BF-1496
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RESEARCH ARTICLE Indian Journal of Animal Research

Transcriptomic Response to Sudden Salinity Drop in the Liver of Juvenile *Scatophagus argus*

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10.18805/IJAR.BF-1496

ABSTRACT

Background: *Scatophagus argus* is a euryhaline fish, but the molecular mechanism of salinity plunge response on liver antioxidant system of *S. argus* is currently poorly understood. Meanwhile, the transcriptome is a powerful tool for studying the effect of stress on physiological mechanisms. Transcriptome analysis of juvenile *S. argus* in salinity slump will provide a reference for future research on the stress resistance of *S. argus*.

Methods: 5 ppt was selected as low salinity and 20 ppt was used as the control group. The experimental treatment time was 6 h. Liver samples were separately obtained for transcriptome sequencing. Transcriptomic data of the liver after salinity plunge for 6 h were analyzed.

Result: 474 differentially expressed genes were generated by the sudden change of salinity. The pathways related to immune defense were enriched, such as 'Antigen processing and presentation' and 'Phagosome', implying that *S. argus* might enhance the immune defense system. Several antioxidant enzyme genes, such as *HAO*, *Trx*, *PHGPx*, were up-regulated to resist oxidative stress caused by low salinity. The down-regulation of *Cul3* also might promote the activation of *Nrf2* to activate the expression of antioxidant enzyme genes. These results indicated that *S. argus* initiated a molecular mechanism to resist low salt stress.

Key words: Antioxidant, *De novo* transcriptome, Salinity, *Scatophagus argus* (spotted scat).

INTRODUCTION

Scatophagus argus (spotted scat) with beautiful shape and gorgeous colors is a valuable brackish water aquarium fish and an important food fish. *S. argus* belongs to euryhaline fish with a salinity tolerance range of 5-35 parts per thousand (ppt) (Xu *et al.* 2019), which is mainly cultured in Hainan province, Guangdong province and other southern coastal areas. The total delicious amino acid and the ratio of delicious amino acid to total amino acids in the muscle of *S. argus* were close to those of *Siganus guttatus* and *Pampus argenteus* in the wild, showing *S. argus* was a kind of high nutritional value and delicious fish (Shi *et al.* 2015). The salinity of fish aquaculture might easily be affected by extreme weather (Xu *et al.* 2005). Therefore, rapid adaptation to the change of salinity in the environment is critical for fish. A previous study has confirmed that *S. argus* can be cultured in freshwater after salinity acclimation depending on the strong salinity adaptability (Lan *et al.* 2005). A recent study has shown that the mechanism of renal dopamine system-induced Na^+ transport was essential in the osmoregulation of *S. argus* (Su *et al.* 2019). Moreover, the liver of fish is also an important metabolic and immune organ, which is closely related to the antioxidant system. However, the molecular mechanism of salinity plunge response on the liver antioxidant system of *S. argus* is currently poorly understood.

Transcriptome analysis is a powerful tool for studying the mechanism of biological growth, stress physiology and disease-resistant immunity (Luo *et al.* 2015; Basang *et al.* 2018). The comparative transcriptome is widely used to analyze the effect of a variety of stress on physiological mechanisms, screening of genes related to environmental stress, growth and development in fish, shrimp and crab (Cui *et al.* 2013; Marie *et al.* 2017; Wang *et al.* 2019). In this study, we performed comparative transcriptome analysis on juvenile *S. argus* underlying low salt stress to identify genes and pathways related to antioxidant and immune which play important roles in *S. argus* liver to resist salinity slump. These results will increase the understanding of stress resistance in *S. argus* and provide insights into the molecular mechanisms of low salt stress.

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How to cite this article: Sun, X.N., Shen, C.C., Feng, G.P., Liu, J.Y., Song, C. and Yu, Y.F. (2022). Transcriptomic Response to Sudden Salinity Drop in the Liver of Juvenile *Scatophagus argus*. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1496.

Submitted: 20-01-2022 **Accepted:** 23-03-2022 **Online:** 12-04-2022

MATERIALS AND METHODS

Experimental animals and low salt stress experiment

The juvenile *S. argus* of the same batch of spotted scat in artificial breeding was selected randomly. The average body length of the selected group was (4.70±0.86) cm and the

average weight of the selected group was (6.27±0.28) g. During the acclimation period, the water temperature remained (22.0±0.5) °C and the salinity was at 20 ppt, 24 h for uninterrupted oxygenation. The juvenile fishes were fed with compound feed twice daily. The 2/3 of water in each tank was replaced every two days and cleaned the bottom dirt daily. After 30 days, healthy and non-mutilated juvenile *S. argus* were selected for the experiment. The experiment and analysis were carried out in the East China Sea Fisheries Research Institute from November 2019 to November 2020.

5 ppt was selected as low salinity group and 20 ppt was used as the control group. Three parallel groups were set for each salinity group and ten fishes were randomly placed in each parallel group. At the beginning of the experiment, individuals randomly taken from the temporary rearing group were directly put into the water of each salinity group. Experimental fishes were put into the water of each experimental group for the initial 0 h and the timing was started. At 6 h, samples were taken from the low salt group and control group, respectively and one fish in each group was taken in parallel. Liver samples were separately excised from treatment and control samples. After dissection, liver samples were frozen in liquid nitrogen and then stored at -80°C for RNA extraction.

RNA extraction, library construction and sequencing

The total RNA was extracted by RNeasy Plus Kit (Qiagen, Germany) following the manufacturer's procedure. The total RNA quality and integrity were analyzed using the Implen Nano Photometer and the Qubit RNA assay kit with a Qubit 2.0 fluorometer. Magnetic bead with Oligo (dT) and poly-A probes was used to isolate mRNAs from total RNA, which were used in downstream library construction, sequencing and transcriptome analysis. The library sequencing was performed on the Illumina HiSeq 2500 platform in Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) with a read length of 150 bp.

De novo sequence assembly and functional annotation

Raw reads were processed to remove reads with sequencing adaptors, unknown nucleotides and low quality by SeqPrep and Sickle software to obtain clean reads. Transcriptome assembly was accomplished by using Trinity. The longest transcript of each gene was used as unigene. The unigenes were annotated by six databases: non-redundant protein sequences (NR), swiss-prot protein, protein family (Pfam), clusters of orthologous groups of proteins (COG), gene ontology (GO) and kyoto encyclopedia of genes and genome (KEGG) databases.

Differential expression and enrichment analyses

To estimate the expression level of each gene, the expression levels of unigenes were measured by the fragments per kilobase of exon model per million mapped reads (FPKM) method. The analyses of differential expression genes (DEGs) between 5 ppt and 20 ppt groups were performed using EdgeR (P-value < 0.05). Additionally, GO and KEGG functional enrichment analyses were performed to identify DEGs that were significantly enriched in GO terms and metabolic pathways (P-value ≤ 0.05).

RESULTS AND DISCUSSION

Sequencing and assembly analyses

Illumina sequencing with *de novo* transcriptome assembly was used to acquire the transcriptome data in the liver of juvenile *S. argus*. 313,351,258 clean reads were obtained after filtering, comprising of 46.71 G bases (Table S1). Subsequently, clean reads were assembled into 103,264 transcripts with 73,721 unigenes of an average length of 1,185.18 bp and N50 of 2,914 bp (Table 1). Transcriptome sequencing is often used in genetic studies of fish, such as *Sebastes schlegelii*, *Pampus argenteus* and *Acrossocheilus fasciatus* (Shi *et al.* 2019; Cao *et al.* 2020; He *et al.* 2022).

Function annotation and classification

27,613 unigenes were annotated in the six databases, accounting for 37.46% unigenes (Table S2). The species distribution revealed that *S. argus* had the highest number of hits to *Larimichthys crocea* (4,812, 18.39%), followed by *Lates calcarifer*, *Perca flavescens*, *Seriola dumerili* and *Collichthys lucidus* (Fig 1). 24,742 unigenes were annotated by COG (Fig S1). 17,293 unigenes were classified into functional groups according to GO categories: biological process (BP), molecular function (MF) and cellular

Table 1: Summary of *de novo* assembly of juvenile *S. argus* transcriptome.

	Total	Total base	Min length	Max length	Mean length	N50	N90
Transcripts	103,264	156,826,789	201	27,185	1,518.70	3,317	3,664
Unigenes	73,721	87,372,868	201	27,185	1,185.18	2,914	4,614

Table 2: DEGs related to antioxidant function in liver.

Gene ID	Genes	Description	log_2FC	Regulation
TRINITY_DN1273_c1_g1	*Trx*	Thioredoxin	2.21	up
TRINITY_DN598_c0_g1	*TXNIP*	Thioredoxin-interacting protein	-1.12	down
TRINITY_DN5929_c0_g2	*PHGPx*	Phospholipid hydroperoxide glutathione peroxidase	1.23	up
TRINITY_DN40783_c0_g1	*HAO*	Hydroxyacid oxidase	2.68	up
TRINITY_DN16595_c0_g1	*Cul3*	Cullin-3	-1.27	Down

Table S1: Basic statistics of RNA-seq reads in juvenile *Scatophagus argus* obtained from Illumina HiSeq 2500.

Sample	Raw reads	Clean reads	Clean bases (G)	Error (%)	Q20(%)	Q30(%)	GC(%)
S5-1	52,800,542	52,509,750	7.82	0.02	99.19	97.14	49.44
S5-2	54,482,526	54,141,010	8.08	0.02	99.12	96.93	49.35
S5-3	54,126,220	53,742,972	8.00	0.02	99.00	96.57	49.57
S20-1	51,794,080	51,451,902	7.68	0.02	99.14	96.99	49.33
S20-2	51,250,542	50,907,020	7.60	0.02	99.10	96.90	49.50
S20-3	50,912,172	50,598,604	7.53	0.02	99.16	97.05	49.47
Total	315,366,082	313,351,258	46.71				

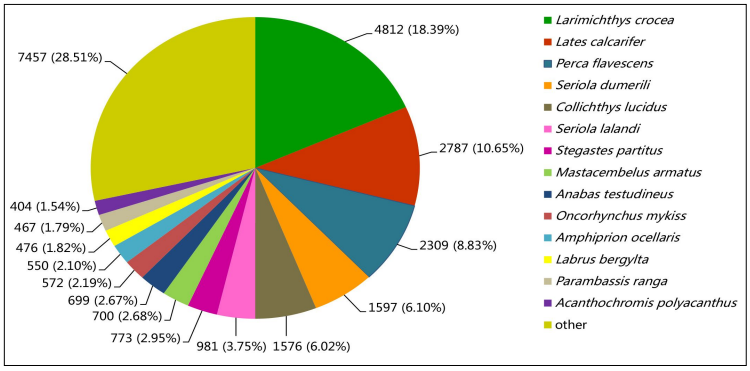

Fig 1: Species distribution of homology search of unigenes against the NR database.

Table S2: The success rate of note records of *S.argus* transcriptome data in the six databases.

Database	Number of unigene	Percentage
NR	26,160	35.49
Pfam	19,793	26.85
COG	23,840	32.34
Swiss-Prot	20,498	27.80
GO	17,293	23.46
KEGG	16,633	22.56
All databases	27,613	37.46
Total unigenes	73,721	100

component (CC) (Fig S2). In BP, the dominant subcategories were 'cellular process' (6,499, 37.58%) and 'metabolic process' (4,034, 23.90%). In MF, a high percentage of unigenes fell into 'binding' (8,685, 50.22%) and 'catalytic activity' (6,698, 38.73%). In CC, 'cell part' (5,754, 33.27%) and 'membrane part' (5,689, 32.90%) were most represented. Additionally, 16,633 unigenes can find significant hits in the KEGG database and the most unigenes were related to the 'immune system' (Fig S3).

Analysis of the expression of antioxidant related genes

474 genes showed significantly different expression in the low salt group vs control group, which included 240 up-regulated genes and 234 down-regulated genes (Fig 2). After salinity plummet, several antioxidant enzyme genes were significantly up-regulated, such as hydroxyl acid oxidase (*HAO*), thioredoxin (*Trx*) and phospholipid hydroperoxide glutathione peroxidase (*PHGPx*) (Table 2). *HAO* exists in peroxisomes, which catalyzes hydroxyl acid with the formation

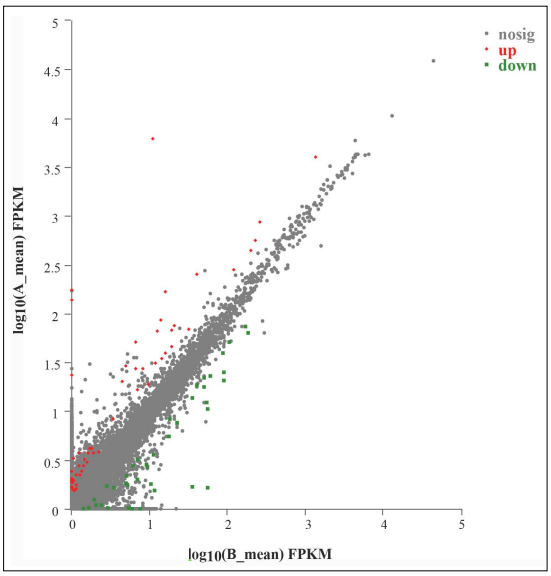

Fig 2: The scatter plot of differentially expressed genes of juvenile *S. argus* under sudden salinity drop. Note: the red dots indicate the genes that are significantly up-regulated, the green dots indicate the genes that are significantly down regulated and the black dots indicate the genes that are not significantly different.

of H_2O_2 (Su *et al.* 2020). *Trx* resists external oxidative pressure by regulating the redox state of cysteine in protein. *PHGPx* can specifically reduce phospholipid peroxide (Zhang *et al.* 2015). Moreover, thioredoxin interacting protein (*TXNIP*) was significantly down-regulated after salinity plummet for 6 h. *TXNIP* is an important regulator of the balance of redox reaction, which participates in the oxidative stress in cells

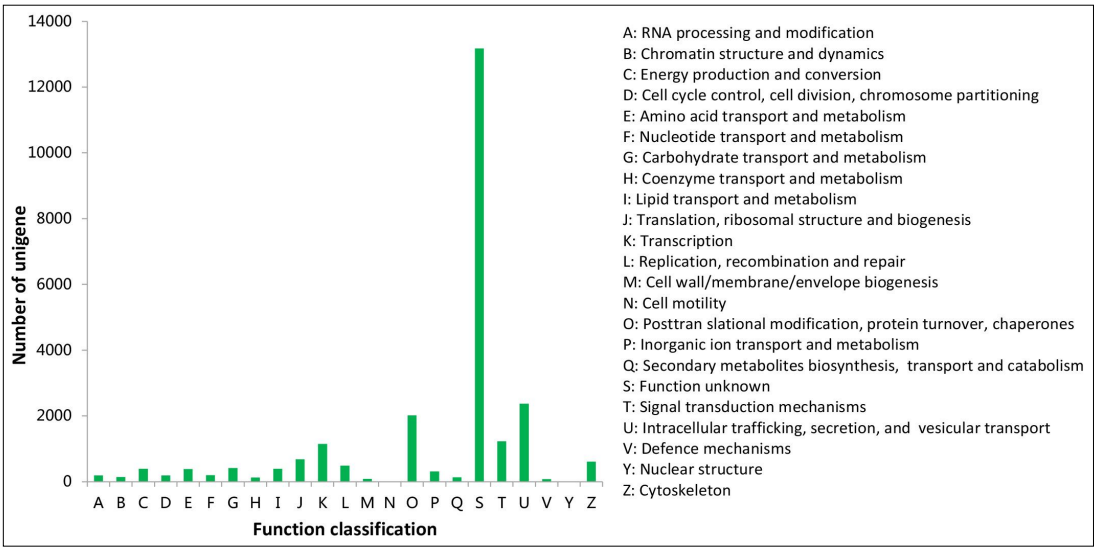

Fig S1: COG classification of the assembled unigenes for juvenile *S. argus* transcriptome. The category represented by each uppercase letter is displayed on the right side of the figure.

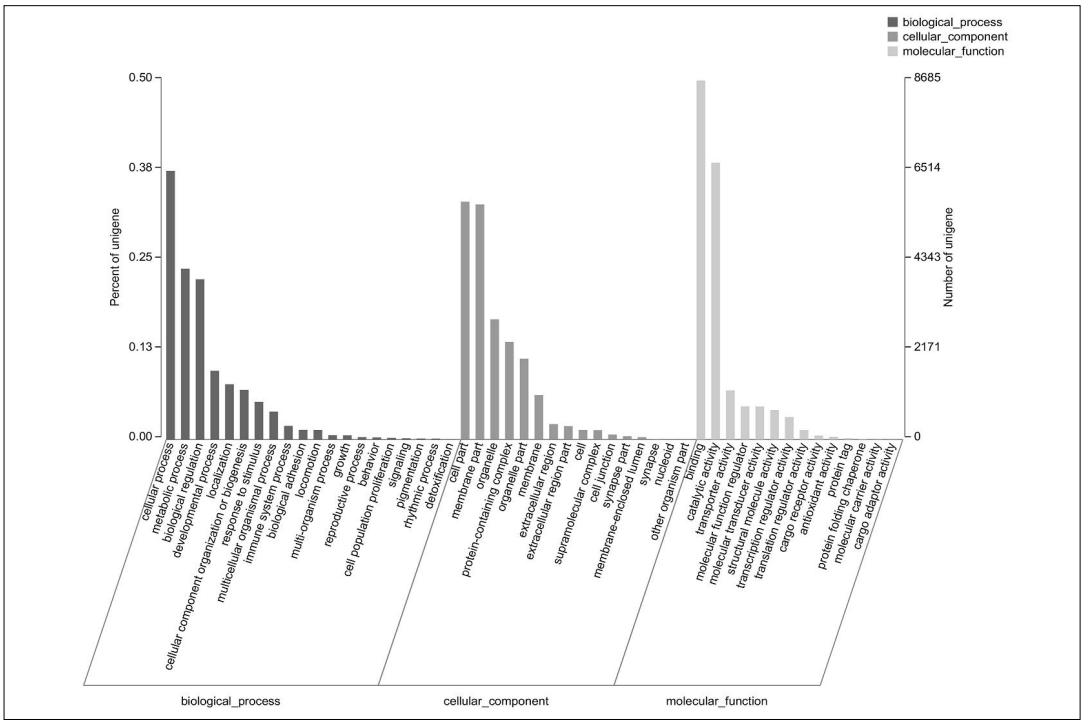

Fig S2: Statistical chart of GO secondary classification of unigenes.

and plays a role in inducing cell apoptosis by inhibiting the activity of *Trx* (Yang *et al.* 2011).These results demonstrated that *S. argus* might be in a state of oxidative stress and enzyme genes with antioxidant function were up-regulated to resist oxidative stress caused by low salinity.

Meanwhile, Cullin-3 (*Cul3*) was significantly down-regulated in this study. It was reported that the overexpression of *Cul3* induced polymorphonuclear ubiquitination of nuclear factor-erythroid 2 related factor 2 (*Nrf2*), thereby reducing the protein level of *Nrf2* and knock-out of *Cul3* or dominant inhibition of *Cul3* expression would reduce the polymorphonuclear ubiquitination of *Nrf2* (Kobayashi and Yamamoto 2006). *Nrf2* can maintain the oxidation balance, which is a master regulator of the antioxidant defense system (Wang *et al.* 2021). Under normal physiological conditions, *Nrf2* is anchored in the cytoplasm by Kelch-like ECH-associated protein-1 (*Keap1*), a substrate for the Cullin 3-dependent E3 ubiquitin ligase complex, in the *Nrf2-Keap1* signaling pathway and *Nrf2* can be ubiquitinated and rapidly degraded by proteasomes (Li

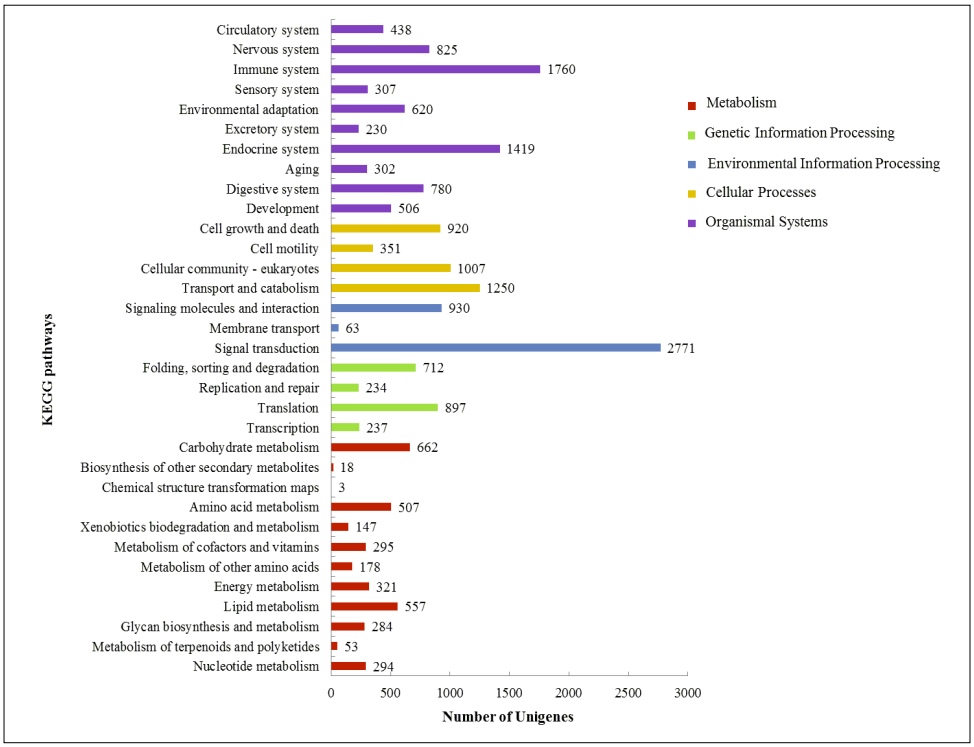

Fig S3: KEGG annotation statistics of Unigenes.

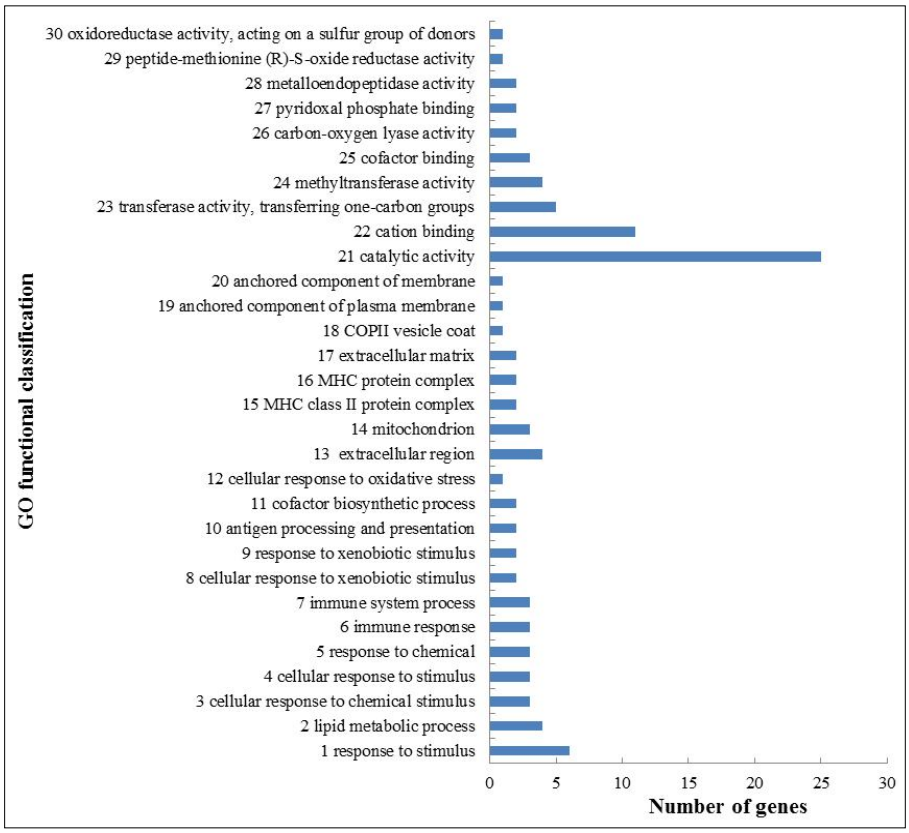

Fig 3: The GO enrichment of differentially expressed genes for juvenile *S. argus* transcriptome (1-12 Biological process; 13-21 Cellular component; 21-30 Molecular function).

Table 3: The top ten pathways of KEGG enrichment.

Metabolic pathway	KO pathway no.	Enrichment factor	Q
Glyoxylate and dicarboxylate metabolism	map00630	0.032	1.1026×10^{-1}
Hematopoietic cell lineage	map04640	0.031	2.0849×10^{-1}
Intestinal immune network for IgA production	map04672	0.033	2.1308×10^{-1}
Glycine, serine and threonine metabolism	map00260	0.032	2.5620×10^{-1}
Selenocompound metabolism	map00450	0.053	2.8065×10^{-1}
Phagosome	map04145	0.021	3.6075×10^{-1}
Ubiquitin mediated proteolysis	map04120	0.018	3.7516×10^{-1}
Th17 cell differentiation	map04659	0.022	3.7691×10^{-1}
Antigen processing and presentation	map04612	0.023	3.7692×10^{-1}
Hedgehog signaling pathway-fly	map04341	0.018	3.7830×10^{-1}

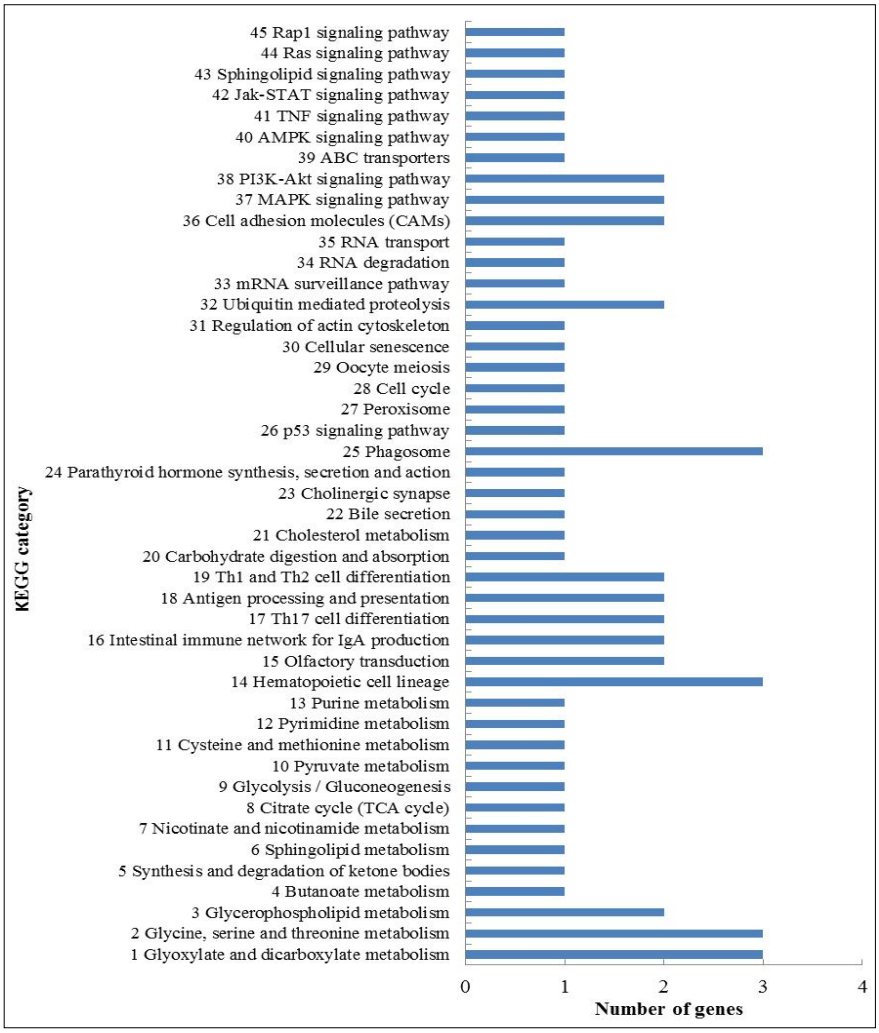

Fig 4: The KEGG enrichment of differentially expressed genes for juvenile *S. argus* transcriptome(1-13, Metabolism;14-24, Organismal systems; 25-31, Cellular processes;32-35, Genetic information processing; 36-45, Environmental information processing).

and Yang 2018; Wang and Zhu 2019). Unfortunately, no significant differences were found in the expression levels of *Nrf2*, *Keap1*, superoxide dismutase and glutathione S-transferase in the downstream signaling pathway in this study. It was speculated that the *Nrf2-Keap1* signaling pathway was in the inactive stage after salinity plunge for 6 h. The reasons can be attributed to several aspects: i) treatment time is too short to activate *Nrf2-Keap1* signaling pathway; ii) 5 ppt of the water environment is adaptable for *S. argus*. Despite this, the down-regulation of *Cul3* also might promote the dissociation of *Nrf2* from *Keap1* and the binding of antioxidant elements and then activate the expression of

antioxidant enzyme genes regulated by *Nrf2*. The specific molecular activation mechanism of the *Nrf2-Keap1* signaling pathway needs further investigation.

Functional enrichment analysis of DEGs

The GO terms 'extracellular region', 'catalytic activity', 'response to stimulus' and 'lipid metabolic process' were enriched in the CC, MC and BP, respectively (Fig 3). Studies on the salinity regulation mechanism of marine organisms, such as *Sinonovacula constricta*, *Nibea japonica* and *Pseudopleuronectes yokohamae*, found that the functions of DEGs were mainly concentrated in the category of 'catalytic activity' under different salinity stress (Cui *et al.* 2019; Ma *et al.* 2019; Meng *et al.* 2021). Moreover, previous studies had shown that the catalytic function of enzymes could regulate osmotic pressure when crustaceans were exposed to low salinity and *Acipenser brevirostrum* could maintain osmotic pressure by the metabolism of non-esterified fatty acids to adapt to higher salinity successfully (Evans 2008; Qian *et al.* 2010). These results indicated that *S. argus* might regulate catalytic and lipid metabolism to adapt to salinity plunge.

In the current study, KEGG pathways in the classifications, such as 'signal transduction', 'immune system' and 'transport and catabolism', played an important role in stress reaction (Song *et al.* 2019). Herein, pathways related to immune defense in the top ten KEGG pathways were enriched, including 'Intestinal immune network for IgA production', 'Phagosome' and 'Antigen processing and presentation' (Table 3 and Fig 4). In addition, 'MAPK signaling pathway' and 'PI3K-Akt signaling pathway', which are related to the activation of the Nrf2-Keap1 signaling pathway, also deserved attention (Chen *et al.* 2016; Ilaria *et al.* 2018). Taken together, these results implied that *S. argus* might enhance the immune defense system to better prepare for survival in low salt stress.

CONCLUSION

Overall, this study represents genetic resources and metabolic pathways related to antioxidant and immune of juvenile *S. argus* under sudden change of low salt based on liver transcriptome data. The antioxidant enzyme genes (*HAO*, *Trx*, *PHGPx*) were up-regulated, which play a crucial function in resisting oxidative stress. GO terms 'catalytic activity' and 'lipid metabolic process' were enriched, showing *S. argus* might regulate catalytic and lipid metabolism to adapt to salinity plunge. Pathways related to immune defense were enriched, including 'Intestinal immune network for IgA production', 'Phagosome' and 'Antigen processing and presentation', indicating that *S. argus* also might enhance immune defense to deal with sudden salinity drop in the environment. These results can provide a reference for exploring the physiological mechanism of environmental stress and the key genes of disease resistance of *S. argus* in the future.

ACKNOWLEDGEMENT

This study was financially supported by grants from the Central Public-interest Scientific Institution Basal Research Fund (NO. 2019Z01) and the National Natural Science Foundation of China (Grant No. 319727952).

Conflict of interest: None.

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