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Transcriptome analysis and the effects of polyunsaturated fatty acids on the immune responses of the critically endangered angtze sturgeon *(Acipenser dabryanus)*

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ABSTRACT

The poor understanding of nutrition needed has become a significant obstruction to artificial conservation of Yangtze sturgeon (*Acipenser dabryanus*) and the relationship between ployunsaturated fatty acid nutrition and the immune response of Yangtze sturgeon is remains unclear. To explore this relationship, the immune response was determined by the activities of serum immune-related enzymes and the transcriptome pattern in the spleen after feeding different fat source diets for 7 weeks. In addition, the gene expression pattern after a lipopolysaccharide (LPS) challenge was investigated in the presence of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Long-term feeding of the fish oil diets increased the serum immune-related enzyme activities, including lysozyme, acid phosphatase, and alkaline phosphatase of Yangtze sturgeon. More than 653,999 transcripts with an N50 length of 1047 bp were obtained and a final set of 280,408 unigenes was generated. After annotating the unigenes, 3549 genes were assigned to the immune system and 2839 were identified to participate in the response to the different fat sources. A transcriptome assay showed the fish oil diets moderately upregulated immune-related signaling pathways in the spleen of Yangtze sturgeon, including NLR signaling, platelet activation, Fc gamma R-mediated phagocytosis, Th17 cell differentiation, and Th1 and Th2 cell differentiation. The quantitative polymerase chain reaction (qPCR) results of candidate genes for these pathways showed similar results. The LPS challenge study revealed that DHA and EPA moderately upregulated the candidate immune-related genes and modulated excessive activation of the immune pathway by the pathogen. This study confirmed the immunomodulatory function of unsaturated fatty acids in Yangtze sturgeon. This research will provide a reference for the preparation of artificial diets for Yangtze sturgeon.

1. Introduction

Yangtze sturgeon (*Acipenser dabryanus*), also known as Dabry's sturgeon, is a freshwater sturgeon only distributed in the mainstream and tributaries of the Yangtze River in China [1]. Due to heavy fishing, damming, and habitat degradation, the natural population of Yangtze sturgeon has decreased dramatically in recent years [1–4]. In addition, Yangtze sturgeon is renowned as a living fossil [5] and has high scientific value.

Many efforts have been made to recover this endangered species [1]. The Yangtze sturgeon has been listed as a critically endangered species and a national-level protected animal in China [6] The Convention on International Trade in Endangered Species of Wild Fauna and Flora [7] and International Union for Conservation of Nature and Natural Resources Red List [8] provide policy support for its protection. In addition, establishing a national nature reserve provides natural conditions for restoration of wild resources. However, due to overfishing and the long reproductive cycle of Yangtze sturgeon, the wild resources have not recovered and have even declined. Controlling reproduction and re-stocking cultured juveniles play an important and crucial role in solving this problem. Although the difficulties of artificial reproduction of Yangtze sturgeon has been overcome [9], the larval mortality rate is as high as 80%, which hinders protective actions. Our observations have shown that nutrition is closely related the mortality

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List of abbreviations

IUCN	International Union for Conservation of Nature and Natural Resources
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
PUFA	polyunsaturated fatty acids
NLR	NOD-like receptor
qPCR	real-time fluorescent quantitative PCR
LZM	lysozyme
ACP	acid phosphatase
AKP	alkaline phosphatase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KASS	KEGG Automatic Annotation Server
TPM	transcripts per million
FOLF	fish oil diet longterm feed
SOLF	soybean oil diet longterm feed
FOPF	fish oil diet postprandial feed
SOPF	soybean oil diet postprandial feed
BUSCO	Bench-marking universal single-copy orthologs
TGICL	TIGR Gene Indices Clustering Tools
GO	gene ontology
COG	clusters of orthologous groups
KOG	euKaryotic orthologous groups
TPM	transcripts per million
EF1-α	elongation factor 1-alfa
MAPK14A	mitogen-activated protein kinase 14A
MAPK8	mitogen-activated protein kinase 8
NFκB1	nuclear factor kappa light polypeptide gene enhancer in B-cells 1
TYK2	Non-receptor tyrosine-protein kinase
LATM1	linker for activation of T-cells family member 1
PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
PKCθ	protein kinase C theta type
PLCB3	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3

of the Yangtze sturgeon, particularly deaths caused by nutritional diseases. However, studies focusing on the relationship between the immune system and nutrition of the Yangtze sturgeon and other sturgeons are absent.

Fats, particularly polyunsaturated fatty acids (PUFAs) are involved in the regulation of various immune pathways. In mammals, PUFAs regulate multiple pathways involved in inflammation through the interaction between free fatty acid receptor 4 (FFAR4) and β-arrestin [10–12]. However, the FFAR4 gene has not been identified in fish [13]. Therefore, whether PUFAs affect the immune response in fish is worthy of attention. In addition, the PUFA requirements vary greatly among different fish species [14]. Due to the unclear nutritional requirement of PUFAs, it is common to use rainbow trout diets to feed the Yangtze sturgeon, which is highly likely to cause nutritional disease. Therefore, it is necessary to study the relationship between polyunsaturated fatty acids and immune responses in Yangtze sturgeon.

Transcriptome sequencing is one of the most powerful and efficient ways to understand functional genes and pathways [15], particularly in species without a reference genome. Large numbers of genes have been identified in many fish using transcriptome sequencing, such as *Cynoglossus semilaevis* [16], *Oncorhynchus mykiss* [17], *Oreochromis niloticus* [18], *Liza heamatocheila* [19], and *Oncorhynchus nerka* [20]. In Yangtze sturgeon, Liu et al. used transcriptome sequencing to analyze the head kidney response to *Aeromonas hydrophila*, and the results showed that the Toll-like receptor signaling pathway, the chemokine signaling pathway, the complement and coagulation pathway, the RIG-I-like and the NOD-like receptor (NLR) signaling pathway are involved in the immune response [21]. Furthermore, our colleagues have used transcriptome sequencing technology to interpret the difference in gene expression between male and female gonads of Yangtze sturgeon and identified sex-differentiation related genes, simple sequence repeats, and single nucleotide polymorphism [22]. However, the spleen transcriptome and the effect of fat source on the immune system of Yangtze sturgeon has not been reported.

In the present study, the effect of different fatty acids on the immune system was evaluated by immunoenzyme activity, transcriptome sequencing and real-time fluorescent quantitative polymerase chain reaction (qPCR) in Yangtze sturgeon. Two semi-purified diets with different fat sources (fish oil and soybean oil) were formulated and used to feed two groups of Yangtze sturgeon for 7 weeks. At the end of the feeding experiment, the spleen and serum were collected at 15:00 (1 h before feeding) and the spleen at 17:00 (1 h after feeding). The serum activities of lysozyme (LZM), acid phosphatase (ACP), and alkaline phosphatase (AKP) in serum were determined. Transcriptome sequencing of the spleen considering the different fat sources was performed using the Illumina sequencing platform. The original data were assembled, and the longest sequences were preserved and designated as the unigenes. Functional annotation, classification, and expression of the unigenes were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server, the R program (version 3.3, package: topGO) and transcripts per million (TPM) methods in RSEM software (version 1.0). Subsequently, qPCR was used to verify that the transcriptome results were credible. In addition, key genes screened by the transcriptome were used to assess the immunomodulatory effect of PUFAs (docosahexaenoic acid [DHA] and eicosapentaenoic acid [EPA]) on lipopolysaccharide (LPS) challenge. The results will provide a valuable reference of nutrient content and the immune system and will stimulate more colleagues studies on the relationship between different fat sources and the immune system in critically endangered sturgeons.

2. Material and methods

2.1. Experimental fish and the experimental process

All Yangtze sturgeons used in this study were cultivated by artificially bred and provided by The Fishery Institute of the Sichuan Academy of Agricultural Sciences. The fish were acclimated and fed a commercial feed for 1 week before use in the study. The fish were held under natural lighting and flowing water (22 ± 3 °C) in the Fisheries Department Laboratory of Sichuan Agricultural University. All animal experimental procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (approval numbers B20172101–1803 and B20172101-1804).

Forty Yangtze sturgeon (initial weight 358.64 ± 37.1 g) were randomly sorted into 2 round fish ponds (2 m in diameter, 2.5 m^3) and fed the different fat source diets once daily at 16:00. The control group was fed a diet with a fat source of soybean oil (diet A, Table 1), and the test group was fed a diet with a fat source of fish oil (diet B, Table 1). The feeding trial lasted 7 weeks. At the termination of the feeding trial, 6 individuals from each pond (fish oil diet longterm feed, FOLF; soybean oil diet long-term feed, SOLF), were caught and sampled 1 h before feeding (15:00). After feeding for 1 h (the most significant time point for response to nutrition after ingestion) [23], another 6 fish from each group (fish oil diet postprandial feed, FOPF; soybean oil diet postprandial feed, SOPF) were captured and sampled. The spleen samples were rapidly collected, immediately frozen in liquid nitrogen, and stored at −80 °C for RNA extraction. The blood samples were aliquoted

Table 1
Formulation and nutritional composition of perimental diet.

Ingredient	Nutrient composition	
	diet A(%)	diet B(%)
Casein	49	49
Gelatin	5	5
α-starch	25	25
Soybean oil	8	0
Fish oil	0	8
choline chloride	0.25	0.25
vitamin and mineral premix	1	1
CMC-Na	4	4
microcrystalline cellulose	2.75	2.75
potassium sorbate	0.1	0.1
Nutrient levels		
Moisture	5.88	6.35
Crud protein	43.27	42.55
Crud lipd	10.24	10.53

Vitamin premix (mg/kg diet or IU/kg diet): vitamin E, 100; vitamin K3, 40; vitamin A, 5000 IU; vitamin D, 2000 IU; vitamin Bl, 50; vitamin B2, 200; vitamin B6, 50; vitamin B12, 0.5; vitamin C, 325; nicotinie acid, 175; folic acid, 5; inosito, 1000; biotin, 2.5; calcium pantothenat,50.2. Mineral premix (mg/kg diet): NaCl, 5000; Ca(H2PO4)2, 15000; FeSO4·7H2O, 1000; ZnSO4·7H2O, 350; MnSO4·4H2O, 40; CuSO4·5H2O, 12; CoCl2·6H2O, 80; KIO3, 5.

into non-heparinized tubes and left to clot for 12 h (at 4 °C), prior to centrifugation at 1900 × *g* for 5 min in a clinical centrifuge. Isolated sera were stored at −20 °C until further analysis.

To investigate the protective effect of PUFAs (DHA and EPA) on an LPS challenge, 27 fish (88.42 ± 6.75 g) with a similar body weight were selected and randomly divided into 3 groups with 3 replicates in each group and 3 individuals in each replicate. The control group was injected with solvent (DMSO: PBS 1:1, 2 μl/g BW; Sigma, St. Louis, MO, USA). One of the experimental groups was injected with LPS (Sigma, 100 μg/μl PBS, 1 μl/g BW) and DMSO (1 μl/g BW) and the dose was referred from that of a previous study [24]. The remaining group was co-injected with PUFAs (DHA 100 nM and EPA 65 nM, 1 μl/g BW; MedChemExpress, Monmouth Junction, NJ, USA) and LPS (100 μg/μl, 1 μl/g BW). Following the injection procedure, the spleens of 3 fish from each replicate was sampled pooled 48 h after the injection for the immune response assays [24]. The spleen samples were processed as described previously.

2.2. Immune-related enzymes assays

To investigate the effects of fat source on immune function, we evaluated the differences in activity of three immune-related enzymes (i.e. LZM, ACP, and AKP) after 49 days of feeding the different fat diets. The plasma activities of LZM, AKP, and ACP were measured using kits (Nanjing Jiancheng, Nanjing, China). LZM activity was estimated as described by Jia et al. [25]. The activity of AKP and ACP activities were measured as described previously using kits (Nanjing Jiancheng, Nanjing, China) [26].

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted with an Animal Total RNA Isolation Kit (Foregene, Chengdu, China) following the manufacturer's protocol. RNA quality was determined by a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), the Agilent 2100 ((Agilent Technologies, Palo Alto, CA, USA), and 1% agarose gel electrophoresis. Only RNAs with RIN ≥7.0, OD 260/280 ≥ 1.8 and OD260/230 ≥ 1.5 were adopted for the next step. One microgram of total RNA was reverse transcribed to cDNA for qPCR, using the PrimeScript™ RT reagent kit (Takara, Dalian, China) which included the gDNA Eraser to prevent residual genomic DNA, according to the manufacturer's protocol.

For RNA-Seq library preparation, mRNA with poly-A was purified with Magnetic Oligo (dT) Beads and a DNase I digestion step, then randomly fragmented using a fragmentation buffer into a suitable size (~220bp). The fragments were used for first-strand synthesis with random hexamer primers, and double-stranded cDNA was synthesized with an end-reparation step. The double-stranded cDNA was purified with AMPure XP beads (Beckman, Brea, CA, USA), poly (A), and ligated sequencing adaptors. The final cDNA libraries were sequenced on the Illumina XTen platform using the paired-end read module primers (forward: AGATCGGAAGAGCACACGTCTGAAC; reverse: AGATCGGAAGAGCGTCGTGTAGGGA).

2.4. Transcriptome processing and assembly

The sequencing service was provided by Majorbio (Majorbio, Shanghai, China). The raw image data from the Illumina sequencing platform were translated by CASAVA base calling into the original sequence data (raw reads). The raw reads were trimmed and filtered with SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software to discard the adaptor sequences, sequences of low quality (Q < 20 bases), sequences with ambiguous bases "N" > 10% and sequences < 30 bp in length. The processed sequences (clean reads) were *de novo* assembled by Trinity software (https://github.com/trinityrnaseq/trinityrnaseq/wiki). Assembly quality was evaluated using the bench-marking universal single-copy orthologues (BUSCO) analysis [27]. The transcripts were clustered using TIGR Gene Indices Clustering Tools to minimize redundancy [28]. The longest sequences in each cluster were preserved and designated as the unique transcript fragments (unigenes).

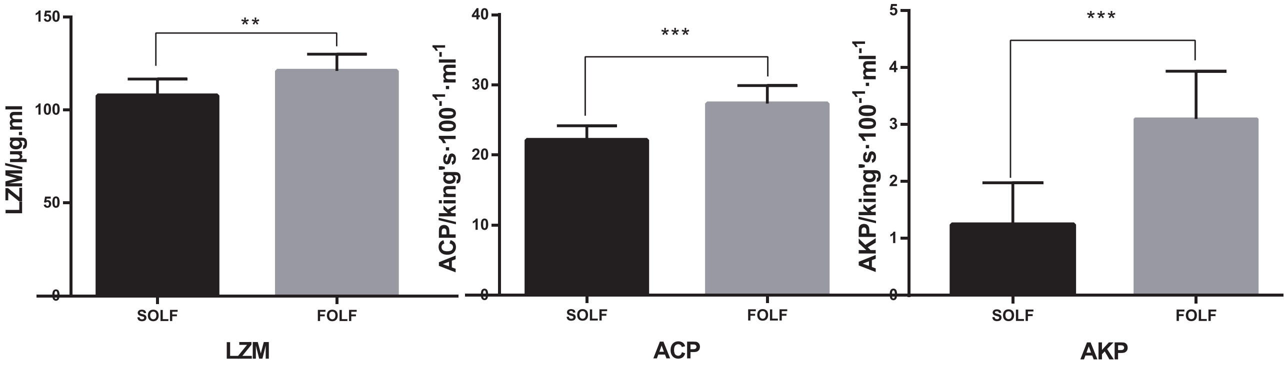

Fig. 1. Immunity-related enzyme activities (mean ± SEM, n = 6) of Yangtze sturgeon feed with different fat sources diets. Different superscripts indicate the significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 2
Statistics of transcriptomic sequences from four libraries.

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Error rate(%)	Q20(%)	Q30(%)	GC content(%)
FOLF	42221012	6375372812	41626756	6194806208	0.024	98.48	95.13	45.87
FOPF	45412992	6857361792	44725602	6646169436	0.0239	98.49	95.23	48.92
SOLF	43556206	6576987106	42910468	6384980810	0.0237	98.56	95.39	47.22
SOPF	44473336	6715473736	43864058	6531186786	0.0238	98.53	95.31	46.93

Table 3
Summary of the A. dabryanus transcriptomes assembly.

Type	Resource
Total transcripts number	653999
Total unigenes number	280408
Total sequence base	440492247
Largest	16413
Smallest	201
Average length	673.54
N50	1047
E90N50	1289
GC percent	42.6
TransRate score	0.131
BUSCO score	89.7%

2.5. *Gene annotation and classification*

The unigenes of Yangtze sturgeon were searched against the Swiss-Prot database (Apweiler et al., 2004), the NCBI non-redundant protein and nucleotide sequence databases (NR and NT: http://ncbi.nlm.nih.gov), Gene Ontology (GO) [59], clusters of orthologous groups (COG) [60], euKaryotic orthologous groups [61] and KEGG orthology (Kanehisa et al., 2004) using the NCBI BLAST + program with an E-value cut-off of 1e-5 and matching to the top hits. HMMER [29] was used to compare the amino acid sequence of the unigenes with the Pfam database to obtain the annotation information.

2.6. *Differential expressionand GO and KEGG enrichment*

To overview the effect of different fat sources on the expression pattern, the expression of unigenes for thedifferent fat sources was calculated rigorously using the TPM method in RSEM software (version 1.0). A differential expression analysis was performed using edgeR [62] in I-Sanger tools, a free online platform for analysis (www.i-sanger.com). Unigenes with a log2 fold change ≥1, Padjust ≥0.001 were defined as differentially expressed genes. In addition, only unigenes that were reverse regulated during postprandial treatment with the different fatty acid diets and that showed differences in expression during the long-term treatment with different fatty acids diet were considered to be involved in the responses to the different fatty acid treatments. The functional groups and pathways of the candidate unigenes were annotated using GO and KEGG analyses with I-Sanger tools (www.i-sanger.com).

2.7. *qPCR*

The expression levels of the candidate transcriptome genes, were determined by qPCR. Previous research has shown use of a single gene for normalization leads to relatively large errors and wrong normalize RNA levels [30,31]. In our preliminary study, we found EF1-α and β-actin stable expression in the Yangze sturgeon [32]. Therefore, the results were normalized using the reference genes, β-actin and elongation factor 1-alpha (EF1-α). qPCR was performed using a volume of 20 μl containing 2 μl of cDNA template, 10 μl of 2 × TB Green qPCR Master Mix (Takara), 1 μl of each target gene primer (10 mM), and 6 μl ddH_2O. The PCR cycling conditions were: 3 min at 95 °C; 40 cycles of 20 s at 95 °C, 15 s at different temperatures for different genes (see Table 4), and 20 s at 72 °C; finally, 70–95 °C with a ramp-up of 0.5 °C/s to generate a melting curve. Each sample was run and analyzed in triplicate. The $2^{-\triangle\triangle CT}$ method was used to analyze the expression levels of the differentially expressed genes [33].

2.8. *Statistical analysis*

Data are presented as mean ± standard error. GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. After the F-test, the unpaired *t*-test was performed to detect differences between the groups. Statistical significance was considered as * $p\text{-value} < 0.05$, ** $p\text{-value} < 0.01$, *** $p\text{-value} < 0.001$.

3. Results

3.1. *Effect of feed fat source on immunity*

The effects of the different fat diets on the activities of the immune-related enzymes are shown in Fig. 1. The activities of all immune-related enzymes were significantly higher in the FOLF group than those in the SOLF group. LZM activity increased significantly ($p < 0.01$) in the FOLF group compared to the SOLF group. ACP and AKP activities were significantly higher ($p < 0.001$) in the FOLF group than the SOLF group.

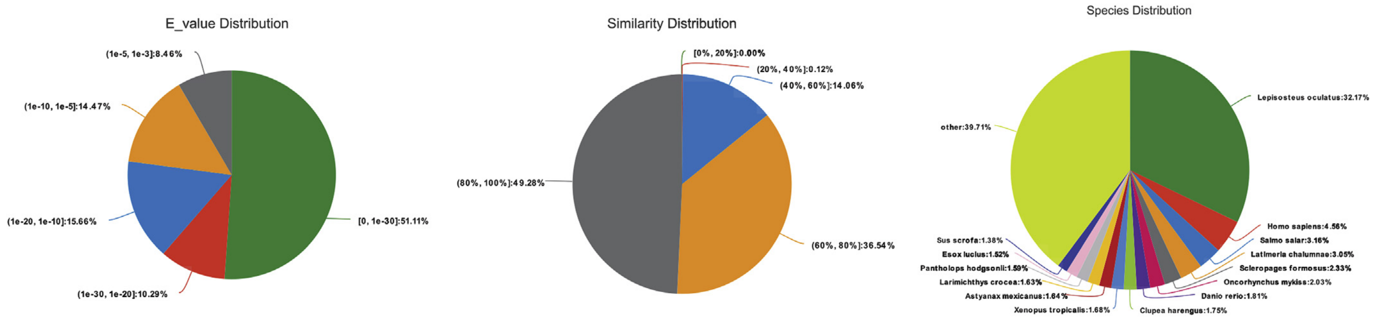

Fig. 2. Comparison of Yangtze sturgeon transcriptomic sequences with known sequences in NR databases. (A) the e-value distribution of unigenes with a cutoff e-value of 1e-5. (B) similarity distribution of blast hits for unigenes in NR database. (C) the species distribution of blast hits for each unigens.

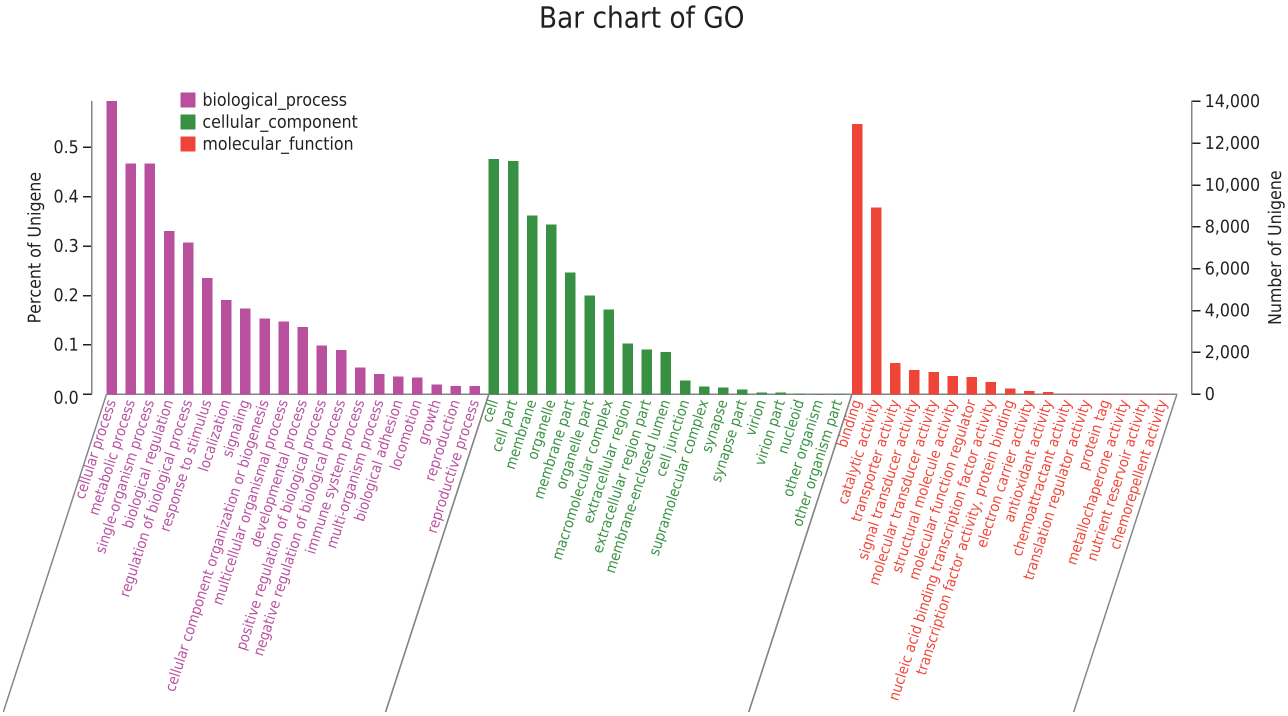

Fig. 3. Distribution of GO classifications at level 2. In total 280408 unigenes were assigned to three categories: biology, cellular component, and molecular function. The horizontal axis indicates the GO terms. The left and right vertical axis represent the percentage of unigenes and number of unigenes that are classified in the corresponding term, respectively.

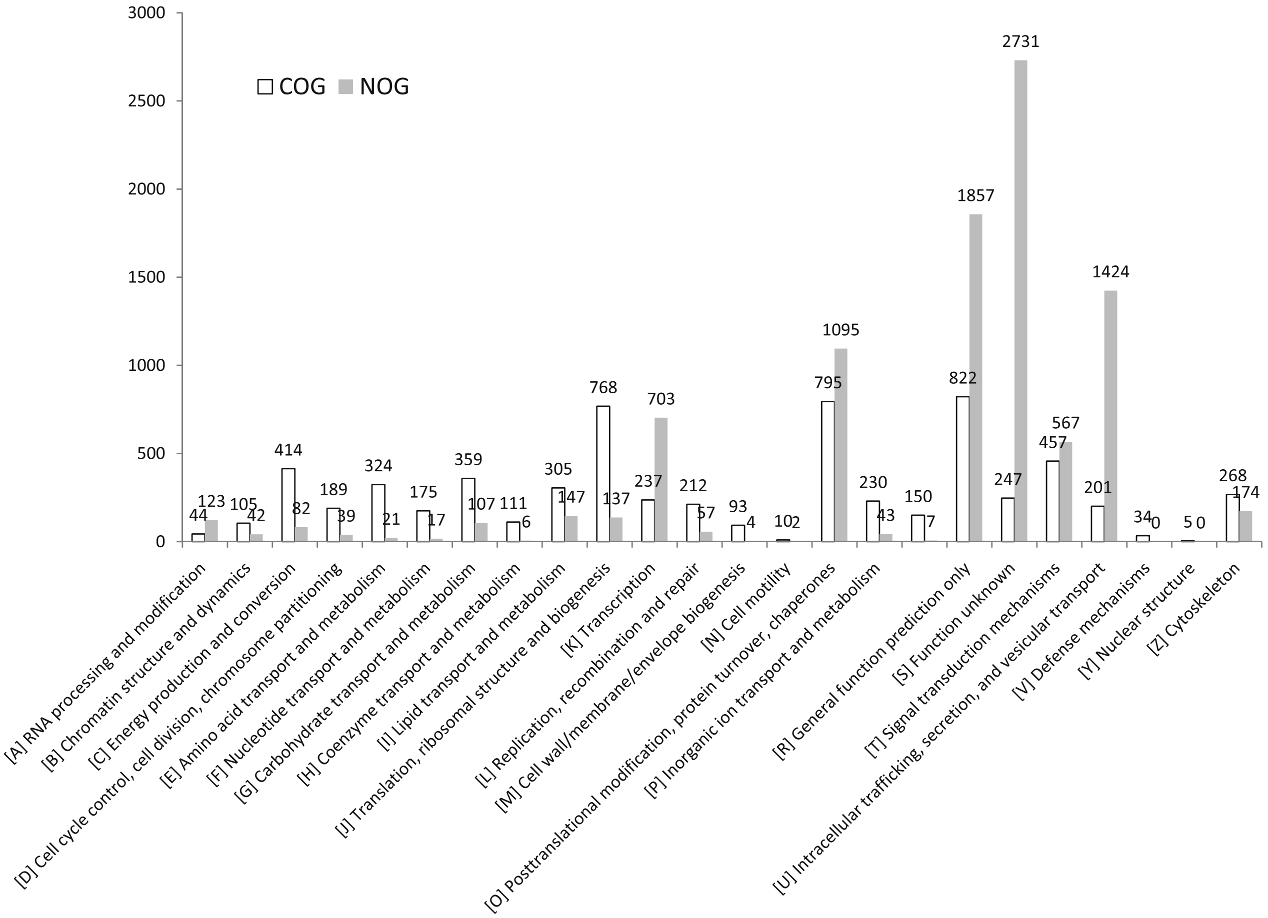

Fig. 4. Cluster of KOG annotations of assembled unigenes. 6555 and 9385 were clustered into 26 functional categories

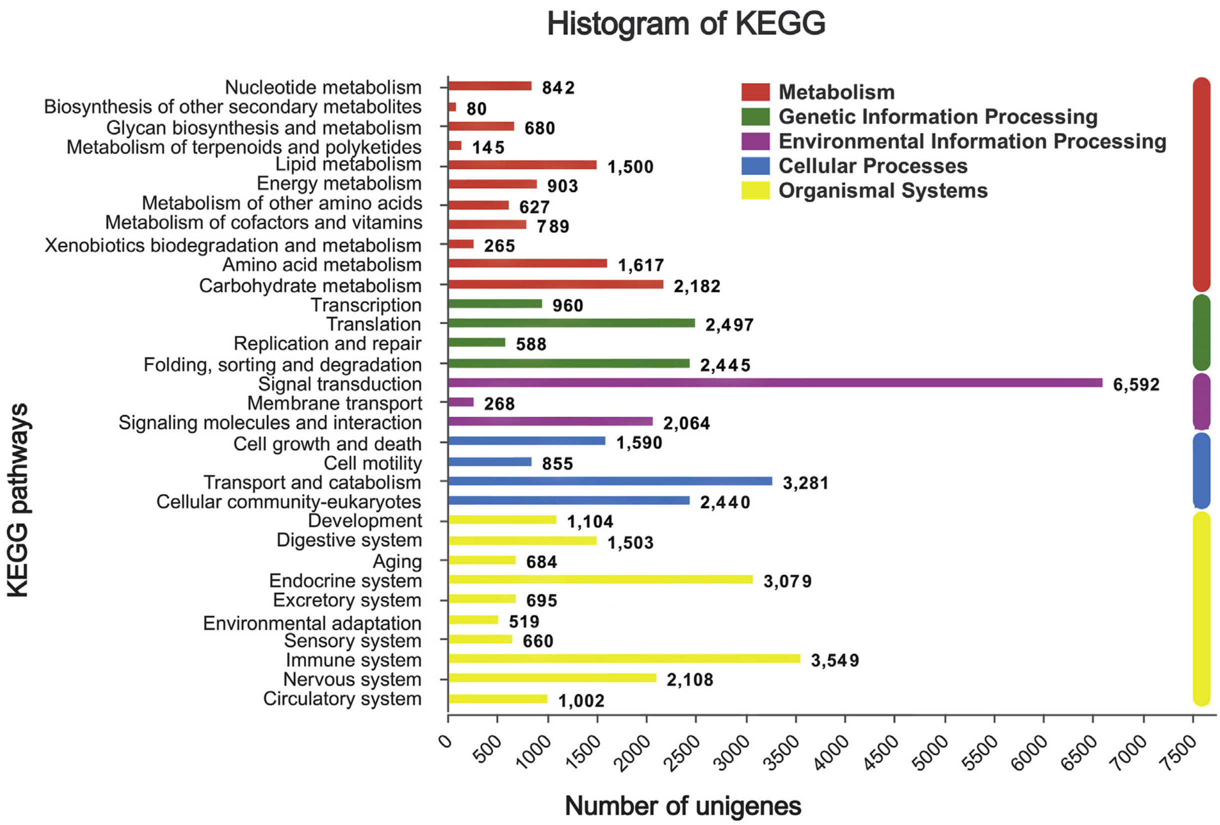

Fig. 5. KEGG pathways assignment of the assembled unigenes. unigenes were assigned to 335 pathways belonging to six main categories: organismal systems. Metabolism, environmental information processing, and cellular process. Five main categories were shown, with the exception of human diseases because of the Yangtze sturgeon in not belong to human species.

3.2. De novo transcriptome assembly and analysis

The transcriptome sequencing of the FOLF, SOLF, FOPF, and SOPF groups was performed using the Illumina XTen platform. All raw sequence data is deposited in the NCBI GEO database with the corresponding accession number GSE136237. After quality filtering, 41,626,756, 44,725,602, 42,910,468, and 43,864,058 clean reads were obtained in the FOLF, SOLF, FOPF, and SOPF groups, respectively, with Q20 (nucleotides with quality values > 20) $> 98\%$, Q30 $> 95\%$ and error rate $< 0.03\%$ (Table 2). These clean reads were assembled into 653,999 transcripts with an N50 length of 1047 bp, and a final set of 280,408 unigenes was generated (Table 3). The scores for the quality assessment of transcriptome assembly using TransRate and BUSCO were 0.131 and 89.7% (Table 3).

3.3. Annotation of unigenes

A sequence similarity search was performed against the NR, Swiss-Prot, Pfam, COG, GO, and KEGG, which annotated 65,393 (23.32%), 63,423 (23.62%), 44,876 (16.00%), 15,180 (5.41%), 23,599 (8.42%), and 45,471 (16.22%) unigenes, respectively. More than 51% of the unigenes had an E-value less than 1e-30 (Fig. 2A), and more than 85.82% of the unigenes shared more than 60% identity compared with the NR database (Fig. 2B). The total of 20,758 clusters (32.17%) was similar to *Lepisosteus oculatus* (Fig. 2C).

All 280,408 unigenes overlapped in the GO, COG, and NOG databases. All unigenes were assigned to the three main GO categories: biological processes (85,734, 30.57%), cellular components (61,994, 22.11%), and molecular functions (28,413, 10.13%) (Fig. 3). After mapping the unigenes to the COG database, 6555 and 9385 were clustered into 26 functional categories in the COG and NOG databases, respectively (Fig. 4).

We also mapped the unigenes to the 5 categories of KEGG metabolic pathways, i.e., metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems. Most of the genes were from signal transduction (6592 unigenes), followed by the immune system (3549 unigenes), transport and catabolism (3281 unigenes), and the endocrine system (3079 unigenes) (Fig. 5). The immune system unigenes were further classified into 20 immune-related pathways. The 10 largest immune pathways were Toll-like receptor signaling (696), RIG-I-like receptor signaling (547), NLR signaling (519), Toll and Imd signaling (477), hematopoietic cell lineage (432), Fc gamma R-mediated phagocytosis (402), natural killer cell mediated cytotoxicity (383), interleukin-17 signaling (381), Th17 cell differentiation (354), and Th1 and Th2 cell differentiation (341) (Fig. 6).

3.4. Differentially expressed genes in sturgeon fed different fatty acids diets

A rigorous differential gene screening condition was employed to identify the key genes involved in the response of the immune-related genes to the different fatty acids diets. The unigenes with p-adjusted < 0.001 and $|\text{fold change}| > 2$ were identified as differentially expressed genes. The differentially expressed genes during long-term feeding of the different fat source diets, postprandial fish oil diet, and postprandial soybean oil diet were screened, and 6192 upregulated unigenes and 300 downregulated unigenes, 363 upregulated unigenes and 18,252 downregulated unigenes, and 4125 upregulated unigenes, and 521 downregulated were screened, respectively (Fig. 7). The unigenes were reversely regulated after feeding the soybean oil diet and the fish oil diet and differently expressed after long-term feeding (49 days) of the same diet, were considered involved in the response to the different fat sources. Based on these screening conditions, 2839 unigenes were identified to participate in the response of the different fat

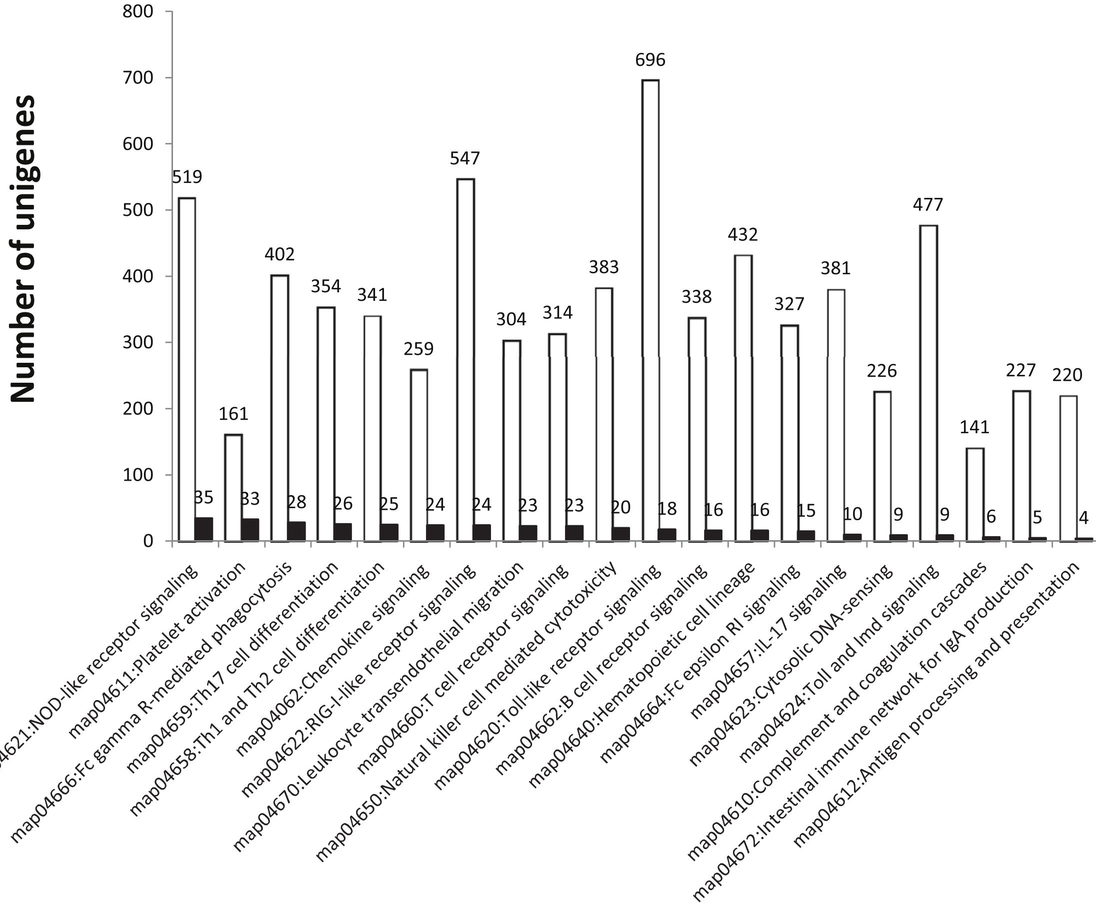

Fig. 6. The number of Unigenes related to immune pathways (white pillar) or immune-related differential gene in response to different fat sources (black pillar) based on KEGG annotation analysis.

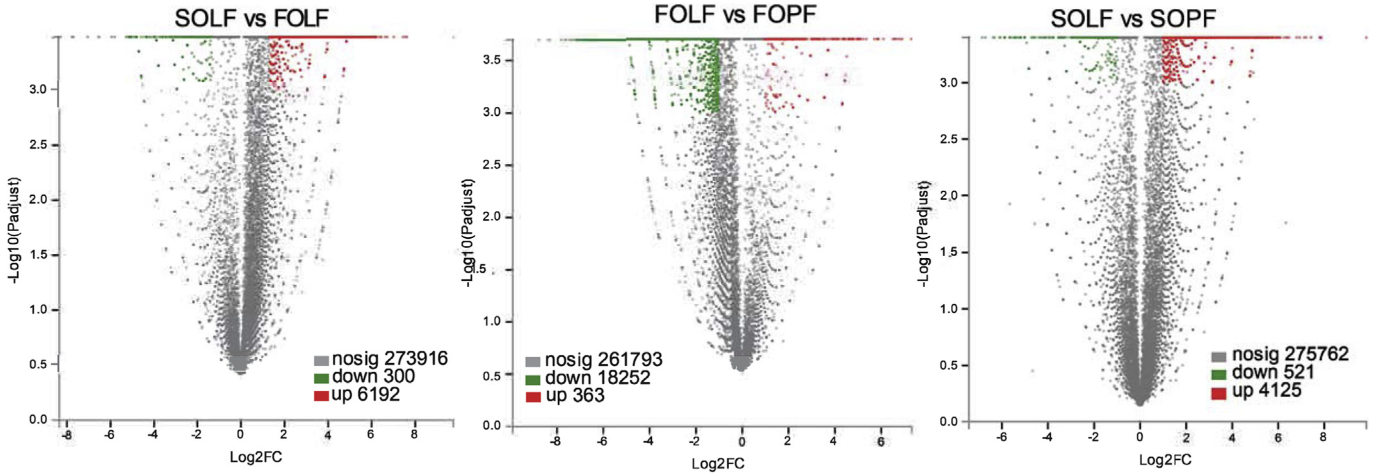

Fig. 7. Volcano plots of differentially unigenes in long-term feeding and postprandial for different fat source diets. (A) long-term feeding for different fat source diet, (B) postprandial for fish oil diet, (C) postprandial for soybean oil diets.

sources (Fig. 8).

3.5. Differentially expressed immune-related genes and pathways

To assign the differentially expressed genes, a KEGG analysis was used for functional annotation. The top 20 enrichment KEGG pathways are shown in Fig. 9, which indicates that the enriched pathways were mainly related to the immune system and human diseases. However, this study focused on the fish immune system, so human disease-related pathways were excluded from the analysis. Differentially expressed genes were enriched for all immune-related pathways in this study, and the five largest enriched pathways were NOD-like receptor signaling (35 unigenes), platelet activation (33 unigenes), Fc gamma R-mediated phagocytosis (28 unigenes), Th17 cell differentiation (26 unigenes), and Th1 and Th2 cell differentiation (25 unigenes) (Fig. 6).

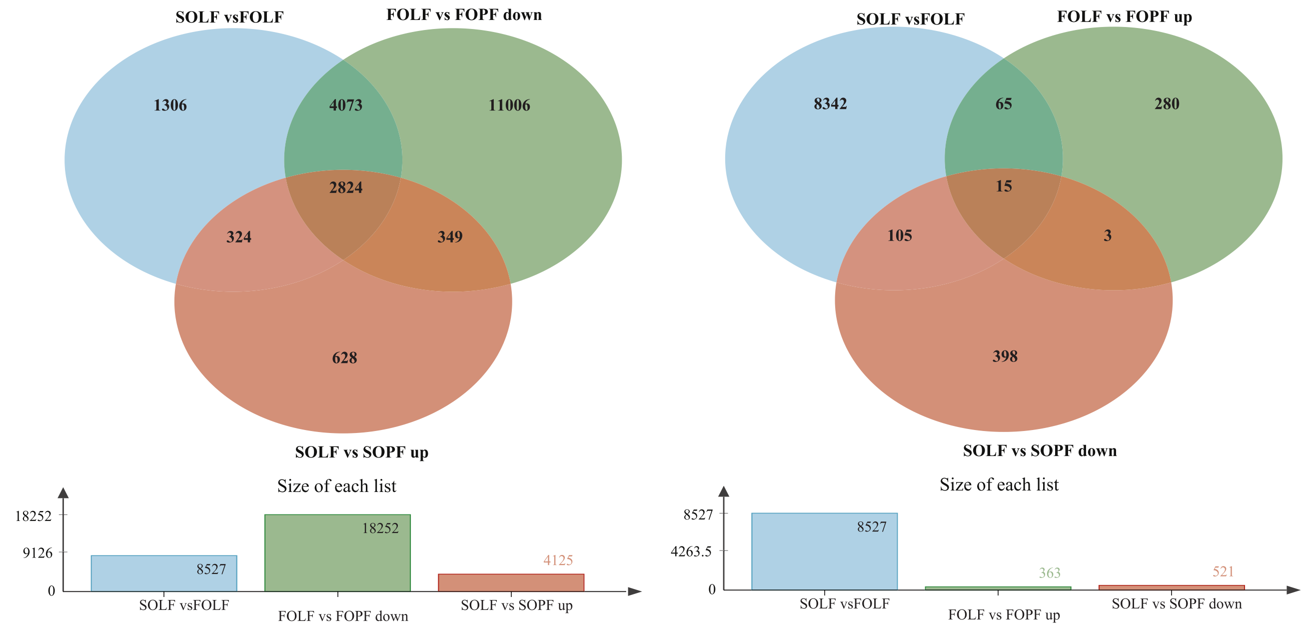

Fig. 8. Screening of key unigenes in response to different fat source diets. (A) Venn diagram of differentially unigenes, down-regulated in postprandial for fish oil diet, up-regulated in postprandial for soybean oil diets and difference in long-term feeding for different fat source diet. (B) Venn diagram of differentially unigenes, up-regulated in postprandial for fish oil diet, down-regulated in postprandial for soybean oil diets and difference in long-term feeding for different fat source diet.

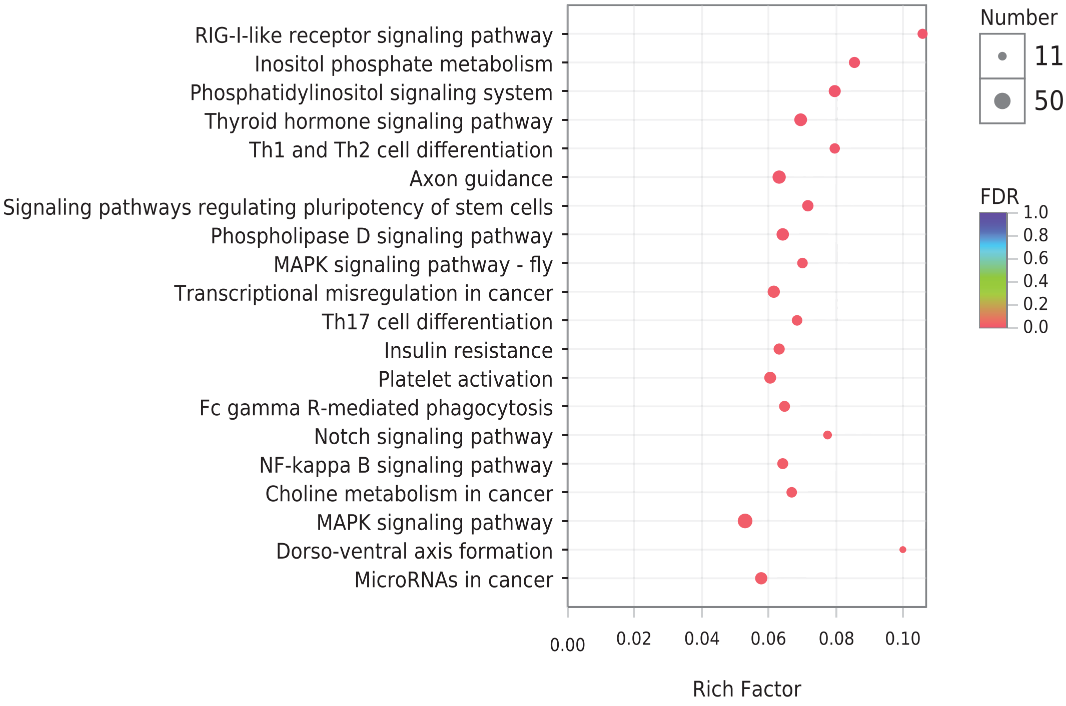

Fig. 9. The top 20 enrichment KEGG classifications of differentially expressed unigenes. The top 20 enrichment KEGG pathways were shown, and the x-axis represents the rich factor, and the y-axis indicates the corresponding KEGG pathway.

3.6. Key immune-related genes in response to the different fat diets

Among the five pathways with the most enrichment, genes involved in two or more pathways were considered to be key genes in this study. Based on a Venn analysis, there was 1 unigene involved in 4 pathways, 7 unigenes involved in 3 pathways, and 17 unigenes involved in 2 pathways (Fig. 10 and Table 4).

3.7. Transcript validation by qPCR

Genes that were involved in differentially expressed genes in the above three pathways or that differed in expression by more than 6-fold in long-term feeding trials were verified by qPCR. Eight immune-related genes, such as mitogen-activated protein kinase 14A (MAPK14A), mitogen-activated protein kinase 8 (MAPK8), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFκB1), non-receptor tyrosine-protein kinase 2 (TYK2), linker for activation of T-cells family member 1 (LATM1), 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase

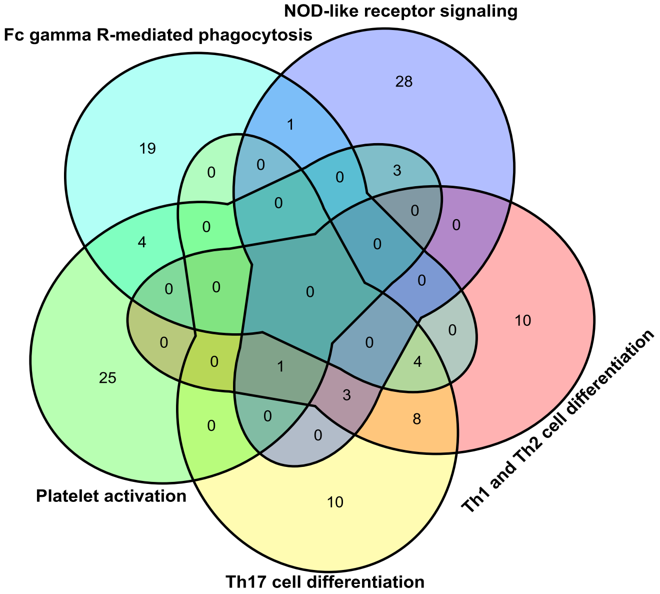

Fig. 10. Venn diagram of five candidate immune pathway genes that respond to different fat sources.

gamma-1 (PLCG1), protein kinase C theta type isoform X1 (PKCθ), and 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3 (PLCB3) were selected to validate the annotated data. The primers (Table 4) of all eight genes were amplified to obtain the correct product, which was verified by sequencing. Next, the standard curve for each pair of primers was prepared to obtain the amplification efficiency of the primers (Table 4), wherein the amplification efficiencies of NFκB1, MAPK14A, MAPK8, TYK2, LATM1, PLCG1, PKCθ, and PLCB3 were 97.9%, 91.1%, 101.0%, 93.2%, 99.8%, 91.0%, 94.0%, and 99.3%, respectively. Information on β-actin and EF1-α was referred to our previous study [32].

In the long-term feeding test, 7 genes in the FOLF group were upregulated except for PLCB3, and MAPK14A, PLCG1, TYK2, and MAPK8 were upregulated significantly, compared with those in the SOLF group (Fig. 11A). In the postprandial test, 6 genes in the FOPF group were upregulated except PKCθ and MAPK8, and the upregulated expression levels of NFκB1, MAPK14A, PLCB3, LATM1, PLCG1, and TYK2 were significant, compared with those in the SOPF group (Fig. 11B).

3.8. Immunomodulatory effect of DHA and EPA on the LPS challenge

A co-injection experiment with LPS, DHA, and EPA was performed to study the function of the selected key immunity genes and to verify the immunoprotection of unsaturated fatty acids, such as DHA and EPA. The results showed that after the LPS challenge, eight candidate genes sharply increased (Fig. 12). However, PLCB3 and TYK2 in the LPS and DHA and EPA co-injection group were significantly up-regulated compared with the control group, whereas PKCθ was significantly down-regulated (Fig. 12), and the other five genes were not significantly different (Fig. 12). Hence, injection of DHA and EPA at the same time as LPS challenge were significantly reduced the expression levels of these candidate genes, and most of the candidate genes (5/8) were returned to the expression level of the control group (Fig. 12).

4. Discussion

Research on the relationship between immunomodulation and nutrition is critical to conservation and germplasm development for extremely endangered species, such as Yangtze sturgeon. Here, we provide evidence for the effects of PUFAs on the immune response of Yangtze sturgeon. After 49 days of feeding two different fats diets, the serum immune indicators of the FOLF group, such as LZM, ACP, and AKP, were significantly higher than those in the SOLF group. Subsequently, RNAseq was used to understand the immune response to the different fat source diets in the Yangtze sturgeon spleen. Based on the RNAseq analysis and expression levels of genes, 8 genes were selected to validate the RNAseq data. The results showed that the majority of the immune-related candidate genes in the fish oil diet group was significantly higher than in fish fed the soybean oil diet, both for long-term feeding and postprandial. After the LPS challenge, 8 candidate genes sharply increased, but this increase was inhibited when co-injecting DHA and EPA. These results are significant for further investigations on the relationship between nutrition and immune function in Yangtze sturgeon.

The effects of different fat diets on animal immune responses have been widely confirmed [34,35]. In the present study, the immune response related enzyme activities of LZM, ACP, and AKP in the FOLF group were significantly higher than those in the SOLF group. After the pathogen phagocytosis process, which is the first line of internal defense, ACP, AKP, and LZM help to completely degrade exogenous substances [36]. ACP and AKP play an extremely important role in

Table 4
Information on candidate genes and their qPCR primers.

Gene name	Abbreviated name	Pathway	Primer for qPCR	TM	Amplification length
mitogen-activated protein kinase 14A	MAPK14A	A, B, D, E	5′ GAAGATGCTGGTTCTGGACAC 3′ 5′ CAAAAGGAGACGGCTCAAAG 3′	61.4	203
mitogen-activated protein kinase 8	MAPK8	A, C, D	5′ TGACTCGGAGCACAACAAG 3′ 5′ TGAAGAGCCTCGTCCACAG 3′	61.4	105
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	nfkb1	A, C, D	5′ GCACAGCCTGGTTGGAAAG 3′ 5′ AGACGCCGAAGTTGTAGCC 3′	61.4	179
tyrosine-protein kinase 2	TYK2	A, C, D	5′ CCCCGAGATCACGCACAT 3′ 5′ CAGCCGTCAGCCGAAAGT 3′	63.3	146
linker for activation of T-cells family member 1	LATM1	C, D, E	5′ GCCCCAGCACTACACTAACC 3′ 5′ GGATGGAACGGACAGGAAG 3′	61.4	121
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	PLCG1	C, D, E	5′ CTCCGCTGACCGAGAACA 3′ 5′ TGGCAAGGGTGAAGACAAA 3′	63.3	123
protein kinase C theta type	PKCθ	A, C	5′ CTGAGATGGAGAGTGAGGGA 3′ 5′ AGAATGTGGGTTGAGGAAAG 3′	61.4	123
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3	PLCB3	A, B	5′ AACGACGAGGAGCCTTTCA 3′ 5′ TCTTCACCTGCTGCTTGGA 3′	61.4	163
Beta-actin	β-actin		5′ CTGTTTCAGCCATCCTTCTTG 3′ 5′ TTGATTTTCATTGTGCTCGGT 3′	59.6	92
elongation factor 1-alfa	EF1-α		5′ ATGTTCACAATGGCAGCGTC 3′ 5′ AAGATTGACCGTCGTTCCG 3′	60	80

A: NOD-like receptor signaling; B: Platelet activation; C: Fc gamma R-mediated phagocytosis; D: Th17 cell differentiation; E: Th1 and Th2 cell differentiation.

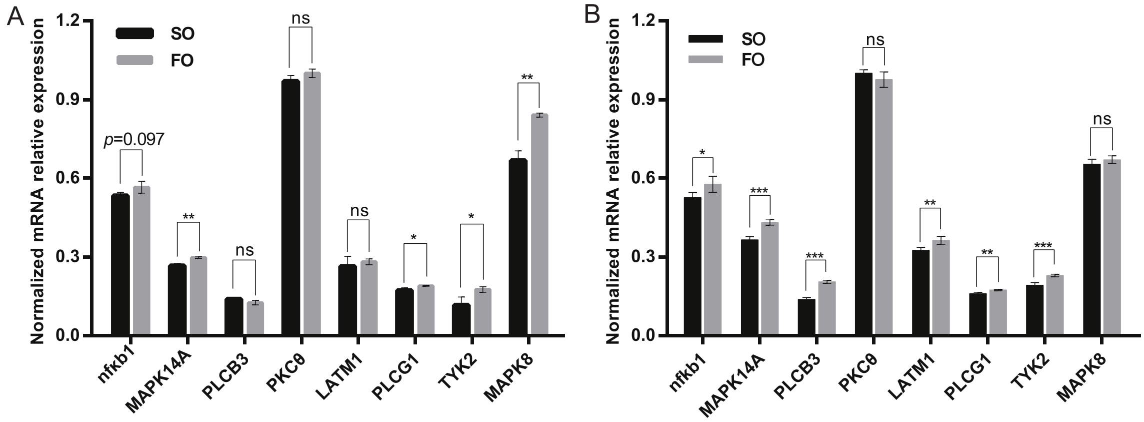

Fig. 11. Validation candidate gene of the transcriptome DEGs in Dabry's sturgeon using qPCR. Gene expression levels were normalized to that of β-actin and EF1-α. Data are presented as the group means ± SEM (n = 6). Statistical comparison of the mRNA levels detected at different group was carried out by t-test of variance (*P < 0.05, **P < 0.01, ***P < 0.001).

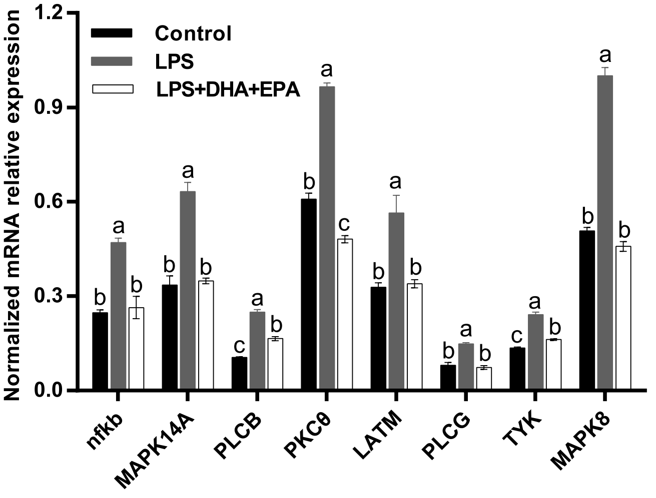

Fig. 12. Expression response of transcriptome candidate genes in the presence of DHA and EPA under LPS challenge. Gene expression levels were normalized to that of β-actin and EF1-α. Data are presented as the group means ± SME (n = 3). Statistical comparison of the mRNA levels detected at the different group was carried out by t-test of variance. Different letters of the same gene indicate significant differences.

dephosphorylation, particularly in performing signal transduction, metabolism, and environmental adaptation [37]. Moreover, increases of ACP and AKP activities indicate that defense against foreign materials is enhanced [38,39]. LZM is an important innate immune factor that kills bacteria and prevents bacterial infections [39,40]. A previous study showed that when environmental factors, such as culture temperature [41], salinity [42] and flow velocity [43], change, LZM levels correspondingly change. In the present study, the increases in LZM, ACP, and AKP activities in the FOLF group indicated that fish oil might increase the immunological activity of Yangtze sturgeon.

Transcriptome sequencing (RNAseq) was an excellent tool to explore the potential mechanisms by which fish oil diets enhance immunological activity in Yangtze sturgeon. RNAseq has been applied in many areas, such as gene expression profiles, alternative splice variants, and novel transcript identification [44]. In addition, RNAseq is a powerful and efficient way to understand genes and their pathways, particularly in species without a reference genome, such as sturgeon [15]. The genes of Yangtze sturgeon have only previously been investigated in head kidney [21], testis, and ovary [22] cDNA libraries and only 123 mRNAs have been recorded in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). In this experiment, 28,0408 genes were identified by sequencing the transcriptomes of the brain, liver, and spleen, which was greater than those identified in the head kidney [21] and gonads [22]. However, in this experiment, the same gene was interrupted into fragments, which is closely related to the sequencing strategy of the second-generation sequencing system. Therefore, use of the latest generation of sequencing [45] such as giant salamander(*Andrias davidianus*) [46], to establish a reference transcription data set will be more conducive to applying RNAseq in Yangtze sturgeon studies.

The transcriptome analysis in the present study revealed that the NLR signaling pathway, FcγR-mediated phagocytosis pathway, platelet activation, Th17 cell differentiation, and Th1 and Th2 cell differentiation were the key pathways for the immune system responseto the different fat sources. NLR signaling is known to mediate the innate immune response to cell damage and stress [47–49]. The FcγR-mediated phagocytosis pathway plays an essential role in the recognition of foreign extracellular materials, formation of phagosomes and digestion of engulfed materials in phagosomes [50–52]. Platelets play a beneficial and critical role in inflammation-associated hemostasis and primary hemostasis after disruption of vessel wall integrity [53]. Balanced differentiation of Th cells is crucial for immunity and host protection, in which Th1 cells stimulate strong cell-mediated immune responses, particularly against intracellular pathogens [54]; Th2 induces humoral responses against parasitic helminths (type 2 immunity) [55], and Th17 differentiation may serve as a protective strategy to “fine-tune” the expression interleukin (IL)-17, so it does not cause excessive inflammation [56]. The qPCR transcriptome validation results showed that the expression of candidate genes in the fish oil diet group increased significantly in 49 day long-term feeding and postprandial groups compared with the soybean oil diet group. In summary, the fish oil diets appropriately upregulated gene expression in immune-related pathways and played an important role in immune regulation.

The main components of fish oil regulating Yangtze sturgeon immunity may be DHA and EPA. The largest difference between fish oil and soybean oil is unsaturated fatty acids, particularly DHA and EPA. In mammals, DHA and EPA activate their specific fatty acid receptor, FFAR4, which leads to the recruitment of β-arrestin-2 and TAB1 (TAK1 binding protein 1) [12]. This process suppresses stimulation of the IKKb/NF-κB and JNK/AP pathways by LPS and NF-κB-mediated pro-inflammatory events, such as secretions of IL-6 and tumor necrosis factor-α [12]. In addition, DHA-mediated FFAR4 coupling with β-arrestin-2 repressees TLR4 signaling via Akt/JNK phosphorylation and nuclear translocation of p65 [57]. DHA activates cytosolic phospholipase A2 (cPLA2)and cyclooxygenase-2, causing prostaglandin E2 release, which inhibites the NF-kB signaling pathway through the prostaglandin E2 receptor 4 (EP4) receptor and LPS induced IL-6 secretion

[58]. Therefore, DHA and EPA play an important role in mammalian immune regulation. In this study, the expression of candidate genes increased sharply after the LPS challenge. However, injectiing of DHA and EPA at the same time as the LPS challenge were significantly reduced the expression levels of these candidate genes, and most of the candidate genes (5/8) returned to the expression levels of the control group. These results indicate that DHA and EPA have immunoregulatory effects after an LPS challenge in Yangtze sturgeon. However, the FFAR4 gene has not yet been identified in fish [13]. In summary, similar to mammals, DHA and EPA have immunoregulatory effects in the Yangtze River, but the mechanism remains unclear. These results will be important for future research on the relationship between fatty acid nutrition and immunity o in Yangtze sturgeon.

5. Conclusion

In this study, long-term feeding of different fish oil diets increased the activities of innate immune-related enzymes in the serum of Yangtze sturgeon. Transcriptome assays showed that the fish oil diets moderately upregulated immune-related signaling pathways in the spleen of Yangtze sturgeon, i.e., NLR signaling, platelet activation, Fc gamma R-mediated phagocytosis, Th17 cell differentiation, and Th1 and Th2 cell differentiation. The qPCR results of the candidate genes for these pathways showed similar results. The LPS challenge revealed that ployunsaturated fatty acids activated immune-related pathways and regulated the inflammatory response caused by excessive activation of immune pathways by pathogens. This study confirmed the immunomodulatory function of DHA and EPA in Yangtze sturgeon and will provide a reference for the preparation of artificial diets for Yangtze sturgeon.

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References

[1] H. Zhang, Q.W. Wei, H. Du, L.X. Li, Present status and risk for extinction of the Dabry's sturgeon (Acipenser dabryanus) in the Yangtze River watershed: a concern for intensified rehabilitation needs, J. Appl. Ichthyol. 27 (2011) 181–5.

[2] J. Li, D. Liu, Q. Ma, X. Zhang, D. Wei, Y. Chen, et al., Erratum to: discriminating Dabry's sturgeon (Acipenser dabryanus) and Chinese sturgeon (A. sinensis) based on DNA barcode and six nuclear markers, Hydrobiologia 762 (2015) 267–268.

[3] H. Du, Q.W. Wei, X. Xie, L.L. Shi, J.M. Wu, X.M. Qiao, et al., Improving swimming capacity of juvenile Dabry's sturgeon, (Acipenser dabryanus Duméril, 1869) in current-enriched culture tanks, J. Appl. Ichthyol. 30 (2015) 1445–1450.

[4] J. Wu, Q. Wei, H. Du, C. Wang, H. Zhang, Initial evaluation of the release programme for D abry's sturgeon (Acipenser dabryanus D uméril, 1868) in the upper Y angtze R iver, J. Appl. Ichthyol. 30 (2014) 1423–1427.

[5] S.H. Zhang, H. Luo, H. Du, D.Q. Wang, Q.W. Wei, Isolation and characterization of twenty-six microsatellite loci for the tetraploid fish Dabry's sturgeon (Acipenser dabryanus), Conserv. Genet. Res. 5 (2012) 409–412.

[6] W. Sung, Y. Peiqi, C. Yiyu, E.S.S. Commission, China Red Data Book of Endangered Animals: Pisces, (1998).

[7] J.M. Wu, Q.W. Wei, H. Du, C.Y. Wang, H. Zhang, Initial evaluation of the release programme for Dabry's sturgeon (Acipenser dabryanus Dumeril, 1868) in the upper Yangtze River, J. Appl. Ichthyol. 30 (2014) 1423–1427.

[8] Q. Wei, F.E. Ke, J. Zhang, Z. Ping, J. Luo, R. Zhou, et al., Biology, Fisheries, and Conservation of Sturgeons and Paddlefish in China, (1997).

[9] Q. Gong, Y. Liu, D.U. Jun, G. Zhao, X.J. Chen, L.I. Jun, et al., Study on artificial reproduction of cultured Acipenser dabryanus dumeril, Southwest China J. Agric. Sci. 26 (4) (2013) 1710–1714.

[10] Y.Q. Yan, W. Jiang, T. Spinetti, A. Tardivel, R. Castillo, C. Bourquin, et al., Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation, Immunity 38 (2013) 1154–1163.

[11] L. Wellhauser, D.D. Belsham, Activation of the omega-3 fatty acid receptor GPR120 mediates anti-inflammatory actions in immortalized hypothalamic neurons, J. Neuroinflammation (2014) 11.

[12] O. DY, S.T., B. EJ, T.I., H.M., W.F., et al., GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects, Cell 142 (2010) 687–698.

[13] M. Sawzdargo, S.R. George, T. Nguyen, S. Xu, L.F.K. Jr, B.F. O'Dowd, A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1, Biochem. Biophys. Res. Commun. 239 (1997) 543–547.

[14] D.R. Tocher, Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective, Aquaculture 449 (2015) 94–107.

[15] M. Vidotto, A. Grapputo, E. Boscari, F. Barbisan, A. Coppe, G. Grandi, et al., Transcriptome sequencing and de novo annotation of the critically endangered Adriatic sturgeon, BMC Genomics 14 (2013) 407.

[16] X. Zhang, S. Wang, S. Chen, Y. Chen, Y. Liu, C. Shao, et al., Transcriptome analysis revealed changes of multiple genes involved in immunity in Cynoglossus semilaevis during Vibrio anguillarum infection, Fish Shellfish Immunol. 43 (2015) 209–218.

[17] M. Long, J. Zhao, T. Li, C. Tafalla, Q. Zhang, X. Wang, et al., Transcriptomic and proteomic analyses of splenic immune mechanisms of rainbow trout (Oncorhynchus mykiss) infected by Aeromonas salmonicida subsp. salmonicida, J. Proteom. 122 (2015) 41–54.

[18] Z. Jiajie, L. Chao, A. Qiuwei, T. Yun, L. Yongju, G. Yafen, et al., Trancriptomic profiling revealed the signatures of acute immune response in tilapia (Oreochromis niloticus) following Streptococcus iniae challenge, Fish Shellfish Immunol. 46 (2015) 346–353.

[19] Z. Qi, P. Wu, Q. Zhang, Y. Wei, Z. Wang, M. Qiu, et al., Transcriptome analysis of soiny mullet (Liza haematocheila) spleen in response to Streptococcus dysgalactiae, Fish Shellfish Immunol. 49 (2016) 194–204.

[20] M.P. Polinski, J.C. Bradshaw, S.M. Inkpen, J. Richard, C. Fritsvold, T.T. Poppe, et al., De novo assembly of Sockeye salmon kidney transcriptomes reveal a limited early response to piscine reovirus with or without infectious hematopoietic necrosis virus superinfection, BMC Genomics 17 (2016) 848.

[21] K. Luo, J. Di, P. Han, S. Zhang, L. Xia, G. Tian, et al., Transcriptome analysis of the critically endangered Dabry's sturgeon (Acipenser dabryanus) head kidney response to Aeromonas hydrophila, Fish Shellfish Immunol. 83 (2018) 249–261.

[22] Y. Chen, Y. Liu, G. Quan, J. Lai, M. Song, J. Du, et al., Gonadal transcriptome sequencing of the critically endangeredAcipenser dabryanusto discover candidate sex-related genes, Peerj 6 (2018) e5389.

[23] J. Qi, N. Tang, Y. Wu, H. Chen, S. Wang, B. Wang, et al., The transcripts of CRF and CRF receptors under fasting stress in Dabry’s sturgeon (Acipenser dabryanus Dumeril), Gen. Comp. Endocrinol. 280 (2019) 200–208.

[24] Z. Xie, C. Niu, Z. Zhang, B. Lei, Dietary ascorbic acid may be necessary for enhancing the immune response in Siberian sturgeon (Acipenser baerii), a species capable of ascorbic acid biosynthesis, Comp. Biochem. Physiol., A 145 (2006) 152–157.

[25] J. Rui, B.L. Liu, H. Cen, B. Huang, J.L. Lei, Effects of ammonia exposure on stress and immune response in juvenile turbot (Scophthalmus maximus), Aquacult. Res. 48 (2017).

[26] H.U. Bergmeyer Jr., G.N. Bowes, M. Hørder, D.W. Moss, Provisional recommendations on IFCC methods for the measurement of catalytic concentrations of enzymes Part 2. IFCC method for aspartate aminotransferase, J. Clin. Chem. Clin. Biochem. Z. Klin. Chem. Klin. Biochem. 70 (1977) F19–F42.

[27] F.A. Simao, R.M. Waterhouse, I. Panagiotis, E.V. Kriventseva, E.M. Zdobnov, BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs, Bioinformatics 31 (2015) 3210–3212.

[28] G. Pertea, X. Huang, F. Liang, V. Antonescu, R. Sultana, S. Karamycheva, et al., TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets, Bioinformatics 19 (2003) 651–652.

[29] S.R. Eddy, Profile hidden Markov models, Bioinformatics 14 (1998) 755–763.

[30] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034.

[31] C. Tricarico, P. Pinzani, S. Bianchi, M. Paglierani, V. Distante, M. Pazzagli, et al., Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies, Anal. Biochem. 309 (2002) 293–300.

[32] L. Zhi-Hai, C. Hu, W. Bin, W. Yuan-Bing, T. Ni, Q. Jin-Wen, et al., Cloning and expression stability of reference genes β - actin, GAPDH and EF1-α in Acipenser dabryanus, J. Agric. Biotechnol. Nong Ye Sheng Wu Jishu Xuebao 26 (2018) 1846–1855.

[33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (2001) 402–408.

[34] P.C. Calder, Polyunsaturated fatty acids, inflammation, and immunity, Eur. J. Clin. Nutr. 36 (2001) 1007–1024.

[35] B.C. Do Amaral, Effect of Supplemental Fat Source on Production, Immunity, Hepatic Gene Expression, and Metabolism of Periparturient Dairy Cows, University of Florida, 2008.

[36] CANICATTI. Lysosomal, Enzyme pattern in Holothuria polii coelomocytes, J. Invertebr. Pathol. 56 (1990) 0–74.

[37] X. Kong, S. Wang, H. Jiang, G. Nie, X. Li, Responses of acid/alkaline phosphatase, lysozyme, and catalase activities and lipid peroxidation to mercury exposure during the embryonic development of goldfish Carassius auratus, Aquat. Toxicol. 120–121 (2012) 119–125.

[38] Y. Zang, X. Tian, S. Dong, Y. Dong, Growth, metabolism and immune responses to evisceration and the regeneration of viscera in sea cucumber, Apostichopus japonicus, Aquaculture 358–359 (2012) 50–60.

[39] D. Huo, L. Sun, X. Ru, L. Zhang, C. Lin, S. Liu, et al., Impact of hypoxia stress on the physiological responses of sea cucumberApostichopus japonicus: respiration,

digestion, immunity and oxidative damage, Peerj 6 (2018) e4651.

[40] C. Canicatti, P. Roch, Studies on Holothuria polii (Echinodermata) antibacterial proteins. I. Evidence for and activity of a coelomocyte lysozyme, Experientia 45 (1989) 756–759.

[41] A.L. Langston, R. Hoare, M. Stefansson, R. Fitzgerald, H. Wergeland, M. Mulcahy, The effect of temperature on non-specific defence parameters of three strains of juvenile Atlantic halibut (Hippoglossus hippoglossus L.), Fish Shellfish Immunol. 12 (2002) 0–76.

[42] H. Zheng, L.I. Bin, X.J. Rong, M.J. Liao, G.P. Chen, Z. Zhang, et al., Effects of salinity and dissolved oxygen variation on the non-specific immune response of Apostichopus japonicus, Prog. Fish. Sci. 1 (2014) 118–124.

[43] X. Li, L. Ji, L. Wu, X. Gao, X. Li, J. Li, et al., Effect of flow velocity on the growth, stress and immune responses of turbot (Scophthalmus maximus) in recirculating aquaculture systems, Fish Shellfish Immunol. 86 (2019) 1169–1176.

[44] W. Huang, G. Marth, EagleView: a genome assembly viewer for next-generation sequencing technologies, Genome Res. 18 (2008) 1538–1543.

[45] M.J. Levene, J. Korlach, S.W. Turner, M. Foquet, H.G. Craighead, W.W. Webb, Zero-mode waveguides for single-molecule analysis at high concentrations, Science 299 (2003) 682–686.

[46] X. Geng, W. Li, H. Shang, Q. Gou, F. Zhang, X. Zang, et al., A reference gene set construction using RNA-seq of multiple tissues of Chinese giant salamander, Andrias Davidianus Gigascience 6 (2017) 1–7.

[47] P. Broz, V.M. Dixit, Inflammasomes: mechanism of assembly, regulation and signalling, Nat. Rev. Immunol. 16 (2016) 407–420.

[48] M. Lamkanfi, V.M. Dixit, Inflammasomes and their roles in health and disease, Annu. Rev. Cell Dev. Biol. 28 (2012) 137.

[49] L. Mohamed, V.M. Dixit, Mechanisms and functions of inflammasomes, Cell 157 (2014) 1013–1022.

[50] F. Nimmerjahn, J.V. Ravetch, Fcγ receptors: old friends and new family members, Immunity 24 (2006) 19–28.

[51] F. Nimmerjahn, P. Bruhns, K. Horiuchi, J.V. Ravetch, FcγRIV: a novel FcR with distinct IgG subclass specificity, Immunity 23 (2005) 41–51.

[52] M. Daëron, Fc receptor biology, Annu. Rev. Immunol. 15 (1997) 203–234.

[53] B. Ho-Tin-Noé, Y. Boulaftali, E. Camerer, Platelets and vascular integrity: how platelets prevent bleeding in inflammation, Blood 131 (2018) 277–288.

[54] R.B. Moss, T. Moll, M. El-Kalay, C. Kohne, W. Soo Hoo, J. Encinas, et al., Th1/Th2 cells in inflammatory disease states: therapeutic implications, Expert Opin. Biol. Ther. 4 (2004) 1887–1896.

[55] N. Harris, W.C. Gause, To B or not to B: B cells and the Th2-type immune response to helminths, Trends Immunol. 32 (2011) 80–88.

[56] A. Peck, E.D. Mellins, Precarious balance: Th17 cells in host defense, Infect. Immun. 78 (2010) 32–38.

[57] X. Li, Y. Yu, C.D. Funk, Cyclooxygenase-2 induction in macrophages is modulated by docosahexaenoic acid via interactions with free fatty acid receptor 4 (FFA4), FASEB J. 27 (2013) 4987–4997.

[58] Y. Liu, L.Y. Chen, M. Sokolowska, M. Eberlein, S. Alsaaty, A. Martinez-Anton, et al., The fish oil ingredient, docosahexaenoic acid, activates cytosolic phospholipase A2 via GPR120 receptor to produce prostaglandin E2 and plays an anti-inflammatory role in macrophages, Immunology 143 (2014) 81–95.

[59] M. Ashburner, C.A. Ball, J.A. Blake, D. Blake, H. Butler, J.M. Cherry, et al., Gene ontology: tool for the unification of biology, Nat Genet 25 (2000) 25.

[60] R.L. Tatusov, M.Y. Galperin, D.A. Natale, E.V. Koonin, The COG database: a tool for genome-scale analysis of protein functions and evolution, Nucleic Acids Res 28 (2000) 33–36.

[61] E.V. Koonin, N.D. Fedorova, J.D. Jackson, A.R. Jacobs, D.M. Krylov, K.S. Makarova, et al., A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes, Genome Biol 5 (2004) R7.

[62] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (2010) 139–140.