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Molecular Mechanism of Extreme Hypoxia Tolerance Difference between Male and Female Adult Fish and Juvenile Fish of *Acrossocheilus fasciatus* by Transcriptomics

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ABSTRACT

Background: Dissolved oxygen in water is an important limiting factor for fish. In this study, the suffocation point and transcriptomes analysis of *Acrossocheilus fasciatus* with different age and gender are helpful to analyze the effects of gender and age on extreme hypoxia tolerance.

Methods: First of all, we compared the difference in tolerance to extreme hypoxia among 15 fish from male adult fish, female adult fish and juvenile fish of *Acrossocheilus fasciatus* in each group by asphyxiation point experiment. Then, we analyzed the molecular mechanism of extreme hypoxia tolerance difference between male and female adult fish and juvenile fish by transcriptomics.

Result: Female adult fish of *Acrossocheilus fasciatus* showed the strongest tolerance to extreme hypoxia in the asphyxiation point experiment. In transcriptomes experiments in all samples, we found that the expression of *ncoa4* and *facl4* was significantly down regulated and the expression of jnk, gpx4 and jip-1 was significantly increased in females adult fish.

Key words: *Acrossocheilus fasciatus*, Asphyxiation point, Rranscriptomes.

INTRODUCTION

It is always worth thinking about the adaptability of different organisms to extreme hypoxia in nature. Different individuals have different responses to extreme hypoxia. Abundant researches showed that fish size has significant effect on hypoxia tolerance (Nilsson *et al.* 2010). Research discovered that small-sized *Megalobrama amblycephala* performed better in hypoxia tolerance than their fellows with larger sizes (Chen *et al*. 2017). Nonetheless, Sloman got an opposite result when studying *Astronotus ocellatus* (Sloman *et al.* 2006). As a result, the relationship between fish size and hypoxia tolerance has always been in the spotlight. The influence of gender on extreme hypoxia tolerance cannot be underestimated. Abundant researches showed that hypoxia-treated male rats show obvious heart damage and arrhythmias compared with females. However, there are few studies on the effects of gender and age on acute hypoxia adaptation in fish at the same time, especially in the molecular field, so it is necessary to continue intensive study.

Asphyxiation point and oxygen consumption rate are important evidences to represent fish metabolism, with asphyxiation point being a limit index of fish hypoxia tolerance (Jia-Er *et al*. 2008). Therefore, asphyxia point is a good experimental condition to study the difference of extreme hypoxia tolerance caused by different gender and age. Fish of different genders and sizes possessed different metabolic demand, resulting in diverse molecular mechanisms towards hypoxia response. Up to now, molecular mechanism related to hypoxia in *A. fasciatus* remains unknown. So further research on the molecular mechanism of extremely low oxygen is needed.

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Acrossocheilus fasciatus (*A. fasciatus*) is termed as Cypriniformes, Cyprinidae, Acrossocheilinae, which is a small-sized fish in mountain streams, widely distributed in areas south of the Yangtze River. The requirement of water quality for *A. fasciatus* is too demanding that the drawback of hypoxia intolerance badly inhibits the promotion of breeding well-bred *A. fasciatus*.

MATERIALS AND METHODS

Acrossocheilus fasciatus (*A. fasciatus*) used in this experiment were obtained from artificial breeding populations from fishery research Department of Zhejiang Ocean University. The experimental site was carried out in the Fisheries Laboratory of Zhejiang Ocean University in 2021. Experimental subjects are juvenile *A. fasciatus* with body length of (3.8±0.23 cm) and body weight of (0.97±0.23 g) in the juvenile stage and genderually mature adult fish with female groups with body length of (13±1.17 cm) and body

weight of (19.45±1.86 g). Male groups with body length of (12±1.05 cm) and body weight of (16.13±1.57 g).

Total 100 *A. fasciatus* in each of the three groups were bred in three separate culture ponds with biofilter circulation system. The domestication experiment lasted for 1 month in the biofilter circulation system under the condition of 24°C and dissolved oxygen (DO) of 7.8 mg/L±0.2 mg/L (normoxia). During domestication, fish were fed twice per day with commercial fish feed. Daily inspection of water quality was performed and dead animals as well as particles were removed immediately.

Ethics statement

This study was conducted in accordance with the guidelines and approval of Institutional Animal Care and Use Committee at the Zhejiang Laboratory Animal Research Center and Zhejiang Ocean University. The approval number is 20210207.

Measurement of asphyxiation point

We have prepared 45 transparent glass bottles (500 mL) with completely sealed lids as breathing chambers for three sizes of fish. Before the experiment, the 45 transparent glass bottles were filled with water. The initial dissolved oxygen in the water was maintained at 7.8 mg/L±0.2 mg/L and the water temperature was maintained at 24 degrees. We randomly selected 15 items from each group of female adult fish, male adult fish and juvenile fish in the previous breeding ponds and a total of 45 fish was placed in these 45 experimental glass bottles independently and then sealed. When the fish was observed in a dying state, YSI Pro20 Oxygen Dissolver (USA) was used to record the DO of the water in the breathing chamber at that time. We defined the dissolved oxygen value at this time as the asphyxiation point of each fish. After recording the asphyxiation point, take out the fish in each breathing room, measure and record the weight and body length. The experimental data were analyzed by SPSS 20.0 software and each data was expressed as mean ± SD. Duncan test was used to compare the mean of each treatment and the difference was statistically significant ($P<0.05$).

Extreme hypoxia test

In the formal experiment, randomly selected 15 healthy *A. fasciatus* in each of the three groups were transported from culture ponds to three sealed glass containers (5L) filled with water, with the DO of 7.8 mg/L±0.2 mg/L, water temperature constant at 24 degrees as extreme hypoxia treatment groups. In each of the groups, the first 5 fish to reach death stage were divided into hypoxia sensitive group and the last 5 fish to reach death stage were divided into hypoxia tolerant group. DO in glass containers dropped with oxygen consumption by fish. Fish were immediately brought out for sampling when the first 5 fish and the last 5 fish of each group displayed death stage. Subsequently, 5 fish in each of the three groups in the nomoxia culture pond with constant DO of 7.8 mg/L±0.2 mg/L, as nomoxia control group, were brought out and bathed in eugenol (dilution 1:1000) for anaesthetic. After measuring body weight and length, fish were dissected to get their liver tissues, which were immediately frozened in liquid nitrogen and stored in -80°C till RNA extraction.

Extraction of RNA, synthesis of cDNA and transcriptome sequencing

After gathering all of the samples, TRIzol® reagent (Invitrogen) was used in accordance with the instruction. Total liver RNA of 9 groups were extracted and the 9 groups were hypoxia sensitive, hypoxia tolerance and nomoxia control groups of each of the three groups respectively Each group of RNA is a mixture of five individuals and labeled as F1 (adult female fish group 1), F2 (female adult fish group 2), FC (Normoxic control group of adult female fish), M1 (adult male fish group 1), M2(adult male fish group 2), MC (Male adult normoxic control group), J1 (juvenile fish group 1), J2 (juvenile fish group 2) and JC (Normoxic control group) respectively. Extracted RNAs in each of the groups were the mixture of 5 individuals. NanoPhotometer® spectrophotometer (IMPLEN, California, U.S.) was utilized to check RNA purity and Qubit®RNA analyzing kit in Qubit®2.0 fluorophotometer was used to detect RNA concentration (Invitrogen, California, U.S.). According to the recommendation of manufacturer, NEBNext®Ultra™RNA Library Prep Kit for Illumina® (NEB, U.S,) was used to generate sequencing library. Later, RNA-seq library was sequenced by Illumina sequencing technology.

Differentially expressed genes and enrichment analysis

The threshold level to identify significant DEGSs was set as p value (Padj)<0.05 and |log2 fold change|>1.0. Those with $p<0.05$ were considered as significantly enriched. Unigene sequences was aligned with NR, Swiss-Prot, GO, COG, KOG, KEGG by BLAST software. After predicting the amino acid sequences of Unigene, HMMER software and Pfam database was used for alignment, obtaining annotations of Unigene.

Transcriptome data certification by transcriptome data deriving from qRT-PCR

Total RNA reversal was conducted under the guidance of construction. Sequences of related genes were searched based on the results of transcirptome sequencing. And 8 specific primers to be used in qRT-PCR were designed based on sequences in the coding region (Table 1). TB PrimeScript™RT Kit (TaKaRa) was placed in the ABI QuantStudio 3 system for qRT-PCR analysis. Relative expression level of each gene was calculated by $2^{-\Delta\Delta CT}$ method. Three replications were tested for each of the groups and all the samples in triplicate were detected on the same plate. The expression level of target gene was normalized into the expression of β-actin, presented as relative expression of target gene (relative mRNA).

RESULTS AND DISCUSSION

Analysis of asphyxiation point results

Under the condition of water temperature of 24°C, the average asphyxiation point of female adult was 0.56, the average asphyxiation point of male adult was 0.66 and the average asphyxiation point of juvenile was 0.858 (Fig 1). It is obvious that the asphyxiation point of adult fish is lower than that of young fish and the asphyxiation point of female fish is lower than that of male fish. Female adults showed the strongest tolerance to extreme hypoxia. Through regression analysis, we can get that the slope of fitting curve of body weight is larger (Fig 2), The regression equation can be expressed as y=0.880-0.013a-0.002b (y stands for asphyxia point, a stands for body weight, b stands for body height), which indicates that body weight has more influence on asphyxia point than body length.

The difference of hypoxia tolerance caused by different age and gender of organisms has always been the focus of researchers. A great deal of studies argued that size of fish had essential impact on hypoxia tolerant ability. In this research, the asphyxiation point of *A. fasciatus* is higher than hypoxia tolerant fresh water fish such as *Pelteobagrus fulvidraco* and *Carassius aruatus* (Wu *et al*. 2014). In the comparison of asphyxiation point in *Acrossocheilus fasciatus* (*A. fasciatus*) with different sizes, an decreasing tendency was observed with the increase of age and weight. The asphyxiation point of female adult fish was the lowest among the three specifications, showing the strongest extreme hypoxia tolerance.

Table 1: Primers used in the qRT-PCR.

Gene	Primer name	Sequence (5'-3')
OSBP6	OSBP6-F	GTCTTGAGGCGTCGCACAT
OSBP6	OSBP6-R	TGGCATCTTGTCACCCACTT
BCO1	BCO1-F	CCTCTGAGCCAGTGTTTGTAGC
BCO	BCO1-R	TGAATAGCAGTATCAATGGTAGCG
MRC2	MRC2-F	CTCTTAATATGGAGCAAACAAACCC
MRC2	MRC2-R	CTCAGCTCAGTTAAAGGCAGAAATA
KLF5	KLF5-F	CTCAGAAGCCTCGGAAACG
KLF5	KLF5-R	TGGCTCCAGTAAACAGACAAGAAG
HIF1A	HIF1A-F	CAGCACGCAGACAGAAGAATC
HIF1A	HIF1A-R	GGAGGCATCGGAAACGACA
NR	NR-F	GCTGACTGCCACTCTTTCCA
NR	NR-R	CAAACTCTTCCTCGCCCTCT
CFH	CFH-F	TGTATGAATCTTTCGCAACTGG
CFH	CFH-R	CCTCGGCTTCGGCTTTT
HO	HO-F	GGCACCGTCATCTTCTCCCT
HO	HO-R	CGTGGCACCCATTTACTTCC
β-actin	β-actin-F	GAACTGCTGCCTCTTCTTCCTC
β-actin	β-actin-R	ATACCGCAAGATTCCATACCC

Statistic of transciptome data and analysis of differentially expressed genes

Juvenile fish, male fish and female fish qualified for sequencing were selected and samples were processed under extreme anoxia stress and no stress (control) respectively. Then, transcriptomic sequencing was performed, gathering 80.16 Gb Clean Data. Detailed data of sequenced samples and their quality could be found in (Table 2). According to the Venn (Fig 3) and the volcano maps (Fig 4). It can be seen that the number of DEGSs of adult fish is much higher than that of juvenile. Further analysis showed that the number of DEGS in adult male and female significantly exceeded the number in juvenile. This phenomenon was also reported in other fish such as grass carp (Jin *et al.* 2017), yellow catfish, indicating that improving the number of gene expression was the main response to adapt to acute hypoxia environment.

Differentially expressed genes were further analyzed by GO functional enrichment analysis (Fig 7). Differentially

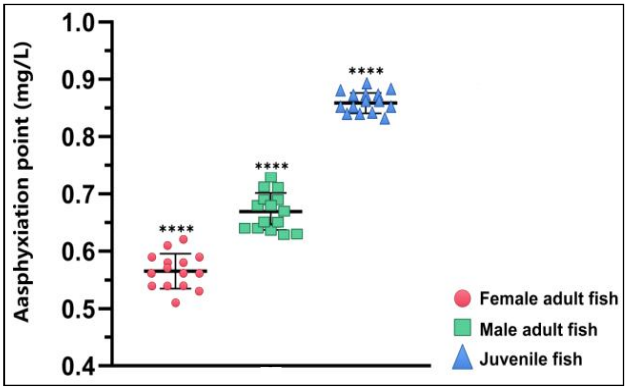

Fig 1: Asphyxiation point of female adult fish, male adult fish and juvenile ****p<0.0001. The y-axis represents the value of suffocation point.

Table 2: Sequencing data table.

	Sample	Read sum	Base sum	GC (%)	Q30 (%)
Juvenile	J1	28,040,933	8,412,279,900	46.09	93.38
	J2	31,918,539	9,575,561,700	46.33	92.88
	JC	29,966,218	8,989,865,400	46.55	93.01
Female	F1	25,605,679	7,681,703,700	46.12	93.09
	F2	35,128,019	10,538,405,700	46.79	93.4
	FC	27,885,539	8,365,661,700	46.71	93.42
Male	M1	30,539,110	9,161,733,000	45.77	93.53
	M2	32,290,681	9,687,204,300	46.27	92.95
	MC	25,828,650	7,748,595,000	47	93.6

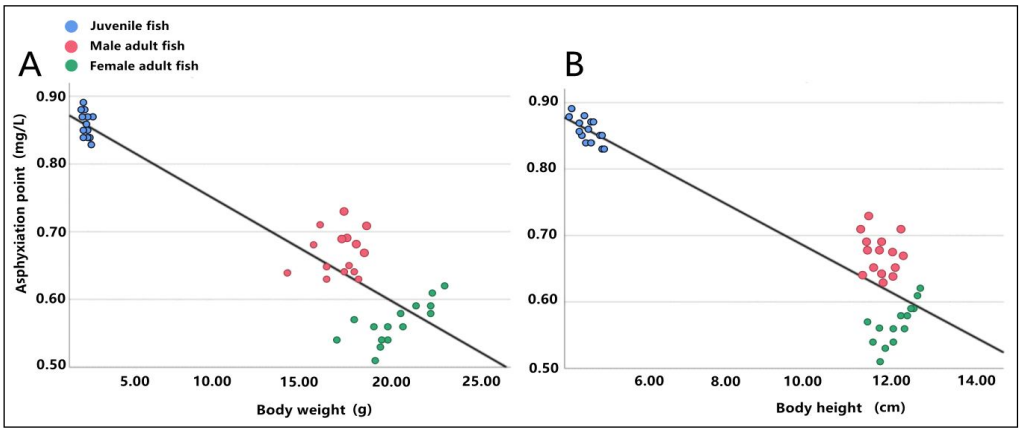

Fig 2: Graphical representation of linear regression equation.y=0.880-0.013a-0.002b (y stands for asphyxia point, A stands for body weight, B stands for body height).

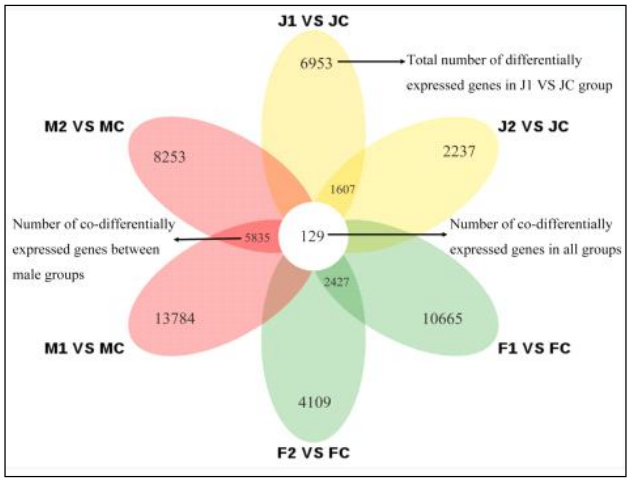

Fig 3: Venn diagram of all DEGSs under treatment group of extreme hypoxia (J1, J2, F1, F2, M1, M2) compared to normoxia group (JC, FC, MC). Genes expressed only in Female adult fish group (light green circle); genes expressed only in Male adult fish group (light red circle); genes expressed only in Juvenile fish group (yellow circle). (log^2 foldchang >1.0 and padj <0.05).

expressed genes across 6 comparison groups were mainly enriched in 3 GO terms (Cell Component: 17, Molecular Function: 14 and Biological Process: 23) and 54 subterms (Fig 5). In general, M1 vs MC clearly had more enriched than the other comparisons and the number of enriched genes was relatively distributed more evenly in each of the terms. The results indicated that under hypoxia stress, processes related to cellular metabolism and synthesis and catalysis were highly activated in the liver and gill of *A. fasciatus*. KEGG enrichment analysis was carried out on the signal pathway of differentially expressed genes (Fig 6). The results showed that the differentially expressed genes in six comparison groups were significantly enriched in Signal transduction, transport and Catabolism and other signaling pathways. The above results showed that acute hypoxia activated endocrine, protein, amino acid and Multiple biological processes such as fat and carbohydrate metabolism to regulate *A. fasciatus* adapt to extreme hypoxia stress (Qian *et al*. 2019). Hypoxia stress changed the endocrine system of *A. fasciatus*.

Screening for key genes under extreme hypoxia stress

In order to further explore the reasons why female fish of *A. fasciatus* are more tolerant to extreme hypoxia, top 10 genes with the greatest significant expression were screened (Table 3), In particular, differentially expressed genes were significantly enriched in the Ferroptosis, MAPK signaling pathway, *etc*. Under hypoxia, a series of responses takes place inside cells to accommodate hypoxia (Jiang *et al*. 1996). We found that the expression of ncoa4 and facl4 was significantly down regulated and the expression of jnk, gpx4 and jip-1 was significantly increased in three sizes of fish under extreme hypoxia. Specially, the increase and decrease of these genes were most obvious in female adult fish. Ferroptosis is an iron-dependent novel manner of programmed cell death, with the essence being intracellular lipid oxide metabolism disorder (Xie *et al*. 2016). Drastic environmental stress makes it easier for the occurance of ferroptosis (Speer *et al*. 2013). In this research, expression of genes related to ferroptosis, such as glutathione gene gcl and gss, transferrin, ferritin heavy chain, was up-regulated in the three groups, which was similar to the results in researches about other fish under stress condition. In this research, expression of NCOA4 was lower in adult groups compared to juvenile groups. The expression level in female groups was even more significantly reduced, suggesting that female groups were equipped with stronger anti-oxidative stress damage ability by down-regulating the expression of NCOA4 (Gryzik *et al*. 2001). Hypoxia stress response was achieved by the interaction of regulatory network based on these pathways and genes involved in the pathways as well as complicated molecular adaptation strategies.

qPCR validation

To verify transcriptome data, sequences of 8 genes (osbp6, bco1, mrc2, klf5, nr, cfh, ho, hif-1a) were randomly selected

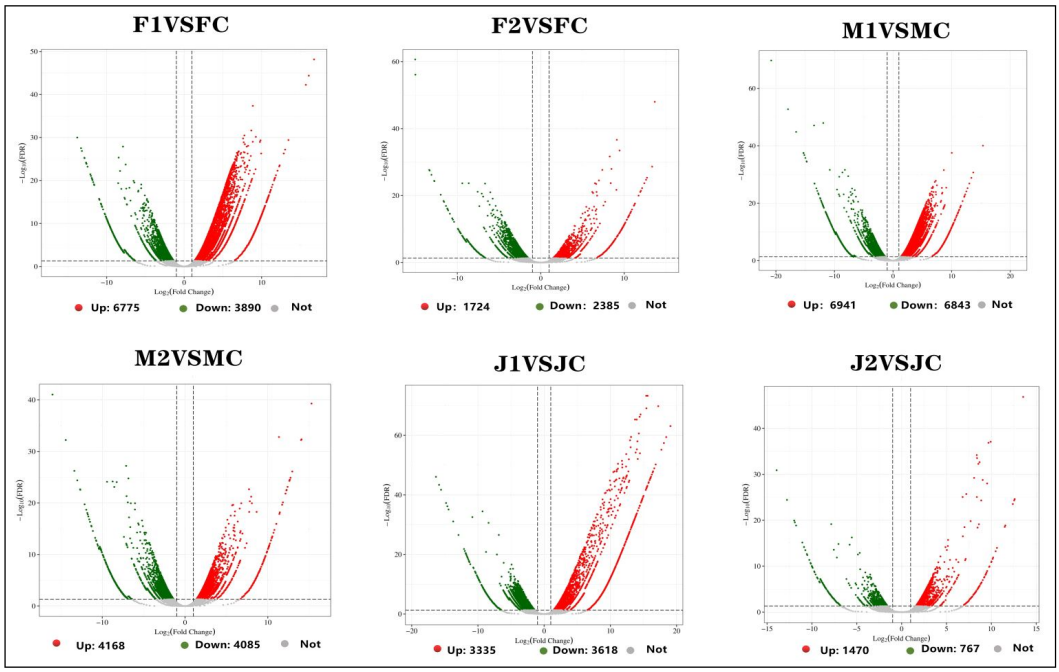

Fig 4: Gene expression profiles in the liver. Differentially expressed genes (DEGSs) were shown in red (up) and green (down). Genes that did not exhibited changes in expression were shown in grey. (log^2 foldchang >1.0 and padj <0.05).

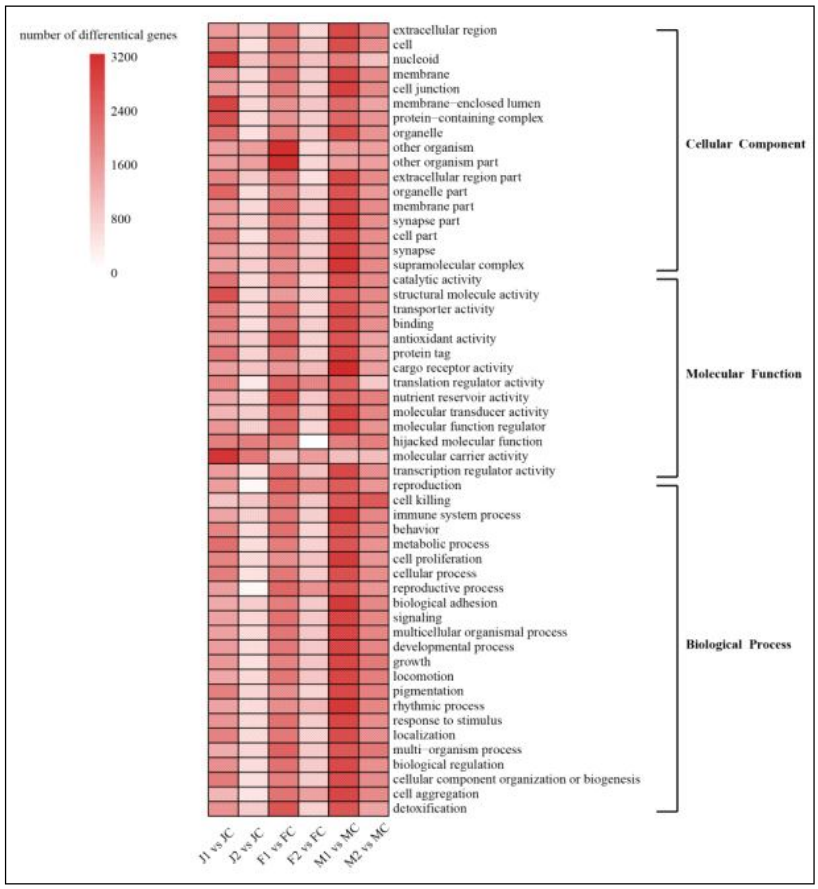

Fig 5: Gene ontology (GO) classification of differentially expressed unigenes in *Acrossocheilus fasciatus*. The abscissa were J1, J2, M1, M2, F1, F2 Compared with the normoxic control group, the color scale in the vertical axis indicated the number of enriched genes and the darker the color, the more the number.

according to the transcriptome data and qRT-PCR was used to detect their relative expression level (Fig 7). Results suggested that the tendency of up-regulation and down-regulation was in accordance with transcriptome data, certificating the reliability of results of the transcriptome data. It is noteworthy that heme oxygenase-1 (HO-1), as the initial enzyme and rate-limiting enzyme of heme degradation, has anti-inflammatory, antioxidant, anti-apoptotic and other physiological effects (Shi *et al*. 2018). The highest expression was observed in juvenile fish under extremely low oxygen stress, which indicated that the damage of juvenile fish was the most serious. As a hypoxia inducible

Table 3: Common differentially expressed genes of the top 10 groups with the largest multiple of expression difference.

Gene ID	Log_2 (Fold Chang)						Decription
	J1 VS JC	J2 VS JC	F1 VS FC	F2 VS FC	M1 VS MC	M2 VS MC	
MUC-5AC	-2.17	-2.95	-12.1	-11.16	-5.06	-11.16	Mucin-5AC
APOL3	8.98	3.63	4.21	9.62	7.44	9.62	Apolipoprotein L3
MR2	-3.7	-4.85	-9.41	-7.05	-7.74	-7.05	C-type mannose receptor 2-like isoform X2
FACL4	-3.72	-3.86	-10.09	-9.76	-2.22	-9.76	Long-chain-fatty-acid-CoA ligase 4
NCOA4	-5.85	-3.63	-9.68	-7.93	-5.73	-3.93	Nuclear receptor coactivator 4
JIP-1	8.67	3.88	10.45	2.57	5.62	2.57	C-Jun-amino-terminal kinase-interacting protein 1
GPX4	5.47	2.52	10.2	4.02	6.43	3.02	Glutathione peroxidase 4
TDO	8.79	2.58	9.7	1.92	6.87	1.92	tryptophan 2,3-dioxygenase B
JNK	6.63	3.08	9.25	3.74	7.16	3.24	C-Jun N-terminal kinase
TRFA	8.83	2.28	8.86	2.53	6	2.53	Transferrin variant A

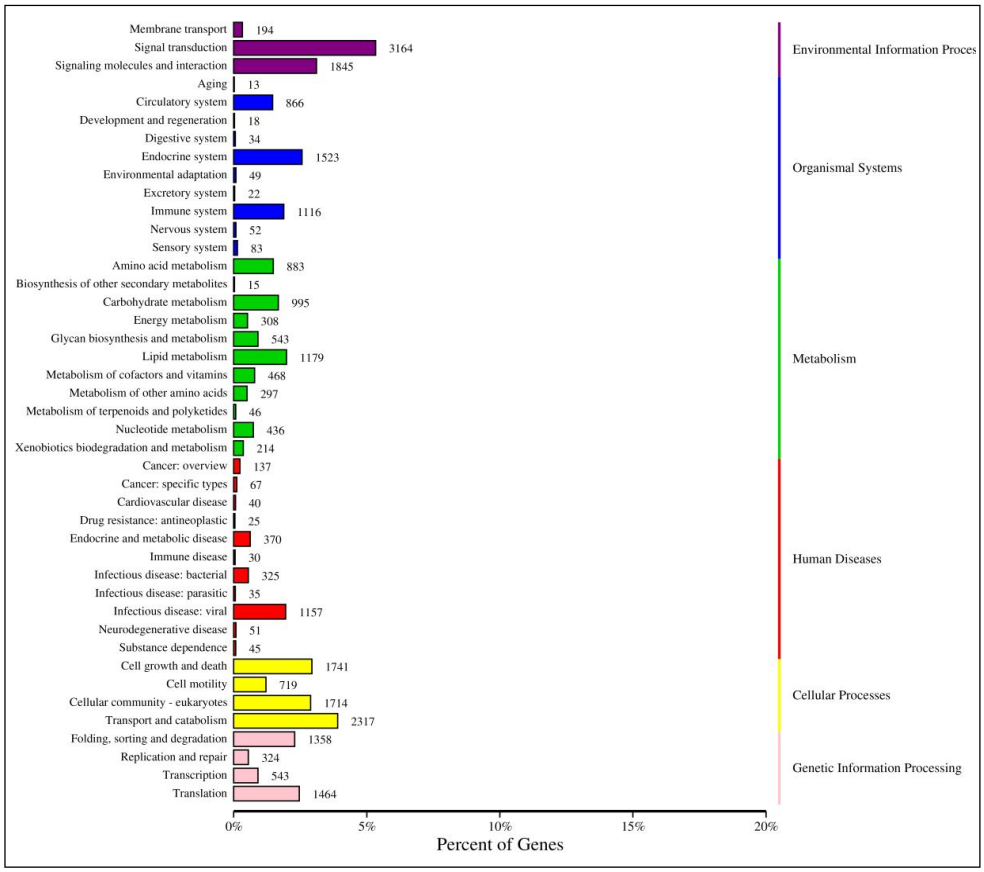

Fig 6: Significantly enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways of differentially expressed genes (DEGSs) in Six groups were treated with extreme hypoxia (padj <0.05).

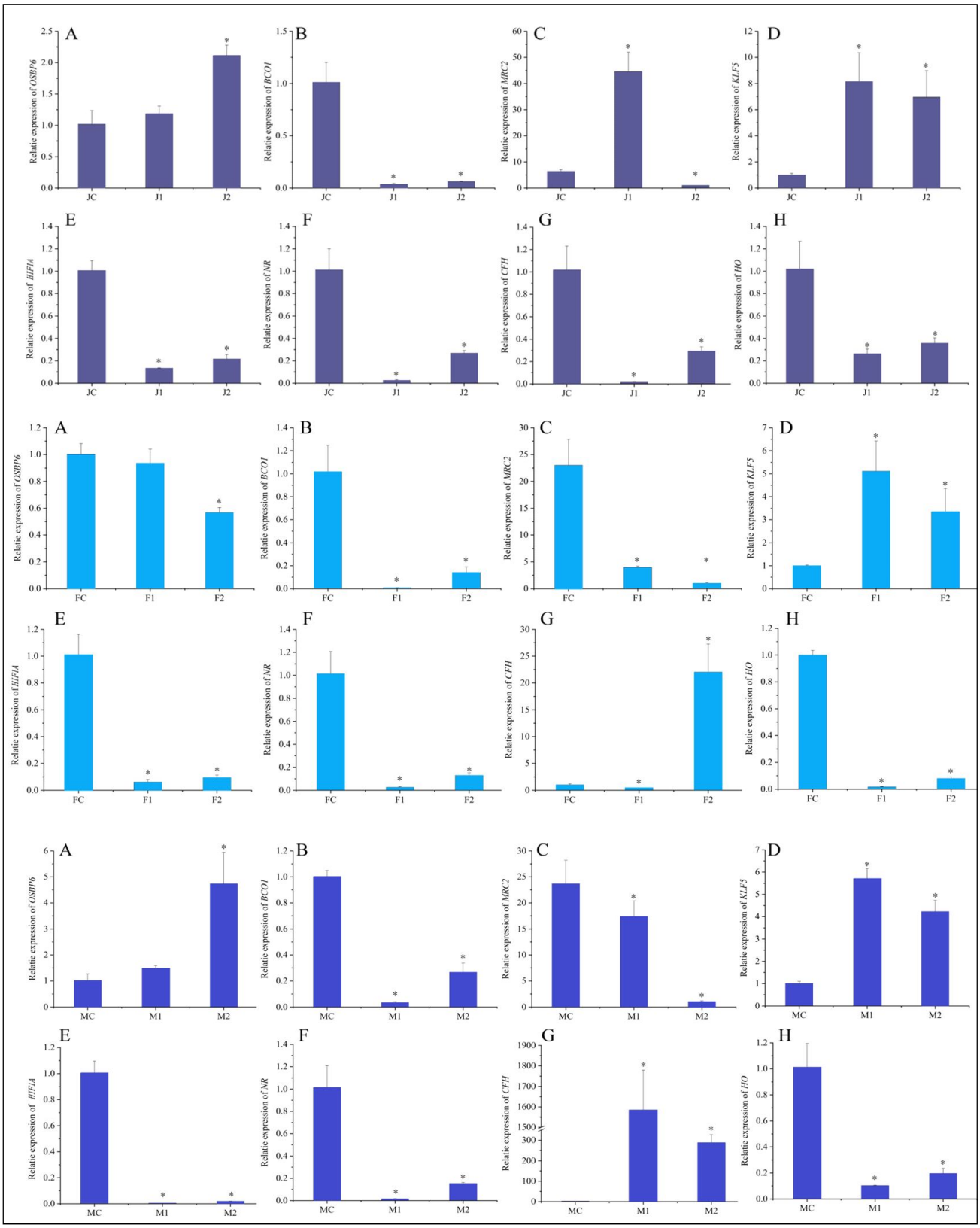

Fig 7: Real-time polymerase chain reaction analysis of the expression of 8 genes in the livers of extreme hypoxia treatment group and normoxic control group, the light blue histogram represents the female adult group, the purple histogram represents the male adult group and the dark purple represents the juvenile group. Asterisks (*) indicated a significant difference 0between control group and treatment group ($p<0.05$).

factor, Hif1a has the highest expression in juvenile fish under hypoxia stress, which also shows that hypoxia stress has a more serious impact on the body of juvenile fish.

CONCLUSION

In a nutshell, by measuring asphyxiation point, understanding exuberant metabolic time span and hypoxia tolerance, it is helpful to better explain the reason why females have stronger hypoxia tolerance. Moreover, by comparative analysis of transcriptome data under hypoxia stress, crucial genes related to extreme hypoxia stress and enhancement of hypoxia tolerance were discovered. This research held a great importance in widening the knowledge of physiological mechanism of *A. fasciatus* under extreme aquatic conditions.

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