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Deep sequencing of the tilapia (*Oreochromis niloticus*) liver transcriptome response to dietary protein to starch ratio

Yuanyan Xiong [b,c,1], Junfeng Huang [b,1], Xiaoxia Li [a,1], Liliu Zhou [a], Fang Dong [b], Hui Ye [a], Lian Gan [a,*]

[a] Animal Science College, South China Agricultural University, Guangzhou, China
[b] School of Life Science, Sun Yat-Sen University, Guangzhou, China
[c] SYSU-CMU Shunde International Joint Research Institute, Shunde, China

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ABSTRACT

To comprehensively understand the effects of dietary protein to starch ratio on alternations in physiologic status of tilapia, fish were fed with high protein to starch ratio (HP, 33.5% protein and 16.62% starch content) and low protein to starch ratio isoenergetic diets (LP, 25.2% protein and 26.82% starch content) for 8 weeks. Our results indicated that the fish fed with LP diet had a significantly poor growth performance and feed utilization. Lower dietary protein to starch ratio also resulted in markedly higher plasma cholesterol, plasma triacylglycerol, liver lipid and muscle lipid content, which indicated more fat deposition in fish. RNA-seq was employed to evaluate the tilapia hepatic transcriptome response to LP diet. RNA-seq data showed that 71 genes were significantly up-regulated and 26 genes were significantly down-regulated by LP diet. Different expression genes were mapped to 47 signaling pathways including glycolysis, gluconeogenesis, biosynthesis of amino acids, lipogenesis and lipolysis etc. The present study gains a comprehensive understanding of the molecular mechanisms underlying the effects of dietary protein and starch ratio on alternations in physiologic of tilapia.

1. Introduction

Dietary proteins are used continuously by fish for maintenance, growth and reproduction functions. By feeding representation of over 50% of the operational costs of aquaculture, protein has been the most expensive nutrient source in aquaculture feeds. Reducing dietary protein content is an important way to formulate cost effective and low pollution diets. Carbohydrates are the lowest cost energy source in practical diet ingredients and efficiently used by fish. Some researchers demonstrated that inexpensive carbohydrate inclusion in the diet can improve growth performance, decrease ammonia excretion and spare some proteins in many fish species (Azaza et al., 2013; Mohanta et al., 2007; Peres and Oliva-Teles, 2002), which may be helpful to reduce fish feed cost. However, excess dietary carbohydrate has been demonstrated to cause negative effect on fish health through metabolic disturbances (Polakof et al., 2012), fish physiological alterations were observed, such as prolonged hyperglycemia (Hatlen et al., 2005), triggering hepatic anti-oxidative response (Azaza et al., 2013), high fat deposition in whole body and liver (Hemre et al., 2002), low red blood cells and hemoglobin (Abdel-Tawwab et al., 2010), increased liver histopathology (Russell et al., 2001) and impaired bone development (Tan et al., 2009).

Tilapia are the most important commercial cichlids, which have been farmed extensively in many tropical and subtropical regions of the word due to their large size, rapid growth, and palatability (EI-Sayed, 2006). By 2010 yearly global aquaculture production of *Oreochromis niloticus* had risen to nearly 2.538 million tonnes (FAO, 2010). Tilapia still continues to increase production. The protein requirement of tilapia is 28%–56%, which varies with fish size and age (Abdel-Tawwab et al., 2010). The omnivorous Nile tilapia can utilize about 40–50% carbohydrate content in the diets (Hemre et al., 2002). Some research had demonstrated that tilapia never compromise growth performance when fed with low protein and high carbohydrate diet (Azaza et al., 2013; Li et al., 2013). But a clear molecular response remains scarcely known when tilapia fed with low protein and high carbohydrate diet. Identification of genes and pathways altered by dietary protein to carbohydrate ratio may contribute towards a better understanding of nutrient metabolism pathways and assessing the effect of nutrition control on fish physiology.

For this reason, a global analysis of gene expression in response to dietary protein to starch ratio is essential for understanding the biological mechanisms. The objective of this study was to identify hepatic genes differentially expressed among tilapia fed with different protein to starch ratio isoenergetic diets.

* Corresponding author. Tel.: +86 02085283529.
E-mail address: ganlian@scau.edu.cn (L. Gan).
[1] Co-first authors.

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2. Material and methods

2.1. Fish and rearing experiment

The two isoenergetic practical experimental diets, a 33.5% protein and 16.62% starch diet (HP) and a 25.2% protein and 26.82% starch diet (LP), were prepared as shown in Table 1. The wheat flour and cellulose content in the LP diet were increased to make it isoenergetic. All experimental diets were prepared as previously described by Gan et al. (2013). After drying, the diets were packed into plastic bags and stored at −20 °C until use. Juvenile tilapia from our facilities were used and their initial wet weight was 15.2 ± 0.06 g. After acclimated to the experimental conditions for 2 weeks with 32% protein and 4% lipid (wet weight) to satiation, fish were randomly divided into 2 groups in a recirculated aquaculture system, and each group had three tanks containing 20 tilapia each. The fish were respectively fed with 7% of body weight thrice a day for 8 weeks. During the trial period, the diurnal cycle was 12-h light/12-h dark. Water quality parameters monitored weekly as follows: temperature, 27.1 ± 1.1 °C; pH, 7.22 ± 0.17, dissolved oxygen, 6.08 ± 0.10 mg L^{-1}; total ammonia–nitrogen, 0.05 ± 0.01 mg L^{-1}, respectively.

2.2. Sampling and analytical methods

At the beginning of the feeding trial, 18 fish were randomly sampled from the initial fish and killed for analyses of whole body composition. After feeding experiment, three fish from each tank were randomly collected for analysis of whole-body composition and six fish were anesthetized with tricaine methane sulphonate (MS222) (50 mg L^{-1}) for blood collection from tail vein and to obtain weights of individual whole body, viscera, liver and intraperitoneal fat. Blood and tissue samples were obtained from fasted (12 h) animals. Serum isolated was stored at −70 °C until analyzed. Liver and white muscle from both sides of the filets without skin were dissected, frozen immediately in liquid nitrogen and then stored at −70 °C until used.

Diets and fish samples (including white muscle and liver) were analyzed in triplicate for proximate composition. Crude protein, crude lipid, moisture, crude ash and gross energy (GE) were determined following standard methods (AOAC, 1984). The concentrations of plasma cholesterol (CHO), triacylglycerol (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were determined using an automatic blood analyzer (Hitachi 7170A, Japan) from a clinical laboratory.

Table 1
Experimental formulation and composition (%).

Diet	HP	LP
Ingredient		
Soybean meal[a]	36	12
Canola meal[a]	17	17
Cotton meal[a]	11	11
Rice bran meal[a]	9	9
Wheat flour[a]	19.16	34.77
Mineral mix[b]	0.5	0.5
Vitamin mix[c]	0.5	0.5
Soy oil[a]	3	3
Choline chlorine (50%)[a]	0.3	0.3
Monocalcium phosphate[a]	2	2
78% lysine-HCL[d]	0	0.17
84% MHA-Ca[e]	0.44	0.24
98% L-Threonine[f]	0	0.12
Cellulose	0	8.3
Phospholipid[a]	1	1
VC ascorbic acid	0.1	0.1
Total	100	100
Proximate analysis (% DM)		
Moisture	8.02	6.03
Crude protein	33.5	25.2
Crude fat	5.40	5.23
Starch	16.62	26.82
Gross energy, kJ g^{-1}	14.4	14.4

[a] Zhuhai Shihai Feed Corporation Ltd., Zhuhai, China.
[b] Mineral mix (mg kg^{-1} of diet): $MgSO_4.7H_2O$, 4061.5; $ZnSO_4.7H_2O$, 525.46; $FeSO_4.7H_2O$, 238.83; $MnSO_4.H_2O$, 49.22; $CoCl_2.6H_2O$, 0.2; KI, 5.23; $CuSO_4.5H_2O$, 11.82; Na_2SeO_3, 0.66; KCl, 600; NaCl, 400 (Guangzhou Chengyi Aquatic Technology Ltd., Guangzhou, China).
[c] Vitamin mix (mg kg^{-1} of diet): thiamin, 20; riboflavin, 20; vitmin A, 2; vitamin E, 50; vitamin D_3, 0.05; menadione, 10; pyridoxine, 10; cyanocobalamin, 0.02; biotin, 1; calcium pantothenate, 50; folic acid, 5; niacin, 100; inositol, 500 (Guangzhou Chengyi Aquatic Technology Ltd., Guangzhou, China).
[d] L-Lysine. HCL contained L-lysine ≥ 78%(CJ Co., Ltd., Liaocheng, China).
[e] MHA.Ca contained DL-HMTBA (2-hydroxy-4-methylthio butanoic acid) ≥ 84% (Novus International Inc., Zhibo, China).
[f] Supplied as L-form (Shanghai Cangda Amino acid Company Ltd., Shanghai, China).

2.3. RNA extraction, cDNA library construction and RNA-seq

Total liver tissue RNA was extracted using TRIzol Reagent (Life Technologies, US) according to the manufacturer's instructions and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent Technologies, US). Qualified total RNA was purified by RNeasy micro kit (QIAGEN, Germany) and RNase-Free DNase Set (QIAGEN, Germany). Poly(A) mRNA was isolated from qualified total RNA using Oligotex mRNA mini kit (QIAGEN, Germany) and then broken into short fragments which were used as templates for synthesis of the first- and the second-strand cDNA. Two paired-end libraries were synthesized by using the Genomic Sample Prep kit (Illumina, US) following the manufacturer's instructions. Short fragments were purified with the Qubit™ dsDNA HS Assay kit (Invitrogen, US) and connected with different sequencing adapters. Each of the two libraries had an average insert size of 400 bp and was sequenced by Shanghai Biotechnology Corporation (Shanghai, China) using Illumina HiSeq™ 2000.

2.4. Mapping reads to the tilapia genome

The Nile tilapia genome was produced by Broad Institute (http://www.broadinstitute.org/) and downloaded from the Ensemble website (version Orenil1.0.72). Clean reads were aligned to the reference genome using Tophat (Trapnell et al., 2009) with default parameters. To reduce the influence of low quality bases in the tail and maximize the read utilization, we set up an iteratively mapping step. After each round's mapping, the paired un-mapped reads were extracted and trimmed the final 10 bp, these processed reads were used for the next round's mapping. The process was stopped while the reads shorter than 30 bp, and all the BAM files were merged as the final mapping output.

2.5. Detecting novel transcripts

A genome-free strategy for transcriptome reconstruction was implemented as described by cufflinks suite (Roberts et al., 2011). This novel assembly was compared to the known transcripts recorded in Ensemble. Those that were not overlapped with the known transcripts were extracted as candidate novel transcripts for further analysis. Four criterions must also be met for novel transcripts: (a) length > 200 bp, (b) away 10 kb upstream or downstream from any known transcripts to reduce the possibility that these sequences derived from extended exons of the known one, (c) with two or more exons, and (d) predicted to be 'coding' both by CPC (Kong et al., 2007) and CNCI (Sun et al., 2013) programs.

2.6. Differentially expressed gene analysis

Both known and novel genes were used to detect the differential expression. Read counts for each gene were assigned by HTSeq (http://www-huber.embl.de/users/anders/HTSeq/). Fish fed with