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Research Article

Transcriptomic Analysis and Histological Alteration of Black Sea Bream (*Acanthopagrus schlegelii*) Liver Fed Different Protein/Energy Ratio Diets

Lei Wang,[1] **Gladstone Sagada**,[2] **Bin Wang**,[1] **Bingying Xu**,[2] **Lu Zhen**,[2] **Yunzhi Yan**,[1] **and Qingjun Shao**[2]

[1]*School of Ecology and Environment, Anhui Normal University, Wuhu 241000, China*
[2]*College of Animal Sciences, Zhejiang University, Hangzhou 310058, China*

Correspondence should be addressed to Lei Wang; leiwang@ahnu.edu.cn and Qingjun Shao; qjshao@zju.edu.cn

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To systematically investigate the effects of different protein/energy (P/E) ratio diets on the microstructure and metabolic response of black sea bream liver, fish (initial weight 11.20 ± 0.20 g) were fed three isoenergetic (20.9 kJ/g) diets with varying protein/lipid levels (LR, 28.8% protein/26.3% lipid; MR, 37.5% protein/20.9% lipid; and HR, 42.8% protein/17.3% lipid, P/E ratio increased gradually) for 8 weeks. The lipid and glycogen contents in the liver decreased with increasing dietary P/E ratios ($P < 0.05$). Histological observation found that the liver of the LR group presented many adipose cells, along with polarized nucleus, massive lipid droplets, and unclear organelle structure. The liver cellular structures of MR and HR groups were normal with some small unmatured lipid droplets. RNA-Seq was applied to investigate the hepatic transcriptome response to different P/E ratio diets of black sea bream. Different levels of gene expressions were identified in the signaling pathways related to energy metabolism, amino acid metabolism, lipid metabolism, carbohydrate metabolism, immune system, etc. The LR diet inhibited the genes related to lipid oxidation, glycolysis, and protein metabolism, whereas the HR diet inhibited those of glycolysis and protein catabolism. The present study provides a comprehensive understanding of the molecular mechanisms behind the effects of different P/E ratio diets on the liver metabolism of black sea bream.

1. Introduction

The global aquaculture industry has been rapidly expanding in recent decades, which intensifies the demand for protein sources, especially fishmeal, straining the supply of protein ingredients. As a necessary way to reduce protein inclusion levels in aquaculture feed, lipids are extensively used in fish feed. Lipids can serve as an energy source with high energy density, and fish has good absorption and utilization abilities for lipids [1]. A suitable protein/energy (P/E) ratio may favor the growth performance of fish due to the protein-sparing effect of lipid. Previously, protein-sparing effect of lipid has been reported in Atlantic cod (*Gadus morhua* L.) [2], rainbow trout (*Oncorhynchus mykiss*) [3], cobia (*Rachycentron canadum*) [4], and brown-marbled grouper (*Epinephelus fuscoguttatus* (Forskal)) [5]. In recent years, there is a popular trend to increase dietary lipid levels in fish feed to improve protein sparing, growth performance, and farm productivity. However, the excessive dietary lipid may cause massive lipid deposition in the tissues, inducing fatty liver disease and eventually depressing the growth, as seen in the results obtained in grass carp (*Ctenopharyngodon idella*) [6] and giant croaker (*Nibea japonica*) [7].

Black sea bream (*Acanthopagrus schlegelii*) is an important species cultured in Asia, especially alongside the coastline of China, due to its favorable characteristics of fast growth rate, and high ability in resisting environmental changes and diseases [8–10]. High-lipid diet is widely applied in farmed black seabream and brings promising benefits. Previously, we have found that reducing dietary P/E ratio by increasing dietary lipid inclusion level significantly reduced the dietary protein requirement (by about 4%) of

black sea bream. However, fish fed the high ratio (HR) and low ratio (LR) of P/E diets obtained significantly lower growth performance than the fish fed moderate ratio (MR) of P/E diet [11]; the molecular mechanisms behind this phenomenon have not been well illustrated. There is a hypothesis that excessive dietary lipid in the LR diet might cause hepatic lipid accumulation and disturb the normal function of the liver, thus inhibiting the growth of fish. On the other hand, the high protein diet may induce more protein for oxidation rather than for protein deposition, which may also retard the growth performance of fish [12]. These hypotheses require corroboration with molecular analysis. Up to now, most of the studies on the protein-sparing effect of lipid have focused on the growth performance, feed utilization, or histological structures, whereas few studies focused on the molecular or genome responses.

The liver is one of the most important organs in charge of metabolism, immunologic defense, and excretory and secretory functions [13, 14]. It plays a central role in protein, lipid, and carbohydrate metabolism, making it a foremost choice for investigating nutrient metabolism [15]. Molecular tools have been used for studying protein and lipid metabolism in fish and mammals to get a deep understanding of the mechanisms. However, the liver expresses thousands of genes in hundreds of pathways; it is apparently impractical to just focus on a few pathways or enzymes when aiming to comprehensively investigate the effects of formulated diets on nutrient metabolism [14]. With the rapid development of high-throughput sequencing, transcriptomics has been widely applied to investigate the nutritional metabolism of fish [16–19]. In the present study, comparative transcriptomics was applied to investigate the response of hepatic genes to different P/E ratio diets of black sea bream couples with histological observation, aiming to explain the molecular mechanisms behind the retarded growth performance of fish fed the LR and HR diets.

2. Materials and Methods

2.1. Experimental Diets. Three isoenergetic (20.91 kJ/g) diets are prepared in Table 1 [11]. White fish meal, wheat gluten meal, and casein were the main protein sources. Fish oil and corn oil were the main lipid sources. The casein content was replaced with corn oil to achieve the same energy level. The protein and lipid levels of experimental diets were low ratio (LR), 28.8% protein/26.3% lipid; moderate ratio (MR), 37.5% protein/20.9% lipid; and high ratio (HR), 42.8% protein/17.3% lipid. Based on the analysis of amino acid contents of all the protein ingredients, crystalline arginine, lysine, and methionine were added to maintain the same levels. The diets were made following the methods and processes described by Wang et al. [11]. Representative samples were taken for proximate analysis.

2.2. Feeding Trial and Experimental Conditions. All the experimental processes followed the Committee on the Ethics of Animal Experiments of Zhejiang University. The fish were obtained from the Marine Fisheries Research Institute of Zhejiang Province in Zhoushan, China. Before the feeding trial, all the fish were fed commercial feed for two weeks to acclimate to experimental conditions. Then, 180 healthy fish (initial weight: 11.20 ± 0.20 g) were randomly assigned to nine tanks (400 L water volume) for three treatments. Purified seawater flowed into each tank at the rate of 2 L min^{-1}. Water quality was monitored all the time throughout the feeding trial, and the indexes were maintained at dissolved oxygen concentrations > 5.0 mg L^{-1}; temperature $27 \pm 1^{\circ}C$; pH 8.2 ± 0.1, and salinity 28 ± 2 g L^{-1}. All the fish were fed by hand twice daily at 8:00 and 16:00 until visual satiation. The mortality and feed intake of all the tanks were monitored and recorded daily.

Table 1: Formulation and proximate compositions of the experimental diets.

Ingredients (g kg^{-1})	LR	MR	HR
White fish meal	200	200	200
Wheat gluten meal	50	50	50
Casein	139.9	249.9	323.3
Corn oil	170.0	108.2	67.0
Fish oil	40	40	40
Alpha starch	223.2	223.2	223.2
Soy lecithin	20	20	20
L-Carnitine	3	3	3
$CaH_2PO_4 \cdot 2H_2O$	15	15	15
Alpha cellulose	61.6	24.9	0.0
Vitamin mixture[1]	7.5	7.5	7.5
Mineral premix[2]	7.5	7.5	7.5
Y_2O_3	1.0	1.0	1.0
Lysine	10.4	5.0	1.5
Arginine	12.1	6.5	3.0
Methionine	6.3	5.8	5.5
Phytase	0.5	0.5	0.5
Carrageenan	2.0	2.0	2.0
Carboxymethyl cellulose	30.0	30.0	30.0
Total	1000	1000	1000
Proximate composition (%)			
Moisture	7.29	7.00	8.67
Crude protein	28.85	37.46	42.87
Crude lipid	26.32	20.94	17.35
Ash	7.90	8.28	8.68
Gross energy (kJ g^{-1})[3]	21.04	20.95	20.81
P/E ratio (mg kJ^{-1})[4]	13.71	17.88	20.60

[1]Vitamin mixture (mg kg^{-1}): α-tocopherol, 80; retinyl acetate, 40; cholecalciferol, 0.1; menadione, 15; niacin, 165; riboflavin, 22; pyridoxine HCI, 40; thiamin mononitrate, 45; D-Ca pantothenate, 102, folic acid, 10; vitamin B-12, 0.9; inositol, 450; ascorbic acid, 150; Na menadione bisulphate, 15; thiamin, 5; choline chloride, 320 and *p*-aminobenzoic acid, 50. [2]Mineral (mg kg^{-1}): Na_2SiO_3, 0.4; $CaCO_3$, 544.9; $NaH_2PO_4 \cdot H_2O$, 200; KH_2PO_4, 200; $MgSO_4 \cdot 7H_2O$, 10; $MnSO_4 \cdot H_2O$, 4; $CuCl_2 \cdot 2H_2O$, 2; $ZnSO_4 \cdot 7H_2O$, 12; $FeSO_4 \cdot 7H_2O$, 12; NaCl, 12; KI, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.5; $AlCl_3 \cdot 6H_2O$, 1; and KF, 1. [3]Gross energy = (crude protein × 23.6 + crude lipid × 39.5 + starch × 17.2)/1000.
[4]P/E = crude protein/gross energy.

Table 2: Effects of different dietary P/E ratio diets on the liver proximate composition and hepatic glycogen content of black seam bream.

Index	LR	MR	HR
Moisture (%)	49.47 ± 1.34^{a}	54.13 ± 1.08^{b}	55.94 ± 0.98^{b}
Crude protein (%)	18.29 ± 3.89^{a}	25.16 ± 5.06^{ab}	30.80 ± 0.84^{b}
Crude lipid (%)	27.83 ± 3.81^{b}	19.36 ± 2.15^{a}	17.82 ± 0.96^{a}
Glycogen (mg/g tissue)	34.94 ± 3.18^{b}	35.59 ± 0.82^{b}	20.44 ± 6.80^{a}

Mean values ± standard deviation (SD) are presented for each group ($n = 3$), and values with different superscripts in the same row differ significantly ($P < 0.05$). Means in the same column sharing the same superscript are not statistically different ($P > 0.05$).

2.3. Sample Collection and Analytical Method. At the end of the feeding trial, all the fish were fasted for 24 hours and then anesthetized (MS-222; 60 mL/L). Three fish from each tank were dissected to obtain the liver and put into liquid nitrogen immediately and then stored at -80°C. The liver samples from three fish in each tank were stored in 10% formalin and 2.5% glutaraldehyde in phosphate buffer (pH 7.0) (for 24 hours at 4°C).

The proximate compositions of experimental diets and the liver were assayed following the methods of the Association of Official Analytical Chemists [20] with three replicates. The glycogen contents in the liver were assayed by the diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. The liver samples were stained following the hematoxylin-eosin (H&E) method. The slide microphotography was viewed by an Olympus B202 microscope coupled with a Nikon DXM 1200 digital camera, and the photos were taken by Nikon ACT-1 software. The tissues for transmission electron microscopy (TEM) were fixed by 1% osmium tetroxide (pH 7.0) for 2 hours and desiccated in a graded ethanol sequence placed in a mixture of alcohol and isoamyl acetate ($v : v = 1 : 1$) for about 30 min, then transferred to isoamyl acetate for about 8 hours. Then, the tissues were washed and dried in Hitachi Model HCP-C critical-point dryer using liquid CO_2. Desiccated samples were coated with gold-palladium in Hitachi Model E-1010 ion sputter for 4–5 min and viewed in Hitachi H7650 TEM.

2.4. RNA Extraction, cDNA Construction, and RNA-Seq. The liver of three fish from each treatment was taken for analysis. Total RNAs were extracted from the tissues using RNAiso Plus (Takara, Japan) reagent following the manufacturer's instructions. The quality and quantity of extracted RNA quality were assayed by the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated. After quality control and transcriptome assembly, the functions of unigenes were annotated based on the following databases: Nr (NCBI nonredundant protein sequences), Nt (NCBI nonredundant nucleotide sequences), Pfam (protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database), and GO (Gene Ontology). Differential expression analysis of two groups was performed using the DESeq R package (1.10.1). Genes with an adjusted P value < 0.05 found by DESeq were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based on Wallenius noncentral hypergeometric distribution [21]. KOBAS software was used to test the statistical enrichment of differential expression genes in KEGG (http://www.genome.jp/kegg/) pathways [22].

2.5. qRT-PCR Validation. Nine genes related to lipid, protein, and glucose metabolism were selected for qRT-PCR validation: Lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor alpha (*PPARα*), glucokinase (*GCK*), glutamate dehydrogenase (*GLUD*), asparagine synthetase (*AST*), insulin-like growth factor 1 (*IGF-1*), target of rapamycin (*TOR*), alanine aminotransferase (*ALT*), and asparagine synthetase (*ASNS*). The PCR primers of these genes were designed using Primer Premier 5.0 (Table S1) according to the alignment of conserved domains from different species and RNA-Seq results. The housekeeping gene β-actin was used as the reference gene. The mRNA expression levels of the genes in tissues were assayed by qRT-PCR using the SYBR Premix Ex Taq™ II Kit (Takara, Japan). The qPCR process was set as 95°C for 30 s and 40 cycles of 95°C for 5 s followed by 60°C for 30 s. Triplicate samples of each sample were used for qRT-PCR amplification. The relative mRNA expression levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [23].

2.6. Statistical Analysis. The results are presented as the means ± SD. All the data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test) first. SPSS 20.0 (SPSS, USA) was used for all statistical analyses. One-way ANOVA was used for the effects of different dietary treatments. Tukey's multiple range test was used for comparing the means.

3. Results

3.1. Liver Proximate Composition. The proximate compositions of black sea bream liver fed different P/E ratio diets are presented in Table 2. Fish fed the LR diet obtained

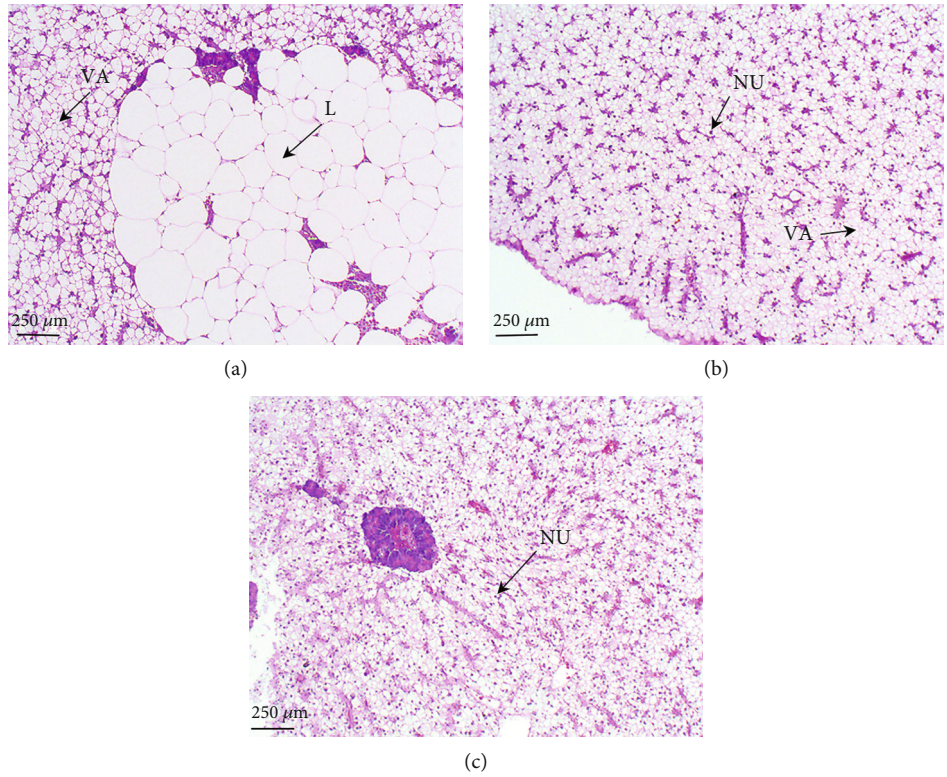

Figure 1: H&E staining of the liver tissue of juvenile black sea bream fed different protein/energy diets for 8 weeks: (a) LR diet; (b) MR diet; (c) HR diet. NU: nucleus; VA: vacuole; L: lipid deposition.

significantly higher crude lipid and glycogen contents than those fish fed the HR diet ($P < 0.05$), while the moisture and crude protein contents in the liver had inverse results ($P < 0.05$).

3.2. Histological Alteration of the Liver. H&E-stained liver of juvenile black sea bream is presented in Figure 1. Fish fed the LR diet had many adipose cells and cytoplasmic vacuolation in the liver. Fish fed the HR diet showed normal structure of the liver, which presented less vacuole and central location of nucleus. Transmission electron microscope images of juvenile black sea bream are presented in Figure 2. In the LR group, many big matured droplets were present in the liver, causing the loss of cytoplasm and translocation of nucleus to the cell periphery. The structure of mitochondria is not clear in the LR group. Fish fed the MR and HR diets had normal structure of the liver cell. Even though in both groups, many small droplets were surrounding the nucleus, it was located in the center of the cell, with clearer mitochondria structure.

3.3. RNA Sequencing and Annotation. In this study, a total of 87.28 GB of clean data with 193,432 unigenes were obtained from the liver transcriptome sequencing of black sea bream via the Illumina HiSeq™ 2000 platform. The basic liver transcriptomic result is presented in Table 3. These assembled unigenes were annotated in the seven functional databases, yielding 115,809 (59.87%) Nt; 143,977 (74.43%) Nr; 45,868 (23.71%) KOG; 96,293 (49.78%) Swiss-Prot; 94,254 (48.72%) GO; 94,175 (48.68%) Pfam; and 55,104 (28.48%) KO annotated unigenes (Figure 3(a)). The homology of sequencing was distributed in some vertebrate fish, among which large yellow croaker (*Larimichthys crocea*) (49.9%) was the most frequently identified, followed by bicolor damselfish (*Stegastes partitus*) (15.4%), Nile tilapia (*Oreochromis niloticus*) (3.9%), black rock cod (*Notothenia coriiceps*) (3.4%), and Pufferfish (*Takifugu rubripes*) (2.4%) (Figure 3(b)).

3.4. GO and KEGG Enrichment Analysis. GO functional enrichment analysis of different groups proved that the DEGs were identified in the subclass of biological process (26), cellular component (20), and metabolic process (10) (Figure 4(a)). A total of 55,104 unigenes were assigned to 32 KEEG pathways under the five major categories as cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems. The KEGG pathways were mostly assigned to signal transduction (9116; 16.5%), followed by the endocrine system (4696; 8.5%), and the immune system (4650; 8.4%). Besides, many unigenes were also enriched in the lipid metabolism (2254; 4.1%), carbohydrate metabolism (2421; 4.4%), and amino acid metabolism (1908; 3.4%) pathways (Figure 4(b)).

3.5. DEG Analysis. Based on liver transcriptome analysis, there were 282, 417, and 370 different expressed genes in the LR, MR, and HR, respectively (Figure 5(a)). 218 DEGs were upregulated, and 175 DEGs were downregulated in LR vs. MR; 314 DEGs were upregulated, and 257 DEGs were

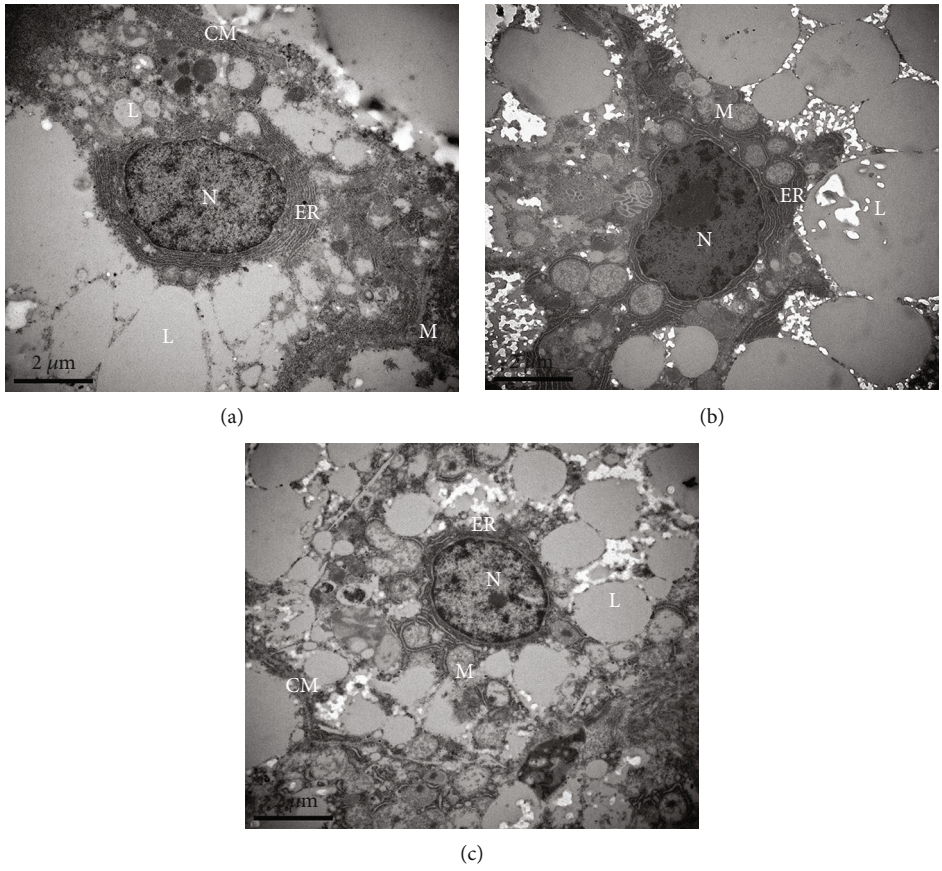

Figure 2: Transmission electron microscope images of juvenile black sea bream liver fed different protein/energy diets for 8 weeks: (a) LR diet; (b) MR diet; (c) HR diet. N: nucleus; M: mitochondria; CM: cell membrane; ER: endoplasmic reticulum; L: lipid droplet.

Table 3: Summary of the *de novo* assembly results of liver transcriptomic results of black sea bream fed different diets.

	Transcripts	Genes
Total nucleotides	337048257	303621941
Max length	21566	21566
Median length	552	1099
Mean length	1128	1570
Min length	201	201
N50	2181	2428

downregulated in LR vs. HR; and 269 DEGs were upregulated, and 256 DEGs were downregulated in MR vs. HR (Figure 5(b)). To investigate the effect of different protein/lipid ratio diets on lipid, protein, and carbohydrate metabolism of black sea bream liver, we summarized the genes related to lipid, protein, and carbohydrate metabolism of different groups in Tables 4, 5, 6 and 7. We found that most of the lipid and protein metabolism-related genes in the LR groups were downregulated compared with those in the MR group, while most of the protein metabolism-related genes in the HR group were downregulated compared with those in the MR group.

3.6. DEG Validation. To validate the transcriptomic results of RNA-Seq, nine genes (*LPL*, *PPARα*, *GCK*, *GLUD*, *AST*, *IGF-1*, *TOR*, *ALT*, and *ASNS*) were selected and analyzed. Their mRNA expression levels by qRT-PCR are shown in Figure 6. The qRT-PCR and RNA-Seq results showed consistency, which confirmed the validity of the obtained RNA-Seq.

4. Discussion

In the present study, the hepatic lipid content of black sea bream was negatively related to the dietary P/E ratio but positively related to dietary lipid level, whereas the liver moisture and protein contents had an inverse trend with that of lipid. Similar results were also reported in *Nibea diacanthus* [24], starry flounder (*Platichthys stellatus*) [25], and large yellow croaker [26]. However, another study on large yellow croaker reported that dietary lipid levels only increased liver lipid content but showed no significant influence on protein content [27], while the liver lipid content of grass carp was not affected by dietary lipid levels [6]. These differences could be related to different fish species having various capacities for utilizing dietary lipid, as well as the different preferences for depositing excess lipid. The proximate composition of the liver was in accordance with the histological results. In H&E-stained liver of the LR group, adipose cells occupied much space and showed many lipid vacuoles,

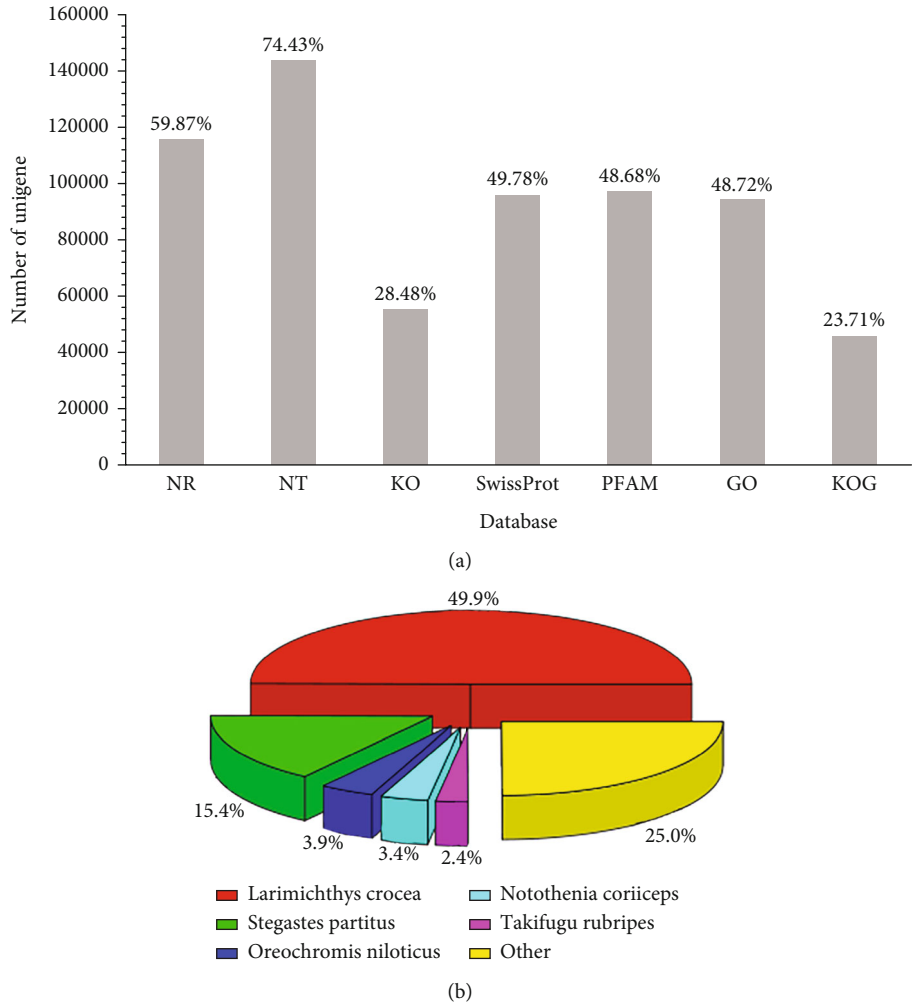

FIGURE 3: Functional annotation of the assembled unigenes (a). All the assembled unigenes were annotated and functionally classified in the seven functional databases: Nt (NCBI nucleotide sequences), Nr (NCBI nonredundant protein sequences), KOG (euKaryotic Ortholog Groups), Pfam (protein family), Swiss-Prot (a manually annotated and reviewed protein sequence database), GO (Gene Ontology), and KO (Kyoto Encyclopedia of Genes and Genomes). Fish species distribution of the combination of best hits from NCBI-NR databases (b).

shown as the fatty-liver disease. Under the TEM observation, the main space of the liver cell was occupied by large matured lipid droplets along with unclear organelle (e.g., mitochondria) and the nucleus translocated to the cell membrane. The nucleus in the liver cells of MR and HR groups were located in the center of cells and surrounded by small unmatured lipid droplets, along with clear structures of organelle, such as mitochondria and endoplasmic reticulum, indicating better health and normal functions. Black sea bream has a high tolerance to dietary lipid level and prefers to accumulate fat in the liver [11]. In the present study, LR diet had high lipid level, which was the main reason for the excessive hepatic lipid accumulation. Previously, excessive dietary lipid was reported to have caused massive hepatic lipid accumulation in black sea bream [28, 29], large yellow croaker [30], blunt snout bream (*Megalobrama amblycephala*) [31–33], and common sole (*Solea solea* L.) [34]. Generally, lipid accumulation is the result of imbalanced relationships between *de novo* fatty synthesis, fat catabolism (β-oxidation), and dietary lipid intake [35], while Jin et al. [36] pointed out that crude lipid accumulation was mainly affected by lipogenesis and lipolysis rather than β-oxidation. The excessive dietary lipid may surpass the hepatic oxidation ability and results in high synthesized triglyceride deposition in vacuoles, eventually causing steatosis [37]. In mammals, when energy intake surpasses the energy expenditure, adipose tissue will enlarge the adipocyte and/or increase the numbers [38]. He et al. [39] reported that Nile tilapia adapt to the high-fat treatment mainly through increasing adipocyte numbers, which is in line with the increased adipocyte numbers in the liver of the LR group. Besides, excessive lipid accumulation was partly because ingested lipid cannot be degraded efficiently, as well as high lipid diet would also reduce insulin sensitivity and eventually impair lipid and glucose homeostasis [40]. Furthermore, the relatively low protein level in the LR diet might also inhibit the synthesis of lipid-transporting protein in the liver, which retards the export of hepatic lipid to other tissues and aggravates the fatty-liver symptoms [11].

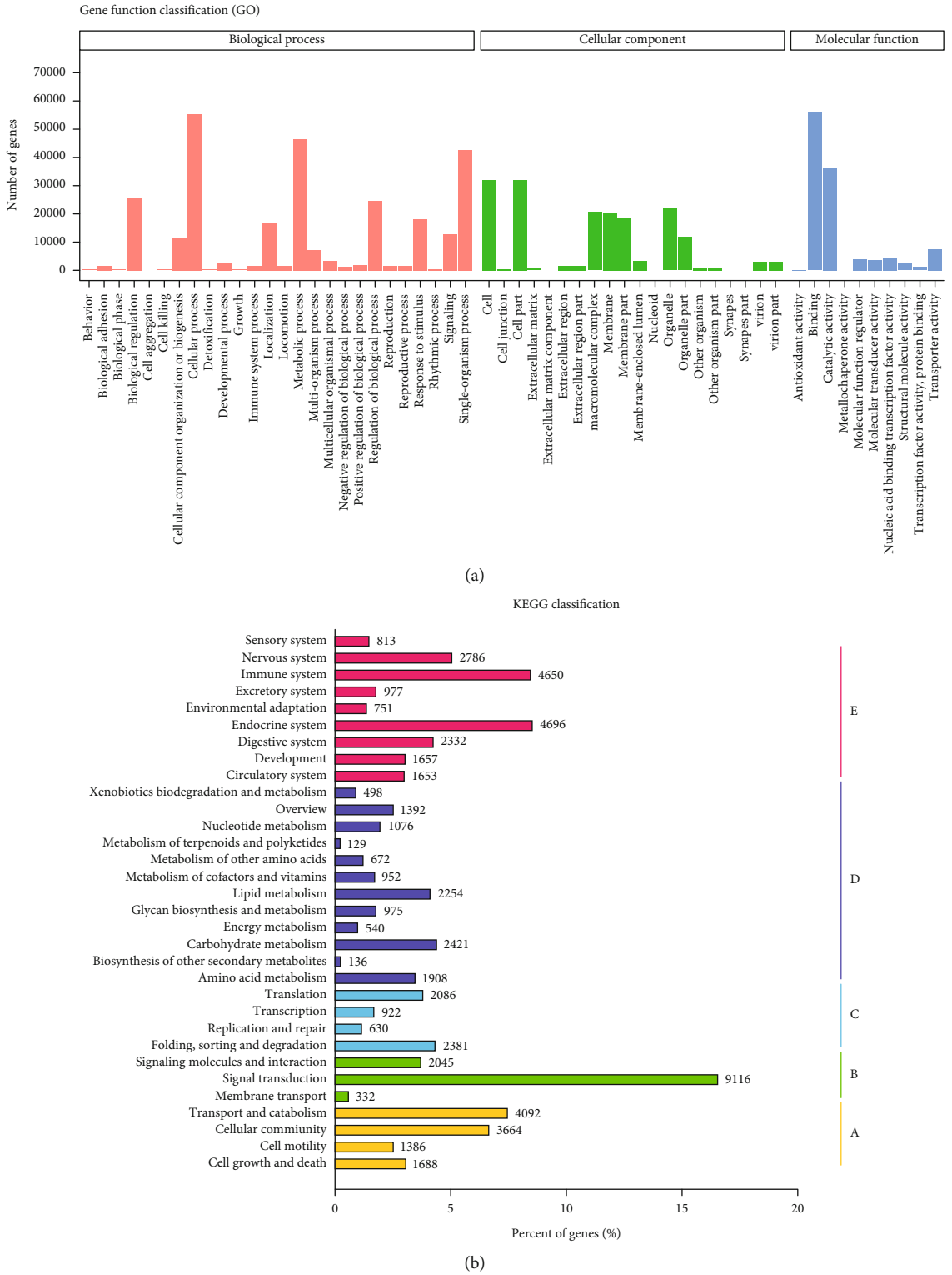

Figure 4: GO functional classification of black sea bream liver (a). The unigenes were annotated and grouped into three categories of GO terms: biological process (orange-red), cellular component (green), and molecular function (blue). KEGG functional classification of black sea bream liver transcriptome (b). A total of 55,104 unigenes were annotated and grouped into five major categories of KEEG terms: A (cellular processes, yellow), B (environmental information processing, green), C (genetic information processing, blue), D (metabolism, pink), and E (organismal systems, red).

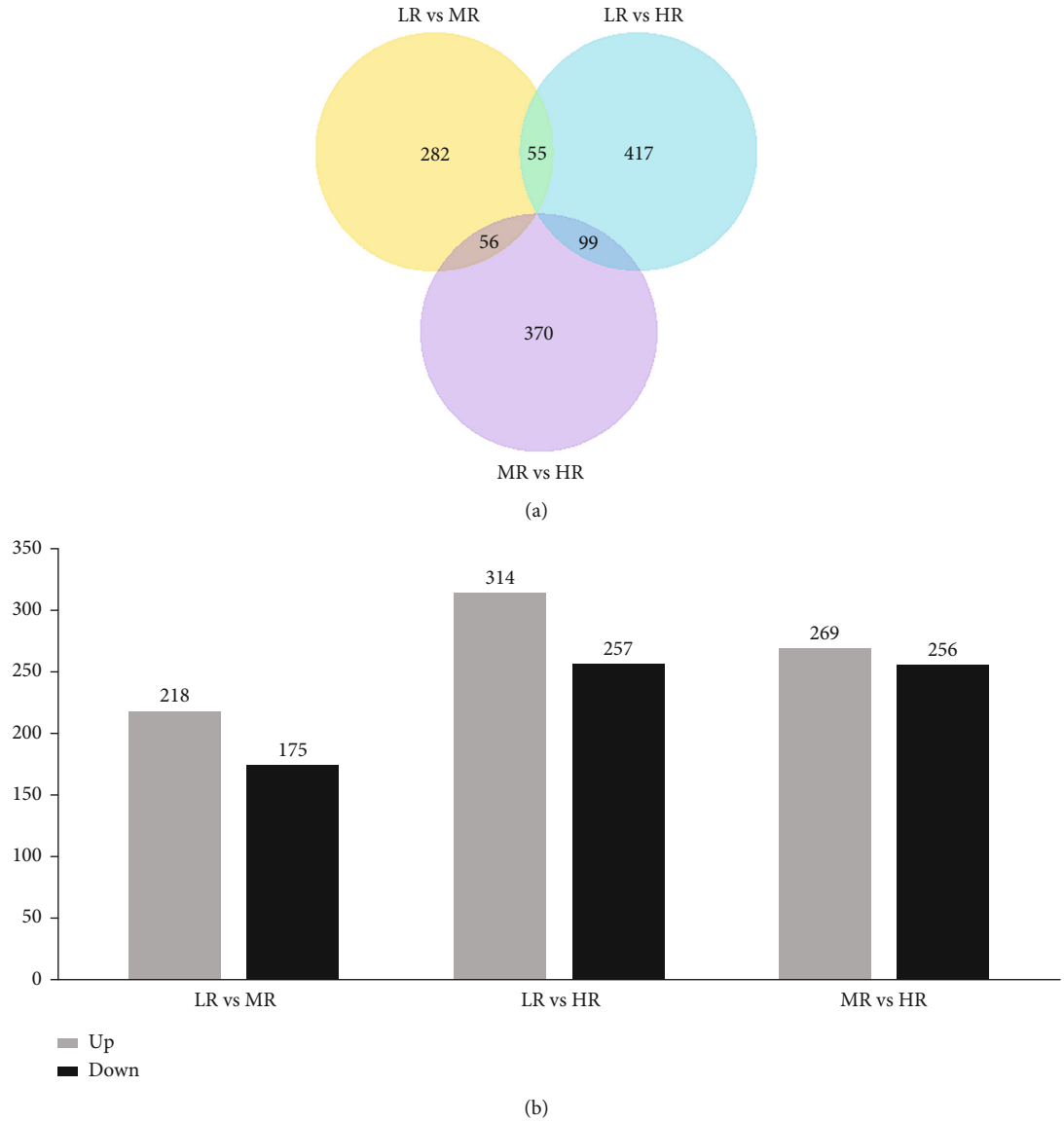

Figure 5: The number of differentially expressed genes (a) and upregulated or downregulated genes (b) in the liver of different groups.

With its development, RNA-Seq has been applied in many aquaculture species to determine the systematic response to nutritional or environmental factors, such as yellowtail kingfish (*Seriola lalandi*) [41], rainbow trout [42], golden pompano (*Trachinotus ovatus*) [43], and Pacific white shrimp (*Litopenaeus vannamei*) [44]. Transcriptome sequencing is a powerful method for obtaining gene transcription information of the nonmodel organism with no reference genome available. High-throughput techniques allow molecular pathways and gene networks to be identified that would not be possible when investigating only a small number of genes at any one time [43]. The Q20 in the assembled results was higher than 97%, indicating the success of transcriptional library construction, as well as the good sequencing quality. The annotation results showed that 80.96% of the unigenes were identified in at least one database, which is higher than that of largemouth bass (*Micropterus salmoides*) (52.98%) [16], gilthead sea bream (*Sparus aurata*) (51%) [45], turbot (*Scophthalmus maximus*) (44.8%) [46], blunt snout bream (44.8%) [47], and common carp (*Cyprinus carpio*) (77.3%) [48]. Collectively, a sum of 193,432 unigenes was assembled in the liver of black sea bream. But only 55,104 transcripts were identified in the KEGG database, which might be related to the limited genomic information of this species and blast tool unable to align sequences effectively with big gaps [43]. A total of 94,254 unigenes were mapped in the GO database, GO annotation indicated that single-organism process, metabolic process, and cellular component organization or biogenesis were the most frequently identified subunits in biological process, indicating the important role of the liver in regulating protein and lipid metabolism.

Compared to the MR group, fatty acid transporter (*FATP*) expression in the liver of the LR group was significantly upregulated. *FATP* plays an important role in transporting long-chain fatty acids, and it has multiple subtypes with specific

Table 4: Summary of the significantly changed genes related to lipid and carbohydrate metabolism between the LR and MR groups.

Gene name	Gene definition	Gene EC number	LR vs. MR
Lipid metabolism			
LPL	Lipoprotein lipase	EC:3.1.1.34	-8.85
LRP1	Low-density lipoprotein receptor-related protein 1	EC:3.4.2.3	-7.89
ADH1-7	Alcohol dehydrogenase 1/7	EC:1.1.1.1	-2.44
DAK	Dihydroxyacetone kinase	EC:2.7.1.29	-5.61
TGL2	Triacylglycerol lipase	EC:3.1.1.3	-7.27
DGAT1	Diacylglycerol O-acyltransferase 1	EC:2.3.1.20	-7.39
PLD1-2	Phospholipase D1/2	EC:3.1.4.4	-6.15
LDLR	Low-density lipoprotein receptor	EC:2.7.11.29	-11.83
FATP1-4	Solute carrier family 27 (fatty acid transporter)	EC:6.2.1.-	+3.90
ANGPTL4	Angiopoietin-like 4	EC:3.1.1.34	+4.59
LYPLA2	Lysophospholipase II	EC:3.1.1.5	-6.82
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	EC:7.1.1.2	+6.56
Carbohydrate metabolism			
UGT	Glucuronosyltransferase	EC:2.4.1.17	-9.02
GCGR	Glucagon receptor	—	+21.44
PFKFB1	6-Phosphofructo-2-kinase	EC:2.7.1.105	-10.09

Table 5: Summary of the significantly changed genes related to protein metabolism between LR and MR groups.

Gene name	Gene definition	Gene EC number	LR vs. MR
SARDH	Sarcosine dehydrogenase	EC:1.5.8.3	-7.13
GNMT	Glycine N-methyltransferase	EC:2.1.1.20	-1.57
GLS	Glutaminase	EC:3.5.1.2	-7.23
P4HA	Prolyl 4-hydroxylase	EC:1.14.11.2	-8.60
DNMT1	DNA (cytosine-5)-methyltransferase 1	EC:2.1.1.37	-6.73
ACMSD	Aminocarboxymuconate-semialdehyde decarboxylase	EC:4.1.1.45	-11.18
CELA2	Pancreatic elastase II	EC:3.4.21.71	-6.67
glyA, *SHMT*	Glycine hydroxymethyltransferase	EC:2.1.2.1	+8.34
glnA, *GLUL*	Glutamine synthetase	EC:6.3.1.2	+5.25
mTOR	Target of rapamycin	EC:2.7.11.1	+7.54
ASH1L	[Histone H3]-lysine 4 N-trimethyltransferase A	EC:2.1.1.354	-8.90
HAL	Histidine ammonia-lyase	EC:4.3.1.3	-2.04

+ means upregulated; - means downregulated.

functions that are still not clear enough in fish [17]. A study in blunt snout bream reported that high-lipid diet could increase *FATP* expression [32, 37]. However, *FATP1* was highly expressed in the low-lipid treatment, while *FATP11* was highly expressed in the high-lipid treatment, which might be because the fatty acids transported by *FATP11* are for triglyceride synthesis, while *FATP1* transports fatty acids for metabolism [26]. Apart from FATP, some other mRNA levels related to lipid metabolism and transportation in the LR group were lower than those in the MR group, such as lipoprotein lipase (*LPL*), LDL receptor-related protein-1 (*LRP-1*), and low-density lipoprotein receptor (*LDLR*). In mammals, triglyceride (TG) in chylomicrons and very-low-density lipoprotein are hydrolyzed by LPL, then absorbed by peripheral tissues, and the hydrolyzed lipoprotein remnants are removed from the plasma *via* LDLR and LRP-1 mainly by the liver but also partially by peripheral tissues [26]. Previously, upregulated expression of LPL by high dietary lipid level has been reported in hybrid snakehead (*Channa maculata* ♀×*Channa argus* ♂) [49], gilthead sea bream [50], and blunt snout bream [32], which is opposite to the results in the present study. The inhibited LPL, LRP-1, and LDLR expressions in the LR group might indicate the disorder of lipoprotein metabolism. Similarly, the expression of LDLR and LRP-1 in large yellow croaker was inhibited by high dietary lipid levels [26]. Besides, upregulated expression of LPL was found in the fish fed high lipid with increasing dietary protein levels; thus, other nutritional factors may influence LPL expression, especially dietary protein level [49]. The high expression level of angiopoietin-like 4 (*AGPTL4*) in the LR group was in accordance with the

Table 6: Summary of the significantly changed genes related to lipid and carbohydrate metabolism between the MR and HR groups.

Gene name	Gene definition	Gene EC number	D4 vs. D6
Lipid metabolism			
PPARα	Peroxisome proliferator-activated receptor alpha	EC:3.1.2.2	-9.46
ALDH	Aldehyde dehydrogenase (NAD+)	EC:1.2.1.3	-7.60
MGAT2	2-Acylglycerol O-acyltransferase 2	EC:2.3.1.22	-2.39
CPT1	Cholinephosphotransferase 1	EC:2.7.8.2	+6.80
EPT1	Ethanolamine phosphotransferase 1	EC:2.7.8.1	+6.80
FATP1-4	Solute carrier family 27 (fatty acid transporter-4)	EC:6.2.1.-	-3.80
NCEH1	Neutral cholesterol ester hydrolase 1	EC:3.1.1.-	+8.76
Carbohydrate metabolism			
GCK	Glucokinase	EC:2.7.1.2	+7.99
HK	Hexokinase	EC:2.7.1.1	+7.99
RAB2A	Ras-related protein Rab-2A	EC:3.6.5.2	+9.16

+ means upregulated; - means downregulated.

Table 7: Summary of the significantly changed genes related to protein metabolism between the MR and HR groups.

Gene name	Gene definition	Gene EC number	D4 vs. D6
MAO	Monoamine oxidase	EC:1.4.3.4	+10.05
HAL	Histidine ammonia-lyase	EC:4.3.1.3	+9.24
PRODH2	Hydroxyproline dehydrogenase 2	EC:1.5.5.3	+8.32
AST1	Aspartate aminotransferase 1	EC:2.6.1.1	+5.34
GNA	Glucosamine-phosphate N-acetyltransferase	EC:2.3.1.4	+7.94
CDO1	Cysteine dioxygenase 1	EC:1.13.11.20	+8.34
AGXT2	Alanine-glyoxylate transaminase	EC:2.6.1.44	+9.17
ASH1L	[Histone H3]-lysine 4 N-trimethyltransferase	EC:2.1.1.354	+7.65
EEF2	Elongation factor 2	EC:2.7.11.20	+11.52
CELA2	Pancreatic elastase II	EC:3.4.21.71	+6.67

+ means upregulated; - means downregulated.

increased adipose cells, which is in charge of adipocyte differentiation. A study in rats reported that *AGPTL4* is the target gene of *PPARγ*, and overexpression of *AGPTL4* resulted in high TG contents in the liver and plasma, along with high hepatic glycogen content [51].

Diacylglycerol O-acyltransferase 1 (*DGAT*) is the final step of TG synthesis. The downregulated *DGAT* in the LR group compared to the MR group indicated that lipid synthesis was inhibited by high-lipid/low-protein diet. Similarly, downregulated *DGAT* expression by high-lipid diet was also observed in large yellow croaker [26, 27] and blunt snout bream [37]. Dietary lipid could suppress lipogenesis [52], gluconeogenesis, and amino acid degradation [53]. Similar to the present result, increasing dietary lipid/protein ratio depressed lipogenesis, while liver crude lipid content was increased in common carp [54]. These downregulated genes were correlated with impaired liver function. In largemouth bass, excessive dietary carbohydrate caused liver damage and resulted in the downregulation of 92.9% of genes compared to the moderate carbohydrate group [16].

Target of rapamycin (*mTOR*) is a central regulator of cell growth and proliferation which is regulated by growth factors and nutrient signaling [55]. Normally, amino acids can activate its upstream gene *Rheb* and promote the expression of *mTOR*. Zhang et al. [49] reported that the expression levels of *mTOR* were positively related to dietary protein levels but not related to dietary lipid level, while significant interaction of protein and lipid influenced *mTOR* expression of hybrid snakehead. However, in the present study, the MR diet contained higher protein level than the LR diet but resulted in decreased expression of mTOR. This result could be because *mTOR* can be activated by AMP-dependent protein kinase (AMPK). When the body is under low energy status (high AMP/low ATP), AMPK is phosphorylated and promotes *mTOR* expression [56]. The excessive lipid accumulation in the liver of the LR group might impair the energy-producing processes such as fatty acid oxidation and glycolysis, resulting in low energy status and causing phosphorylation of AMPK, thus promoting *mTOR* expression. In addition, other protein metabolism-related genes such as glutaminase, sarcosine dehydrogenase, glycine N-methyltransferase, and histidine ammonia-lyase were downregulated in the LR group compared with the MR group. Even though the functions of these genes have not been identified in fish, these results at least proved that the protein metabolism process in the LR group was inhibited.

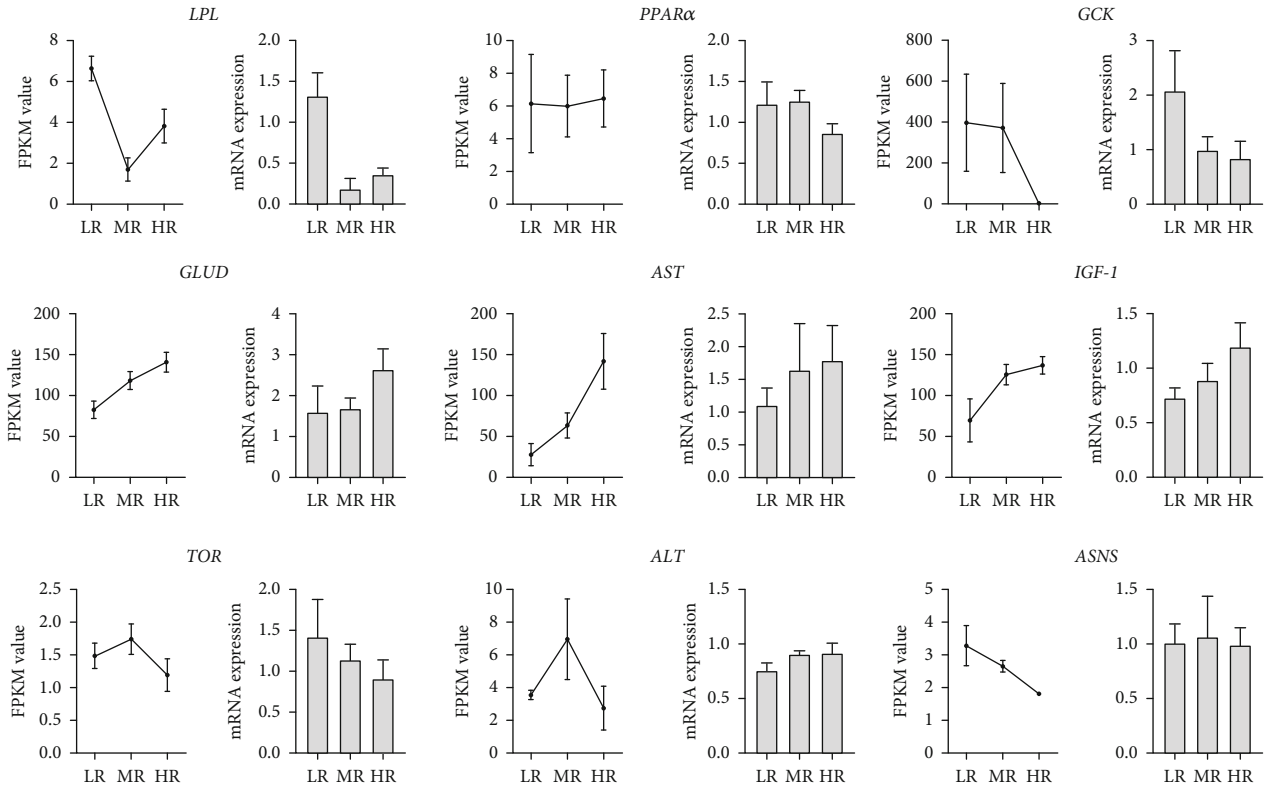

Figure 6: Comparison of the expression of qRT-PCR and RNA-Seq results. Nine genes related to lipid, protein, and carbohydrate metabolism were selected to validate the RNA-Seq data. The mRNA expressions of qRT-PCR are normalized with β-actin. The gene abbreviations are as follows: *LPL*: lipoprotein lipase, *PPARα*: peroxisome proliferator-activated receptor alpha; *GCK*: glucokinase; *GLUD*: glutamate dehydrogenase; *AST*: asparagine synthetase; *IGF-1*: insulin-like growth factor 1; *TOR*: target of rapamycin; *ALT*: alanine aminotransferase; *ASNS*: asparagine synthetase.

In the present study, the expression levels of glucokinase (*GCK*) and hexokinase (*HK*) in the MR group were higher than those in the HR group, indicating that the glycolysis processes in the MR group were more active. GCK phosphorylates glucose to glucose-phosphate, which is the first step of glucose metabolism and occurs in the liver of animals [19], while HK catalyzes glucose phosphorylation at lower glucose levels [16]. Normally, GCK and HK expressions are positively related to dietary carbohydrate level [57]. Besides, upregulation of GCK is also beneficial for insulin secretion and promotes glucose uptake by the liver for glycogen synthesis to maintain the homeostasis of glucose [18]. Studies in gilthead sea bream [58] and Nile tilapia [59] reported that a high-carbohydrate/low-protein diet increased fish liver GCK expression. In the present study, the relatively low protein level in the MR group might have promoted the use of carbohydrate as an energy source and saved protein for growth. Besides, Ras-related protein Rab-2A (RAB-2A) can promote the transport of glucose transporter 4 (GLUT4) to the cell membrane and promote the absorption of glucose. The expression level of *RAB-2A* in the liver of the MR group was 9.16 times higher than that of the HR group, which also proved better utilization of carbohydrate in the MR group. Dietary carbohydrate is absorbed and stored in the liver and muscle in the form of glycogen. Once the liver glycogen stores are replete, further glucose will be converted into lipids in the liver. If the liver is overloaded with glucose, hepatic steatosis will occur with a high glycogen content [60]. Furthermore, glucose as the precursor of acetyl-CoA is the primary substrate for lipid synthesis in the liver, the hepatic lipid, and glycogen levels were negatively related to dietary P/E ratio in the present study, which was also related to the utilization of carbohydrate in the MR and LR groups.

CPT1 and PPARα are two important genes involved in lipid fatty acid β-oxidation [61]. CPT1 is a major regulatory enzyme of fatty acid oxidation. It is located in the outer membrane and catalyzes carnitine-dependent esterification of palmitoyl-CoA to palmitoylcarnitine [18, 29]. PPARα regulates fatty acid β-oxidation by upregulating the expressions of target genes related to peroxisomal and mitochondrial β-oxidation of FAs in the liver [35, 62]. The mRNA level of *CPT1* in the MR group was 6.8 times higher than that in the HR group, indicating that more active lipid oxidation occurred when the dietary P/E ratio decreased. This result is in accordance with our previous conclusion that reducing the dietary P/E ratio improved the use of lipid as an energy source and thus saved protein for growth, showing a "protein-sparing" effect [11]. The expression of *CPT1* was higher in the fish fed the low-protein diet than that in a high protein diet, suggesting that when dietary protein was insufficient, lipid catabolism supplied the main energy [63]. However, the *PPARα* expression in the MR group was downregulated compared to the HR group. In mammals,

PPARα is normally upregulated by high-lipid diet, but in fish, the relationship between dietary lipid and *PPARα* expression is even more complicated [37].

Compared to the HR group, most protein-related genes were upregulated in the MR group, such as aspartate aminotransferase1 (AST1), hydroxyproline dehydrogenase (PRODH2), and cysteine dioxygenase 1 (CDO1). AST works in transferring amino group, aspartate, to α-ketone glutaric acid, for the synthesis of glutamic acid and oxaloacetic acid [64–67]. High AST activity was found in fish fed high-protein/low-carbohydrate diet and those fed low-energy diet [64]. CDO1 takes part in taurine synthesis, which can participate in various processes such as protein anabolism and catabolism, as well as bile salt binding [67]. However, the functions of many protein metabolism-related genes have not been identified in fish yet, but the MR group showed better protein utilization than the HR group.

5. Conclusion

In conclusion, a low protein/energy ratio diet caused excessive lipid accumulation in the liver of black sea bream, resulting in inhibition of protein metabolism, fatty acid synthesis, and lipoprotein transportation. The moderate protein/energy ratio diet induced higher β oxidation of fatty acids, glycolysis, and protein metabolism than the high protein/energy ratio group, which resulted in protein-sparing effects and was beneficial for protein deposition and fish growth. The transcriptome information in the present study provides a better understanding of fish lipid and protein metabolism.

Data Availability

Derived data supporting this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Lei Wang and Qingjun Shao designed this experiment. Gladstone Sagada, Bin Wang, Bingying Xu, and Lu Zhen provided help during the feeding trial, sampling, and data analysis. Lei Wang wrote this manuscript under the supervision of Yunzhi Yan. Gladstone Sagada helped revise it. All authors read and approved the final manuscript.

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Supplementary Materials

Table S1: the primer sequences for q-PCR verification. *(Supplementary Materials)*

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