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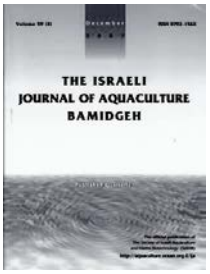

Hepatic Transcriptome Profiling under Shear Stress in *Sciaenops ocellatus*

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Keywords: flow velocity; quantitative PCR; *Sciaenops ocellatus;* shear stress; transcriptome

Abstract

Water flow is basic in the environment of fish. To date, most studies investigating the influence of water flow on fish behavior have focused on swimming speed and associated mechanisms, but few studies have addressed the molecular basis. This study used liver transcriptome profiling of *Sciaenops ocellatus* exposed to shear stress from the aquatic environment. De novo assembly yielded 70,148 genes with 12,465 unigenes > 1 kb. We identified 10,214 simple sequence repeat markers as well as 38,020 and 29,627 homozygous and 57,835 and 66,088 heterozygous single nucleotide polymorphisms in the experimental and control groups, respectively. After screening, there were 1773 differentially expressed genes, of which 204 were upregulated and 969 were downregulated. Gene Ontology analysis revealed 424 genes that were enriched in biological processes (90 in cellular component and 310 in molecular function). A total of 423 genes were annotated to a Kyoto Encyclopedia of Genes and Genome pathways (136 to metabolic pathways; 69 to environmental information processing; 67 to cellular process). There were four significantly enriched pathways, including glycine, serine, and threonine metabolism; tryptophan metabolism; retinol metabolism; and steroid biosynthesis. Some genes were known to be related to flow stress; those in the Hedgehog signaling pathway were upregulated, whereas others related to RNA degradation were downregulated. There was strong correlation between up- and downregulated genes identified by quantitative PCR and RNA sequencing. Thus, shear stress from aquatic environments greatly influences liver function in *S. ocellatus*. The results provide a reference that can be useful for selecting appropriate breeding sites for *S. ocellatus* aquaculture.

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Introduction

Water flow is a basic feature of the environment of fish and this affects growth, breeding, migration, and population distribution (Fu et al, 2013). To date most studies investigating the influence of water quality on fish physiology and behavior have focused on swimming velocity and associated mechanisms (López-Olmeda & Sánchez-Vázquez, 2011), with few studies addressing the molecular basis. Muscle transcriptome analysis, in rainbow trout (*Oncorhynchus mykiss*), revealed that genes related to energy regulation were highly expressed under maximum sustained swimming speed (Magnoni et al, 2013). Under continuous swimming conditions, genes controlling white muscle growth and development were upregulated (Palstra et al, 2013), whereas under the influence of flow velocity, genes in zebrafish (*Danio rerio*) associated with insulin-like growth factor and growth hormone receptors showed altered expression (Palstra et al, 2010). Changes in the levels of genes involved in immune function (Long et al, 2013), low temperature stress, and metabolism (Ulrike et al, 2010) have also been reported under these conditions. However, the effect of shear stress from water on liver physiology in fish has not been adequately addressed.

Sciaenops ocellatus (red drum) is an economically important fish species in China (Wu, 2016). There have been previous investigations concerning the effects of water velocity (Chao et al, 2017). In the present study, we examined changes in the liver transcriptome of *S. ocellatus* under maximum sustained swimming speed. The results provide a reference that can be useful for selecting appropriate breeding sites for *S. ocellatus* aquaculture.

Materials and Methods

Materials and equipment.

S. ocellatus fingerlings were obtained from Zhoushan City in China and acclimated for 2 weeks in laboratory tanks which were disinfected with sodium hypochlorite, aerated, and filled with natural filtered precipitated seawater (seawater is naturally precipitated) so the conditions were comparable and consistent with the natural growth environment of *S. ocellatus*. During the experiment, water temperature was 20°C-21°C; salinity was 27%-28%; dissolved oxygen was > 7.0 mg/L; a natural light source was used for illumination. A total of 25 fish were used for experiment (body length, 31.5-33.8 cm; body weight, 501.3-589.0 g). Basic physiological parameters were measured at the end of the experiment. Fish swimming was monitored and recorded with a camera to avoid the effects of human interference.

Sequencing of S. ocellatus liver transcriptome.

Before the experiment, the flume parameters (the flow rate of the flume was corrected, and flume function was checked (Chao, 2016)). Properties were corrected, maximum sustained swimming speed was determined, and flow rate was set to 0.9 m/s. The fish were acclimated to a flow rate of 0.2 m/s for 1 h (Chao, 2016). The experimental group (T01) was then acclimated to a flow rate of 0.9 m/s. Fatigue was noted when fish touched the net for more than 20 s. At the end of the experiment or when the experimental flow rate was 0 m/s (Liu et al, 2016), there were no statistically significant differences between T01 and the control group (T02) in terms of physiological parameters.

Sampling and processing.

The fish were removed from the tanks immediately after the experiment and the livers were rapidly dissected on an aseptic table, frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted from liver tissue using TRIzol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. RNA purity was assessed using a NanoPhotometer (Implen, München, Germany). RNA concentration was determined using the Qubit RNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was evaluated by 1% agarose gel electrophoresis and with the NA Nano 6000 Assay kit and Agilent Bioanalyzer 2100 (Di Pietro et al, 2011).

RNA library construction.

The RNA library was constructed and sequenced on an HiSeq 2500 platform (Illumina, San Diego, CA, USA) (Zheng et al, 2010). The cDNA library was constructed with Biomarker Technologies (Rohnert Park, CA, USA).

Bioinformatics analysis.

To obtain clean reads (high-quality data), we removed bases with quality < 1 for those shorter than four bases and < 13 for those shorter than six bases; the insert sequence was deleted along with polyA sequences and fragments less than 15 nucleotides in length. The Clean Data sequence was assembled, acquired the Unigene library of *S. ocellatus*, to evaluate the quality of sequencing library.

After qualification, expression analysis and gene structure analysis were performed, and differential expression analysis, differential expression gene function annotation, and functional enrichment analysis were performed according to gene expression in different sample groups.

Results

Sequencing data.

A total of 9.32 Gb of clean reads were obtained by sequencing (4.54 Gb for each group); the Q30 base percentage was > 92.12%. A total of 70,148 unigenes were obtained after de novo assembly, with 12,465 having a length > 1 kb; 31,704 functional annotation results were obtained using the Non-redundant, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups, Eukaryotic Orthologous Groups, Gene Ontology (GO), and Protein Family databases (Fig. 1 and Table 1).

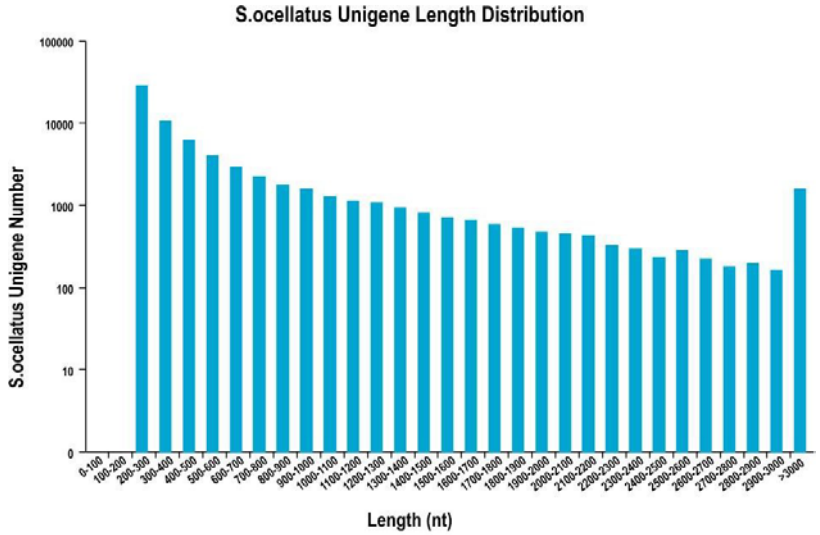

Fig. 1. The length distribution map of Unigene

Table 1. The assembly results.

	Assembly			
	Length range	*Contigs*	*Transcripts*	*Unigenes*
200-300	31303(0.93%)	32391(29.74%)	28527(40.67%)	200-300
300-500	18287(0.55%)	22359(20.53%)	16727(23.85%)	300-500
500-1000	13170(0.39%)	22046(20.24%)	12429(17.72%)	500-1000
1000-2000	8436(0.25%)	18920(17.37%)	8136(11.60%)	1000-2000
2000+	3071(0.09%)	13188(12.11%)	4329(6.17%)	2000+
Total number	3353834	108904	70148	Total number
Total length	188086190	100558459	46361983	Total length
N50 length	48	1654	1101	N50 length
Mean length	56.08	923.37	660.92	Mean length

Simple sequence repeat (SSR) analysis.

A total of 10,214 SSR markers were identified; the maximum number of single-base (mononucleotide) repeats was 6052, and there were at least two six-base (hexanucleotide) repeats. There were many two- and three-base pair repeats (1685 repeats of AC/GT and 404 of AGG/CCT) (Table 2).

Single nucleotide polymorphism (SNP) analysis.

There were 38,020 and 29,627 homozygous and 66,088 and 57,835 heterozygous SNPs in T01 and T02, respectively (Table 3).

Table 2. Length and number distribution of SSR based on the number of repeat units.

	Repeat units			
	single base	*two-base*	*trinucleotide*	*four-base*
5	0	0	789	153
6	0	829	425	25
7	0	483	296	1
8	0	307	21	2
9	0	389	0	0
10	1916	315	1	1
11	1218	104	0	0
12	774	6	2	0
13	536	0	0	0
14	402	0	0	0
15	367	0	0	0
16	304	0	0	0
17	148	0	0	0
18	93	0	0	0
19	102	0	0	0
20	95	0	0	0
21	63	0	0	0
22	26	0	0	0
23	7	0	0	0
24	1	0	0	0
total	6052	2433	1534	182

SSR =Simple Sequence Repeat. It is a simple repeating sequence widely distributed in the genome of eukaryotes.

Table 3. Statistics of SNP number.

	The kinds of SNP		
	homozygous SNP number	*heterozygous SNP number*	*total*
S1	38020	57835	95855
S2	29627	66088	95715

SNP= Single Nucleotide Polymorphism. The polymorphism of a nucleotide sequence caused by a change in a single nucleotide base.

Gene expression analysis.

Obtained reads were compared to those in GenBank using Bowtie5 (Assumpção et al, 2012) and to those in RSEM5 (Herbert & Steffensen, 2005), and their expression levels were evaluated and expressed as fragments per kilobase of transcript per million mapped reads. The abundance of transcripts encoded by the gene fragments varied significantly (Fulton, 2007) (Fig. 2).

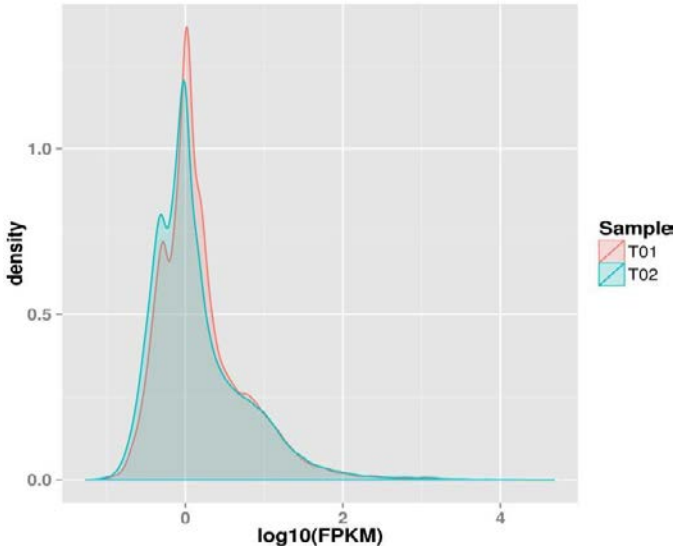

Fig. 2. The comparison of FPKM density in two samples

We analyzed the transcripts that were differentially expressed between T01 and T02 based on a default false discovery rate < 0.01 and fold difference ≥ 2 (Zeng et al, 2008). After screening, we identified 1773 differentially expressed genes, including 204 and 969 that were up- and downregulated, respectively. These are shown as volcano plots (Fig. 3).

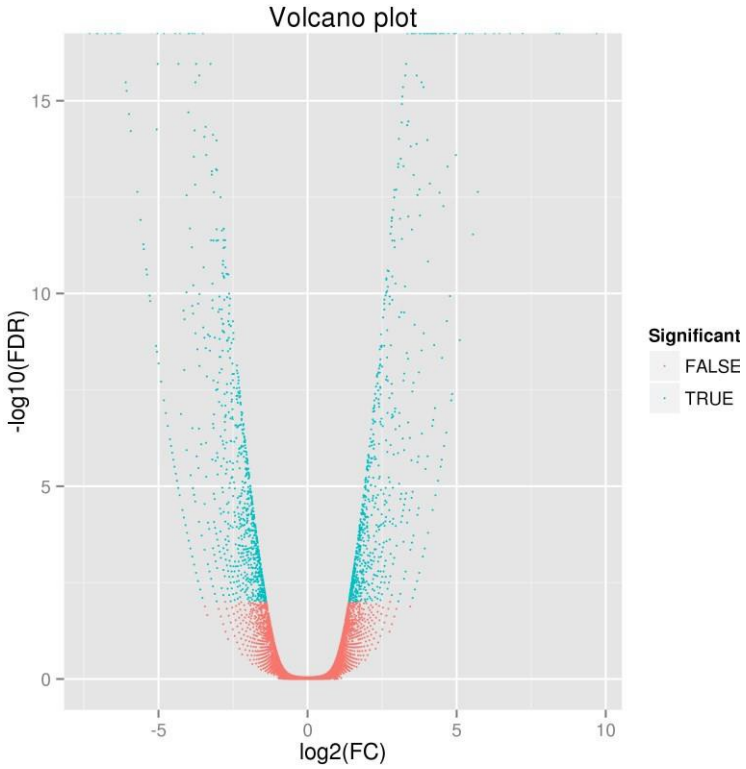

Fig. 3. The Differentially expressed gene volcanic map between T01 and T02

Cluster analysis of differentially expressed genes. Genes with similar expression patterns are in many cases functionally related. The expression patterns in the two groups were analyzed with cluster method. (Fig. 4). The results showed that the two samples had similar gene expression patterns, implying a high degree of functional similarity.

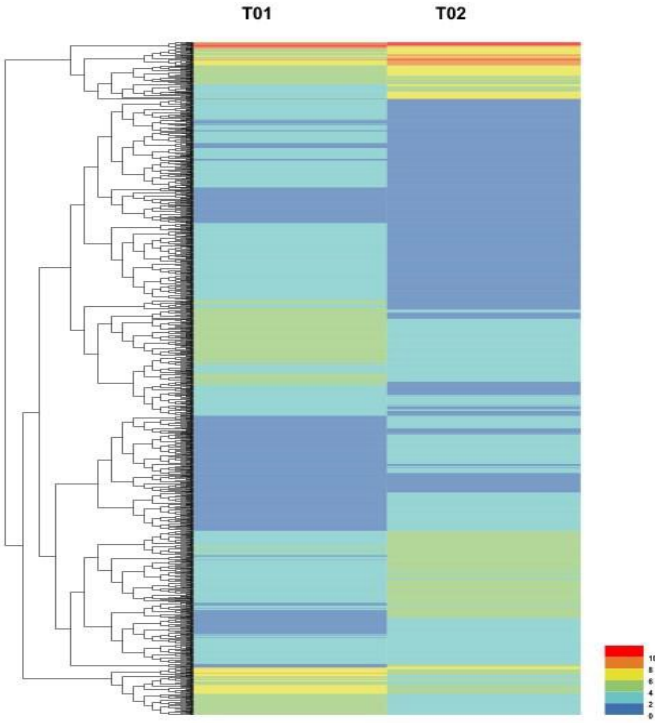

Fig. 4. Cluster analysis of differentially expressed genes in T01 and T02.

GO functional enrichment of differentially expressed genes. The gene ontology (GO) enrichment analysis revealed biological processes in S. ocellatus that were altered in response to shear stress, including 90 in Cellular components and 310 in Molecular function. In total, 80 differentially expressed genes were associated with Metabolic process, Oxidation-reduction, and Negative regulation of endopeptidase activity.

There were 90 different genes enriched in cell components, with significant enrichment in the GO terms extracellular region and extracellular space. In addition, among the 310 differentially expressed genes enriched in the molecular function category, only one (endopeptidase inhibitor activity) was significant. There were obvious differences between T01 and T02 with respect to GO term enrichment (Fig. 5).

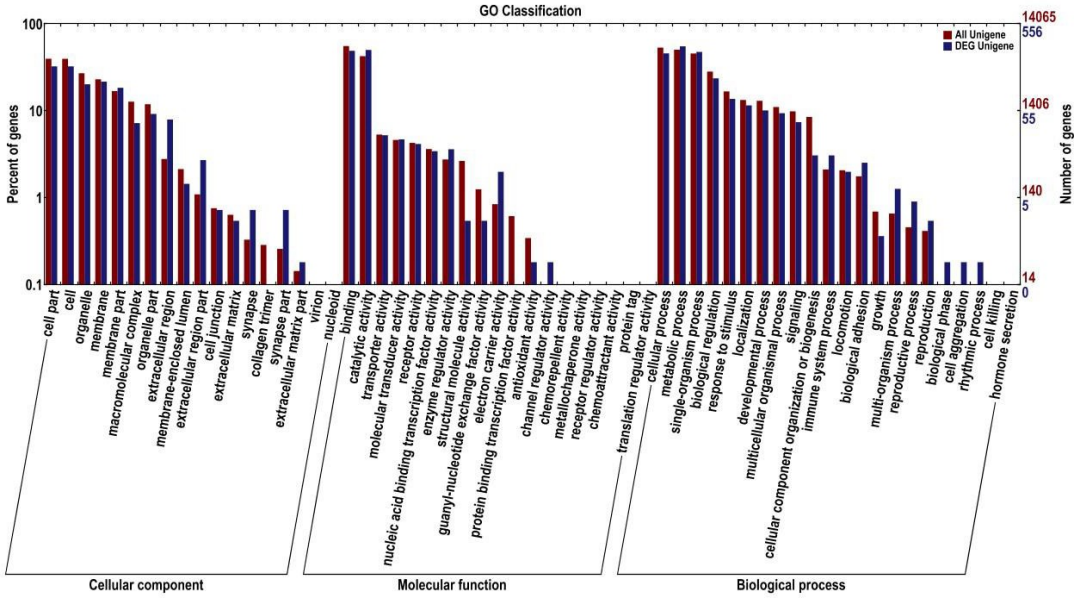

Fig. 5. Gene Ontology classifications of differentially expressed genes

KEGG annotation of differential expressed genes.

With the use of Kyoto Encyclopedia of Genes and Genomes (KEGG) we carried out pathway analysis of differentially expressed genes to clarify their functional significance. Of the 1096 differentially expressed genes that were analyzed, 423 were associated with a KEGG pathway,136 with metabolic pathways, 69 with environmental information processing, and 67 with cellular processes). Four metabolic pathways were significantly enriched (Q value ≤ 0.05 or less) specifically, those related to glycine, serine, and threonine metabolism (P = 0.0009); tryptophan metabolism (P = 0.0036); retinol metabolism (P = 0.0036); and steroid biosynthesis (P = 0.0303) (Fig. 5).

Differentially expressed genes related to swimming physiology.

Genes related to shear stress were identified in the KEGG metabolic pathway enrichment analysis of differentially expressed genes (Fig. 6); those involved in Hedgehog (Hh, protein kinase [PK]A, casein kinase [CK]1) and Wnt signaling were upregulated, while those associated with RNA degradation (decapping protein [Dcp]1 and enhancer of mRNA decapping, which exposes the transcript to 5' to 3' exonucleolytic degradation [EDC]3) were downregulated. The expression of transducer of ErbB-2 (TOB) was also increased. In steroid hormone biosynthesis, 17β-dehydrogenase and other enzymes related to estradiol, testosterone, and NADP+ regulation were downregulated, whereas glucuronic acid transferase was upregulated. In histidine metabolism, aldehyde dehydrogenase (NAD+), histidine ammonia lyase, and aspartoacylase were upregulated. Genes expressed in the liver which were related to fatigue under shear stress were compared to those expressed under normal conditions (Table 4).

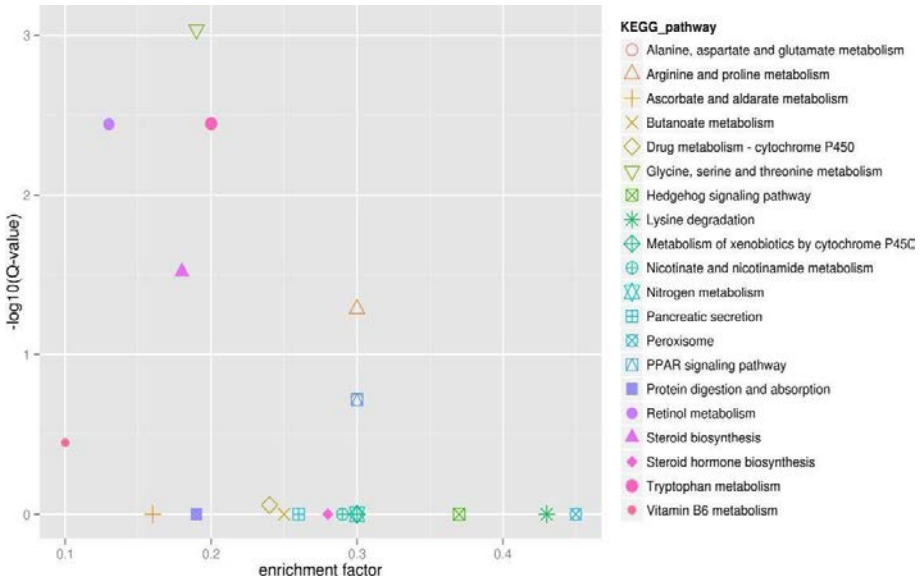

Fig. 6. Scatter chart of top 20 pathway enrichment of differentially expressed genes.

Table 4. GO and KEGG signaling pathways and differentially expressed genes related to swimming physiology.

Gene Ontology	*Differentially expressed*	
	No. of differentially expressed gene	*Corrected P-value*
oxidation-reduction process	53	0.000177
metabolic process	28	0.010358
proteolysis	28	1
transmembrane transport	21	1
extracellular region	29	9.25E-07
integral component of membrane	85	0.643520046
cytoplasm	36	1
nucleus	39	1
extracellular region	29	0.000000025
iron ion binding	17	0.005458
catalytic activity	17	0.521623
oxidoreductase activity	15	1
KEGG Pathway	*No. of differentially expressed gene*	*Corrected_P-value*
Glycine, serine and threonine metabolism	11	0.000922898
Tryptophan metabolism	10	0.003577881
Retinol metabolism	7	0.003610275
Steroid biosynthesis	7	0.030261253
Arginine and proline metabolism	11	0.051781012
Alanine, aspartate and glutamate metabolism	9	0.191261542
PPAR signaling pathway	9	0.191261542
Vitamin B6 metabolism	3	0.356052118

Verification of transcriptome data by sequencing.

The expression profiles obtained by RNA sequencing (RNA-seq) were validated by quantitative (q)PCR analysis of the mRNA levels of 11 differentially expressed genes (Table 5 and Fig. 7). Spearman's rho test revealed a significant correlation between RNA-seq and qPCR data (R2 = 0.9797).

Table 5. Comparisons between RNA-Seq data and RT-PCR results.

Gene symbol	*Comparisons*		
	Gene name	*qPCR*	*RNA-seq*
tshr	thyroid stimulating hormone receptor	-7.89	-8.34
gcgrb	Bglucagon receptor b	-7.02	-7.21
grm6a	6Aglutamate receptor,metabotropic 6a	-7.33	-6.43
mgr	merry-go-round	-5.82	-6.26
abi3b	ABI family,member 3b	5.29	5.99
ndufb8	NADH dehydrogenase NADH dehydrogenase (ubiquinone) 1 beta subcomplex,8	6.89	6.07
srp19	19 signal recognition particle 19	-5.03	-5.43
ewsr1a	1A EWS RNA-binding protein 1a	-6.43	-5.3
ambp	Bikunin alpha-1-microglobulin/bikunin precursorα	-6.13	-5.1
uqcc3	3 ubiquinol-cytochrome c reductase complex assembly factor 3	3.5	2.05
syt9a	synaptotagmin IXa	2.15	2.05

RNA-Seq= Transcription Sequencing. High-throughput sequencing for mRNA of a species.
RT-PCR=Reverse Transcription-PCR. It is a widely used deformation of PCR.
In RT-PCR, an RNA chain is reverse transcribed to complementary DNA, then it used as a template for DNA amplification by PCR.

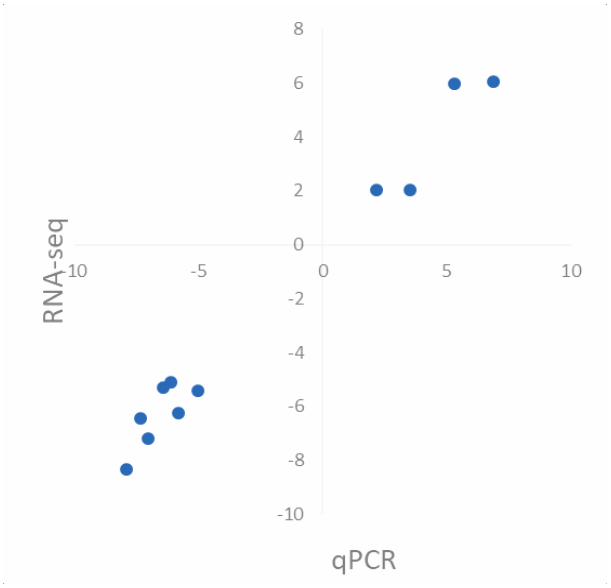

Fig. 7. Validation of RNA-seq data using qPCR

Discussion

The results of this study indicate that *S. ocellatus* has strong swimming ability, as previously reported (Wang et al, 2010). There are no reference genes for *S. ocellatus* (Li et al, 2008), and the effects of shear stress on the organism are unclear. The high-quality sequences in our library and our analyses of gene function provide a valuable resource for future molecular-level investigations on red drum.

Exogenous stressors such as changes in temperature, salinity, pH, and heavy metal concentration influence gene expression in fish (Gao et al, 2013). In this study, we analyzed genes that were differentially expressed in response to shear stress from water flow in *S. ocellatus*. There were 1773 genes whose expression differed between the experimental and control groups, of which 204 were upregulated and 969 were downregulated. These genes were related to processes such as sugar metabolism, RNA splicing, protein decomposition, ribosomal synthesis, and RNA splicing, among others. The highest enrichment of differentially expressed genes was observed for metabolic process, followed by oxidation/reduction and negative regulation of endopeptidase activity. Swimming fatigue in *S. ocellatus* is reflected mainly in liver glycogen levels (Chao et al, 2017). The profile of differentially expressed genes

indicates that *S. ocellatus* is largely dependent on aerobic respiration in the first 200 min of swimming, with glucose stores becoming depleted with sustained activity. Oxidation-reduction directly influences cell ageing and protein function (Wu et al, 2014). The enrichment of extracellular region and extracellular space GO categories indicates that swimming in *S. ocellatus* is accompanied by intense exchange of material with the environment.

Endopeptidase inhibitor activity was significantly enriched in the molecular function category. The hematopoietic and respiratory systems are affected by shear stress; detection of stress signals and intracellular signal transduction are critical for organism adaptation and survival under these conditions. Here we found that the expression of Hh, PKA, CK1, Wnt, and other genes related to Hedgehog signaling were upregulated along with TOB, in the T01 group whereas Dcp1 and EDC3, which are linked to RNA degradation, were downregulated. In the early division of zebrafish muscle cells, Hh signals can control fast and slow muscle fate (Xia et al, 2016). In addition to the decision to regulate the fate of the early two - element (slow and fast fiber type) cells, Hh signals also induce different types of cell formation in these pedigrees (Wolff et al, 2003). In the continuous swimming process of *S. ocellatu*s, it is speculated that the Hh signal will induce the formation of slow fiber and differentiate muscle in the neural tube of Shh and Wnt signal. Wnt signals play an important role in the process of embryonic myogenesis, and Wnt and Shh cooperation induced sarcomere formation (Münsterberg et al, 1995). In mature osteoblasts, the enhanced activity of Hedgehog signaling pathway will increase the expression of parathyroid hormone related proteins in cells. Through the signal regulation axis such as PKA, the binding protein of the cyclic adenosine phosphate response element is combined with the RANKL gene enhancer, promoting the expression of RANKL gene, inducing osteoclast to differentiate into mature osteoclasts, further enhancing bone resorption (Wu et al, 2017), and further improving the swimming ability of *S. ocellatus*. In mice, TOB is specifically expressed in the hippocampus and cerebellum (Wang et al, 2002). When *S. ocellatus* is in the process of continual motion, motion coordination will be significantly decreased in order to better adapt to the environment, with the up-regulated TOB gene. Steroid hormones are lipid-soluble compounds with a cyclopentane polyhydride structure (Hartmann, 1998). When *S. ocellatus* is in the process of swimming, sugar oxidation is reduced in steroid hormone biosynthesis, which increases blood sugar concentrations; moreover, other non-sugar substances such as proteins are converted to sugar, a process known as sugar dysplasia. Glucuronic acid transferase (UGT) is an important phase II metabolic enzyme and in rats and buffaloes (Jiang & Mao, 2003), UGT activity in the liver was decreased by infection. We speculate that in the continuous process of swimming, *S. ocellatus* liver glycogen consumption continued, there was immunity damage. In order to improve the activity of the liver, the expression of UGT and other genes increased, although the functional significance remains to be determined. Histamine 1 receptor is distributed in many cell types including endothelial and smooth muscle cells (Metz et al, 2011). We found that several enzymes related to histidine metabolism including aldehyde dehydrogenase and acyl transferase were upregulated in T01. Decarboxylation of histidine yields histamine, which can stimulate the dilation of blood vessels to accelerate exchange of materials and blood circulation during high-intensity activity such as swimming. We speculate that the reason for this is the strong endurance of *S. ocellatus*.

In summary, our study showed that shear stress alters the liver transcriptome profile of red drum. These results provide a basis for further investigation of the signaling networks that are activated in fish under shear stress, as well as useful information for selecting appropriate breeding sites for *S. ocellatus* aquaculture.

Acknowledgements

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