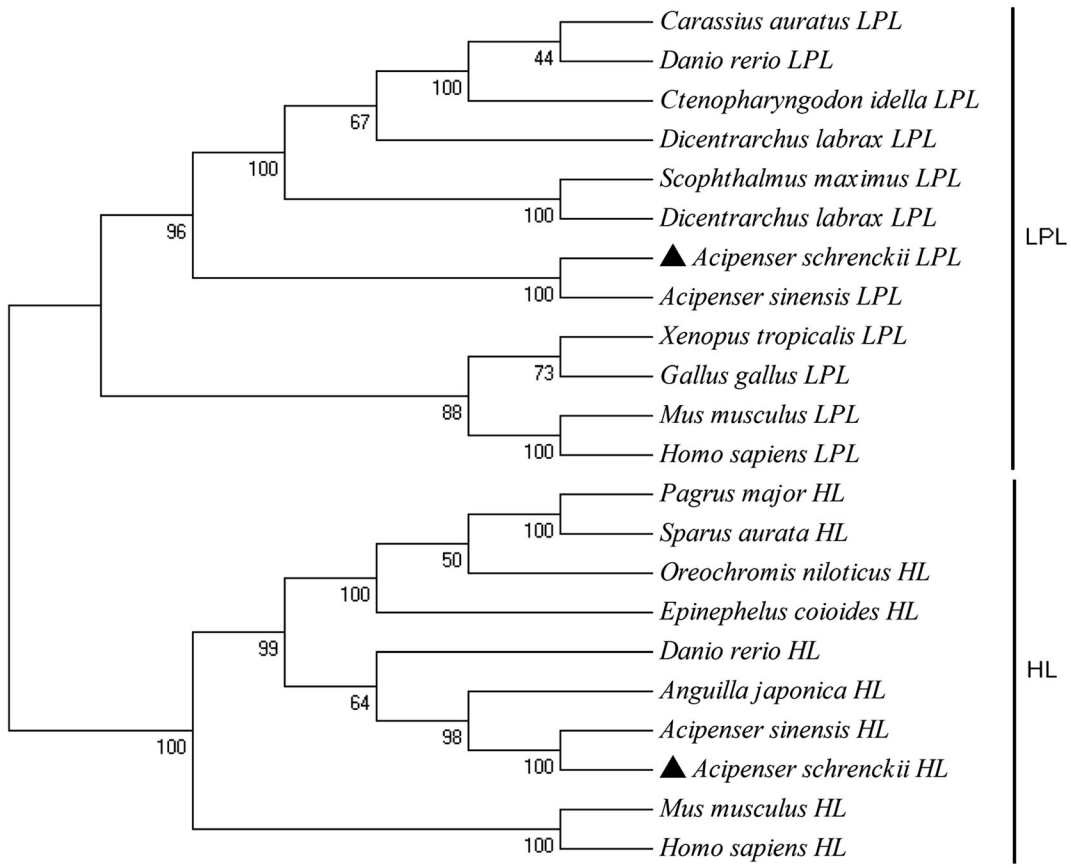

Fig. 3 Phylogenetic analysis of LPL and HL. The phylogenetic tree was constructed by MEGA 4.1 software using the neighbor-joining method following ClustalX. *Scale bar* indicates an evolutionary distance of 0.1 amino acid substitutions per position in the sequence. The GenBank accession numbers used in this analysis are as follows: *Acipenser schrenckii* LPL, AGC24229; *Acipenser sinensis* LPL, ACT22662; *Danio rerio* LPL, AAH64296; *Ctenopharyngodon idella* LPL, ACN66300; *Carassius auratus* LPL, ACN37860; *Oncorhynchus mykiss* LPL, NP_001118076; *Dicentrarchus labrax* LPL, CAL69901; *Scophthalmus maximus* LPL, AFH75405; *Xenopus tropicalis* LPL, XP_002934038; *Gallus gallus* LPL, NP_990613; *Homo sapiens* LPL, CAG33335; *Mus musculus* LPL, CAJ18552; *Acipenser schrenckii* HL, AGI04247; *Acipenser sinensis* HL, ACT22637; *Danio rerio* HL, AAH53243; *Anguilla japonica* HL, ACJ26849; *Oreochromis niloticus* HL, ACT22657; *Sparus aurata* HL, ABY82366; *Epinephelus coioides* HL, ACH53600; *Pagrus major* HL, BAF31236; *Homo sapiens* HL, AAI36496; and *Mus musculus* HL, AAO73443; The pound sign (*black up-pointing triangle*) indicates Amur sturgeon genes. Bootstrap support values appear on the branches

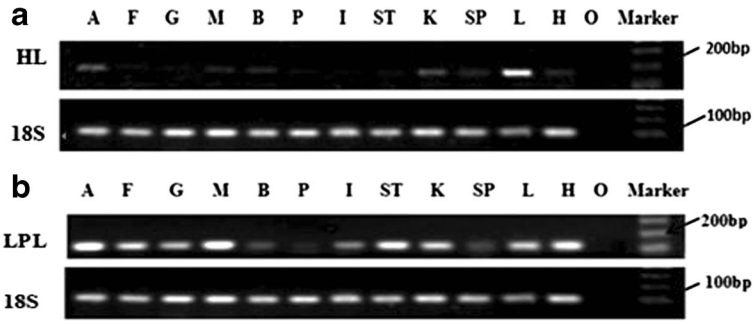

Fig. 4 Tissue expression pattern of LPL (**a**) and HL (**b**) in Amur sturgeon detected by RT–PCR The integrity of the RNA from the each tissue was ensured by uniform amplification of 18S transcripts (*lower panel*). *A* gonad adipose tissue, *F* fin; *G* gill; *M* muscle; *B* brain; *P*: pituitary; *I*: intestine; *ST* stomach; *K* kidney; *SP* spleen; *L* liver; *H* heart; *O* negative control (with water as the template); Marker molecular weight standard

showed no significant difference among fishes at different stocking density (Fig. 5c).

Discussions

In intensive fish rearing, stocking density is considered as an important issue concerning growth, metabolism, and welfare of farmed fish (Ashley 2007; Costas et al. 2008; De Oliveira et al. 2012; Hwang et al. 2014; Heras et al. 2015). In this study, high stocking density adversely affects growth performance of sturgeons, along with the reduction in body weight and decrease in SGR. Similar results were obtained in Nile tilapia *Oreochromis niloticus* (Telli et al. 2014). It has been demonstrated that high stocking density may suppress fish growth due to the deteriorating water quality, decreasing food consumption, and the alteration in metabolic state induced by crowding stress (Ellis et al. 2002; Lupatsch et al. 2010; Tolussi et al. 2010). In our study, water quality was not a limiting factor during the experiment because sturgeons were reared in flowing water with satisfactory physicochemical parameters. Fish were fed three times per day and proportionally to the number of fish in the ponds, which eliminated the effect of food consumption on fish growth performance. Consequently, alteration in metabolic state due to crowded stress induced by high stocking density may contribute to the growth suppression of fish. Combined with the reduced lipids of fish in high stocking density, we deduced the preliminary factors that impair fish growth were increased consumption of energy reserves, especially lipids with reallocation of metabolic energy caused by crowding stress.

In the liver of sturgeons reared at high stocking density, the total amounts of lipids were significantly reduced comparing with that at lower stocking density. This suggested a higher utilization of hepatic lipids when fish reared in a more stressful condition. The HSI in high stocking density show a slight decline. This finding was in agreement with the result observed in *Brycon insignis* (Tolussi et al. 2010) and *Salmo salar* (Basrur et al. 2010) that showed a lower HSI reared in high stocking density, indicating that fish in high density reallocate the energy to cope with the crowding stressor. Fish reared at higher stocking density also presented lower lipid concentration in the muscle and gonad adipose tissue, suggesting these two organs were also the lipid depot sites in sturgeon. Lipids storage in animal tissues is mainly determined by the state of synthesis and decomposition, which depends largely on the transport rate of triglyceride and fatty acid (Tolussi et al. 2010). The decreased lipids storage in sturgeons under crowding stress suggested the enhanced lipid mobilization and utilization. In addition, adipose tissue played a vital role regulating lipids deposition and mobilization. Moreover, gonad adipose tissue performed a crucial function and provides energy and fatty acids in gonad development of fish (Ibáñez et al. 2008; Petochi et al. 2011). It could be speculated that high stocking density was not conducive to gonad development and might affect the profitability in sturgeons farming for possibly affecting caviar producing. Further investigations will be needed to testify these hypotheses.

Plasma lipids, including triglycerides, cholesterol and phospholipids are essential for the metabolism of cells in vertebrates. Triglyceride is mainly involved in energy metabolism, while cholesterol is the fundamental components of cell membrane and participates in the

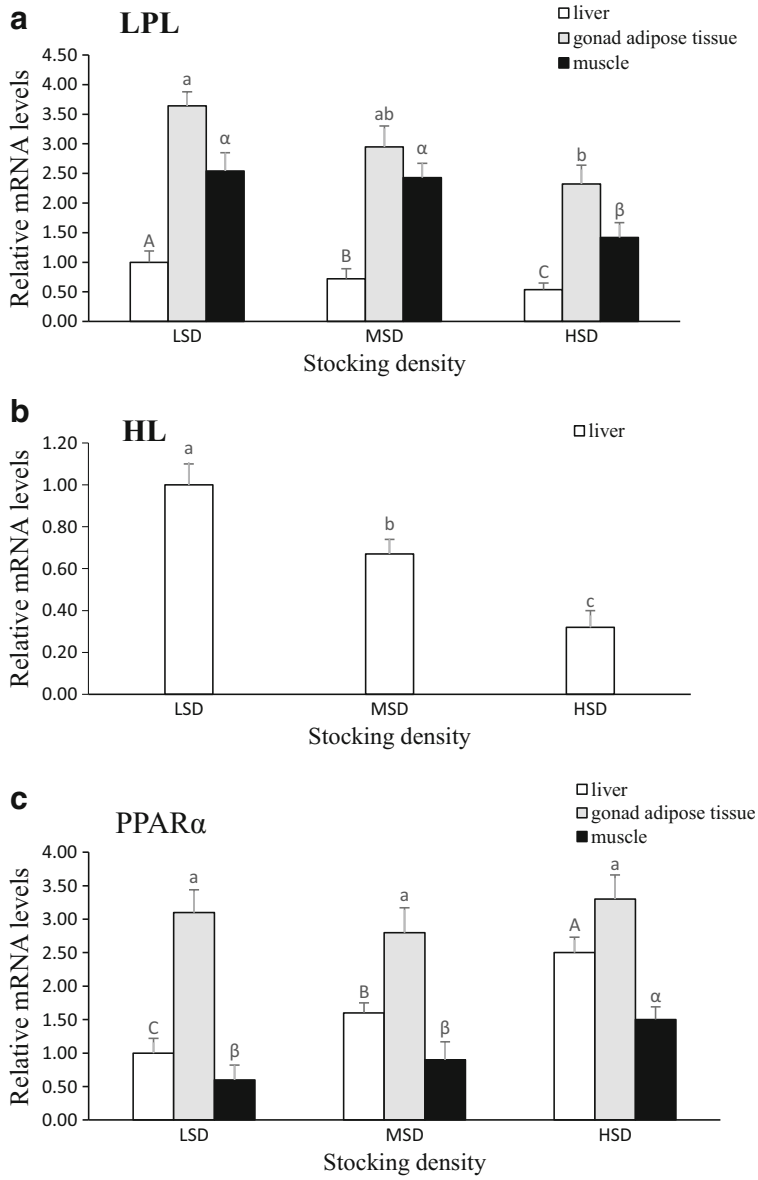

Fig. 5 Quantitative real-time PCR (qRT-PCR) analysis for the expression of the Amur sturgeon LPL (**a**), HL (**b**), and PPARα (**c**) genes in tissues after 70 days breeding at different stocking densities. An 18 s rRNA was used as internal control for qRT-PCR. Values are expressed as mean ± SEM ($n = 4$). *Different letters* indicate statistically significant differences ($p < 0.05$)

synthesis of steroid hormones. Previous study showed that the utilization of triglycerides in fish increased to cope with the more energy demand under crowding stress (Ni et al. 2014). Similar results were obtained in our study, showing that triglycerides concentration declined significantly in high stocking density, suggesting its role in energy metabolism during the stress of high stocking density. Morever, cholesterol and HDL-C levels in our study also decreased in the HSD group, which were similar to the previous study by Ni et al. (2014). In fish, HDL-C transports cholesterol from peripheral tissues to the liver for removal, while LDL-C transports cholesterol from the liver to peripheral tissues for utilization (Zhu et al. 2014). The decreasing of HDL-C in high stocking density suggests the more removal and less consumption of cholesterol in sturgeons under crowding stress.

The significant difference of lipid distribution in sturgeons of different stocking densities prompted our interest in the activities and expression of genes possibly connected with lipid metabolism. LPL and HL, which play critical roles in the absorption and metabolism of lipids and lipoproteins, were selected to analyze their variation in Amur sturgeon at different stocking densities.

Firstly, the full length cDNA sequences of LPL and HL were cloned from liver of Amur sturgeon, and the primary structures were determined. The deduced amino acid sequences contain putative signal peptides, N-glycosylation sites and several other functional features, including conserved catalytic triad, putative lipid-binding region, and polypeptide "lid" (Fig. 1), which play important roles in lipid enzymatic function. Among them, the Ser-Asp-His catalytic triad is the site where hydrolysis of triglyceride takes place (Santamarina-Fojo and Brewer 1994) and the polypeptide "lid" participates in substrate specificity (Saera-Vila et al. 2005). LPL and HL protein of Amur sturgeon shares a highest degree of homology and similar structural features with known LPL and HL of other species. This suggests that LPL and HL protein are highly conserved during evolution and might have similar functions in vertebrates. Though LPL and HL are two members of the lipase gene superfamily, they split to two main branches in the phylogenetic tree based on protein distances. This indirectly explains their differences in structure and suggests the diversity of lipase gene superfamily.

As a key enzyme in lipoprotein metabolism, LPL is expressed mainly in tissues related to lipids oxidation or storage (Ibáñez et al. 2008). Consistent with the observations in gilthead sea bream (Saera-Vila et al. 2005), Pengze crucian carp (Cheng et al. 2009), and red seabream (Kaneko et al. 2013), our results shows that sturgeon LPL gene is ubiquitously expressed in all tissues tested, especially highly in the skeletal muscle and liver, which are the major sites of lipid accumulation in fish (Kaneko et al. 2013). These findings indicated that the LPL is likely to play an important role in the lipid metabolism and deposition. Notably, high expression of LPL was also detected in sturgeon gonad, coinciding with the result in European sea bass (*Dicentrarchus labrax* L.), that LPL is highly expressed and active in the ovary during gonadal development to supply the oocytes with fatty acids (Ibáñez et al. 2008). In the present study, tissue distribution patterns of HL gene, which played important role in hydrolyzing phospholipids and triglycerides of plasma lipoproteins, were also determined. Different from the exclusively expression of HL in the liver in red sea bream (Oku et al. 2006), HL were detected in the liver, gonad, muscle, kidney in Amur sturgeon, suggesting its extensive roles in different tissues of fish.

In our study, the changes of lipid distribution, lipase activities and mRNA levels of lipid-related genes showed strong correlations. The activities and mRNA levels of LPL and HL in liver and gonad adipose tissue were markedly lower in high stocking density, which were consistent with the variation tendency of lipid content. This all together indicates high stocking density can affect lipid deposition in fish by regulating the transcriptional level of LPL and HL. Moreover, fish reared at high stocking density presented lower activities and mRNA levels of LPL in the muscle, which might reduce lipid metabolic rate and provide less energy for muscle growth. Adipose tissue, liver and muscle play central roles in regulating the balance between deposition and mobilization of lipid reserves (Bouraoui et al. 2012). The deposition of fat in tissues was related to the synthesis and decomposition of triglyceride in their fat cells. As important participants in lipids metabolism, LPL and HL controls triacylglycerol partitioning between muscle and adipose tissues, thereby affecting fat deposition or providing energy in the form of fatty acids for physiological processes (Hocquette et al. 1998; Peng et al. 2014). In high stocking density, the lower mRNA levels of LPL and HL suggested a decrease in lipid synthesis in tissues and more energy consumption caused by stress. This alteration of synthesis/oxidation of lipids could partly illustrate the poor growth performance of Amur sturgeon under chronic stress.

Fish reared in high stocking density need more energy expenditure to cope with stress, thus high consumption of energy reserves such as lipids, protein, and carbohydrate are required (Montero et al. 2001; Vargas-Chacoff et al. 2014). PPAR-α regulates lipid metabolism by affecting transcription of genes participating in peroxisomal and mitochondrial β-oxidation pathways and triglyceride catabolism (Philippe et al. 2006; Zhan et al. 2011). PPAR-α of Amur sturgeon was possibly primarily participating in fatty acid oxidation, which has been mentioned in mammals (Braissant et al. 1996). The mRNA expression of PPARα in both the liver and skeletal muscle increased significantly with the increasing density. This suggested enhanced lipid mobilization and utilization in fish under crowding stress, accounting for the reason of decreasing in lipid accumulation in sturgeons in high stocking density.

In conclusion, the present study showed that Amur sturgeon achieved better growth performance and higher lipid deposition in relatively low stocking density. High stocking density could negatively affect growth and the content of lipids in fish by altering the metabolic state and changing the expression patterns of several lipid-related genes. We characterized the cDNA sequences and/or

expression patterns of LPL, HL, and PPARα of Amur sturgeon. Our results indicated that LPL, HL, and PPARα may play obligatory roles in regulating lipid distribution and maintaining lipid homeostasis in fish cultured at different stocking densities, which help further the understanding of their functions in lipid metabolism in teleost. Further studies will be necessary to investigate the specific mechanism and signaling pathways of lipid metabolism that stocking density affects in fish.

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