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Integrative analysis of transcriptomics and metabolomics profiling on flesh quality of large yellow croaker *Larimichthys crocea* fed a diet with hydroxyproline supplementation

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Abstract

A previous study showed that flesh quality of large yellow croaker (LYC) was improved by feeding dietary hydroxyproline (Hyp, 0·69 %). The aim of the present study was to explore the underlying mechanisms using transcriptomics and metabolomics analysis. The metabolomics analysis showed that muscle metabolite profiles could be clearly separated between the basal diet and Hyp supplementation diet. Metabolites including betaine, Hyp, lactate, glucose-6-phosphate, trimethylamine *N*-oxide, taurine, creatine, inosine monophosphate, histamine and serine made significant contribution to the separation. Compared with the control diet, the transcriptomics analysis identified a total of 334 different expressed genes, of which 298 genes were up-regulated and thirty-six genes were down-regulated in the Hyp supplementation group. The altered genes of the Hyp supplementation group were involved in collagen metabolism, lipid metabolism and energy metabolism. The integrated results revealed that the increased muscle collagen content in the Hyp supplementation diet was partly because of its enhancement of biosynthesis and the reduction of degradation. The improvement of muscle quality by dietary Hyp supplementation could also be related to a good utilisation of glucose through enhancement of glycolysis. It was concluded that dietary Hyp supplementation could improve flesh quality because of comprehensive metabolism changes including elevated collagen content, glycolysis, lipid metabolism and flesh flavour of LYC. The present study provided a novel strategy to understand the underlying molecular mechanism of flesh quality of LYC fed diet with Hyp supplementation.

Key words: Flesh quality: Hydroxyproline: Large yellow croaker: Metabolomics: Transcriptomics

Large yellow croaker *Larimichthys crocea* is popularly farmed in southern China. It has become the most large-scale mariculture fish species in China with a production of more than 148 616 metric tons in 2015[1]. Along with the increasing production, quality of the farmed large yellow croaker is also receiving increasing concerns, such as skin colour and muscle texture.

Hydroxyproline (Hyp) is a substrate for the synthesis of glycine, pyruvate and glucose, as well as scavenging oxidants and regulating redox state of cells[2–4]. It is a conditionally essential amino acid in aquatic animals[5]. Meanwhile, 4-hydroxyproline is essential for intramolecular hydrogen bonds, thus contributing to the thermal stability of the triple helical domain, the integrity of the monomer and collagen fibril[6]. In connective tissue of fresh fish muscle, collagen plays a vital role in maintaining fillet integrity and muscle cohesiveness[7]. Collagen content in muscle could be elevated when fish are fed dietary Hyp supplementation[8,9]. Moreover, dietary Hyp could significantly enhance fish flesh quality in turbot *Scophthalmus maximus* L.[9] and salmon *Salmo salar* L.[10]. However, it is unclear regarding the underlying molecular mechanism and metabolism variation *in vivo* of fish flesh quality caused by Hyp supplementation in the diet.

Transcriptomics can enable high-throughput screening of thousands of expressed genes in a given tissue, which is highly efficient and in-depth to study the development of complex phenotypic traits affected by genes[11]. Larsson *et al.*[12,13] used transcriptomics analysis to observe a variety of potential causes of fillet firmness, and revealed a strong positive correlation between firmness and the gene expression of Atlantic salmon (*Salmo salar* L.). Meanwhile, metabolomics is derived from the profiling study of metabolites, which are not merely end products of gene expression but also forming part of the regulatory system in an integrated manner[14]. Metabolomics is a powerful tool to detect the changes in metabolite content of fish treated under different conditions in quality assessment via the

Abbreviations: B-D, basal diet; H-D, Hyp supplementation diet; Hyp, hydroxyproline; TMA, trimethylamine.

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multivariate analysis of metabolic profiles by ^{1}H-NMR spectra[15]. The combination of transcriptomics and metabolomics profiling technologies has been successfully used in analyses for drug development[16], disease diagnosis[17,18] and nutrition health[19,20]. Multi-omics technology was used to assess diverse data sets at different biological levels to exploit the full potential of such information for a holistic improvement of production performance, disease resistance and welfare in animals[21]. Also, the multi-omics method was used to explore the molecular mechanism and metabolism associated with phenotypic characteristics in fish muscle quality.

A previous study found that dietary Hyp significantly improved flesh quality of large yellow croaker[22]. On the basis of the total Hyp content in muscle, the optimal dietary Hyp content for large yellow croaker was estimated to be 0·61 %. To elucidate the underlying molecular mechanism of flesh quality improvement by dietary Hyp, in the present study the comprehensive analysis of gene expression with RNA sequence and metabolomics profile with ^{1}H-NMR was used in muscle of large yellow croaker.

Methods

Feeding trial and sampling

The basal diet (B-D) was formulated to contain a combination of fish meal, maize gluten meal, soyabean meal and beer yeast as the intact protein sources. On the basis of the B-D, the Hyp supplementation diet (H-D) was made by adding 0·6 % Hyp to the diet. The formula and the proximate compositions of the two experimental diets are listed in Table 1.

Large yellow croaker juveniles were obtained from a commercial farm in Ningbo, China. Before the start of the feeding trial, juveniles were reared in floating sea cages (3·0 × 3·0 × 3·0 m) for 2 weeks to acclimatise to the experimental diets and conditions. After that, fish of similar sizes (initial body weight: 189·87 (SEM 0·89) g) were randomly distributed into six sea cages (1·5 × 1·5 × 2·0 m) for two groups, and each cage was stocked with thirty-eight fish. Each group was assigned to triplicate cages, and fed one of the two experimental diets. The feeding trial lasted for 82 d in the sea of Xiangshan Harbour of Ningbo, Zhejiang Province, China. All the procedures were strictly carried out in accordance with the Regulations of the Experimental Animal Ethics Committee of Ocean University of China and in compliance with regulations by Institutional Animal Care and Use Committee.

Sample collection

At the end of the feeding trial, fish were fasted for 24 h and anaesthetised with tricaine methanesulphonate (MS-222, 1:10 000) (purity 99 %, Shanghai Reagent) before sampling. In total, six fish per cage were sampled; three of them were mixed and considered as one replicated. One side of the dorsal fillet, which is above the lateral line and anterior to the dorsal fin, was collected into sterile tubes and frozen in liquid N_2, and then stored at −80°C. Samples in liquid N_2 were ground into a powder with liquid N_2. The powder was sampled into a sterile tube and freeze-dried and used for ^{1}H-NMR analysis.

Table 1. Formulation of the experimental diets (%DM)

	Dietary Hyp levels	
Ingredients	B-D	H-D
Fish meal*	25·0	25·0
Soyabean meal*	25·0	25·0
Wheat meal*	26·0	26·0
Fish oil	6·0	6·0
Soyabean lecithin	2·5	2·5
Mineral premix†	2·0	2·0
Vitamin premix‡	2·0	2·0
Choline chloride	0·2	0·2
Attractant§	1·5	1·5
Mould inhibitor‖	0·1	0·1
Ethoxyquine	0·05	0·05
Amino acid premix¶	5·70	5·70
Microcrystalline cellulose	3·15	3·15
Alanine	0·8	0·2
Hydroxyproline	0	0·6
Total	100·0	100·0
Proximate analysis		
Crude protein	43·56	43·42
Crude lipid	12·82	12·34
Moisture (% wet weight)	5·32	5·36
Hydroxyproline	0·17	0·69

Hyp, hydroxyproline; B-D, basal diet; H-D, Hyp supplementation diet.
* All of these ingredients were supplied by Qingdao Great Seven Biotechnology Co., Ltd. Fish meal, crude protein: 74·31 %, crude lipid: 8·98 %; soyabean meal, crude protein: 57·40 %, crude lipid: 1·70 %; wheat meal, crude protein: 17·39 %, crude lipid: 1·47 %.
† Mineral premix (mg/kg diet): Na_2SeO_3 (1 %), 20, $Ca(IO_3)_2.6H_2O$ (1 %), 60; $CoCl_2.6H_2O$ (1 %), 50; $CuSO_4.5H_2O$, 10; $FeSO_4.H_2O$, 80; $ZnSO_4.H_2O$, 50; $MnSO_4.H_2O$, 45; $MgSO_4.7H_2O$, 1200; $Ca(H_2PO_4)_2.H_2O$, 10 000; microcrystalline cellulose, 8485.
‡ Vitamin premix (mg/kg diet): thiamine, 25 g; riboflavin, 45; pyridoxine HCl, 20; vitamin B_{12}, 10; vitamin K_3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, $Ca(IO_3)_2$, 60; retinyl acetate, 32; cholecalciferol, 5; *α*-tocopherol, 240; ascorbic acid, 2000; wheat middlings, 16 473.
§ Glycine:betaine = 1:2.
‖ 50 % calcium propionic acid and 50 % fumaric acid.
¶ Lysine-H_2SO_4, DL-methionine, L-threonine, L-arginine, L-isoleucine, L-leucine, L-valine and L-phenylalanine.

The sample power was also used for transcriptomic assay and gene expression validation.

Transcriptomic assay

Total RNA from muscle samples was isolated by Trizol® Reagent. The quality and integrity of RNA were examined by agarose gel electrophoresis and 2100 Bioanalyzer. The qualified RNA was treated with DNase (5 U/μl) (Takara) at 37°C for 30 min. The DNase-treated RNA was purified by Dynabeads® Oligo (dT) 25 (Life Technologies): RNA solution is diluted to 100 μl and mixed with 100 μl of binding buffer. The mixture was incubated at 65°C for 2 min, and then cooling on the ice rapidly. After cooling, 100 μl of pre-washed beads were added and mixed at room temperature for 5 min, and then placed on the Magnetic Grate (Life Technologies) for separation for 1–2 min. The supernatant was washed twice with 200 μl of Washing Buffer B followed by the addition of 15 μl of cooled 10 mM Tris-HCl resuspended beads, and incubated at 75–80°C for 2 min, and then placed on the Magnetic Grate for 1–2 min, immediately. The supernatants were collected to obtain the purified mRNA. The library was prepared by NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, American) according to the

manufacturer's instructions. Random Primers and First Strand Synthesis Reaction Buffer (5×) were added to 100 ng of purified mRNA. The mix was incubated at 94°C for 15 min and then immediately placed in ice to break in the mRNA. Murine RNase Inhibitor and ProtoScript II RT were added for the first-strand synthesis, and the reaction procedure was listed as 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. Second-Strand Synthesis Reaction Buffer (10×) and Second-Strand Synthesis Enzyme Mix were added and incubated at 16°C for 1 h to synthesise the second strand. After adding 1·8 times volume AMPure XP Beads (Agencourt) to purify the reagent obtained from the last step, the End Repair Reaction Buffer (10×) and End Prep Enzyme Mix were added and incubated at 20°C for 30 min, and then 65°C for 30 min for end repairing. NEBNext Adaptor and Blunt/TA Ligase Master Mix were added and incubated at 20°C for 15 min for adaptor linkage, followed by the addition of USER Enzyme at 37°C for 15 min to excise U in adaptor. After adding and mixing 0·6 times volume of AMPure XP Beads, the mixture was placed on the Magnetic Grate for 5 min after allowing it to stand for 5 min. The supernatant was collected, and then 0·25 times volume of AMPure XP Beads was added for purification. The 300–500-bp-length ligated cDNA was made. Universal PCR Primer and Index (X) Primer were used for PCR amplification. After preheating at 98°C for 10 s, 12–15 cycles of amplification were performed using a cycle profile as denaturation at 98°C for 10 s, annealing at 65°C for 30 s and extension at 72°C for 30 s. The reagent was purified by one times volume AMPure XP Beads and the library for sequencing was obtained. The 2 % agarose gel electrophoresis, Qubit quantification and High-sensitivity DNA chip were used to obtain qualified cDNA library. The sequencing of cDNA in the library was performed on Illumina Hiseq™ 2500 after using TruSeq PE Cluster Kit (Illumina) performing cluster generation on cBot.

Gene expression validation

Genes obtained from the transcriptomic assay were validated and quantified by quantitative real-time PCR (qRT-PCR). The primers used for qRT-PCR are listed in Table 2. Total RNA used in RT-PCR analysis was isolated with Trizol reagent (Invitrogen) from the same samples as that in transcriptomic assay. The qRT-PCR was performed using SYBR Green as the fluorescent dye according to the manufacturer's protocol (Takara). Each sample was run in triplicate. The mRNA expression levels were normalised by the reference gens and calculated using the comparative cycle threshold (C_t) values expressed as $2^{-\Delta\Delta C_t}$. GraphPad Prism 7.0 was used for the interpretation and analysis of data.

^{1}H-NMR analysis

Sample preparation for ^{1}H-NMR analysis. In brief, lyophilised muscle powder (50 mg) was weighed into a centrifuge tube and suspended in 1 ml of purified water. The tube was then sonicated (VCX-130; Sonics & Materials, Inc.) on ice. The ultrasonic extraction was repeated eight times for 4 and 3-s intervals with the cycling programme. Samples were centrifuged (Eppendorf Centrifuge 5415R) at 13 000 rpm for 15 min at 4°C. The aqueous layer was collected and filtered by amicon Ultra 0·5-ml centrifugal filters with 3-kDa cut-off (Millipore). The filtrate was centrifuged at 13 000 rpm for 30 min at 4°C. The top 450-μl aqueous layer was collected and transferred to a clean 2-ml centrifuge tube. A 50-μl 2,2-dimethyl-2-silapentane-5-sulphonate standard solution (Anachro) was added in the tube. Samples were vortex-shocked well for 10 s and centrifuged at 13 000 rpm for 2 min at 4°C. The 480 μl of supernatant was transferred to ^{1}H-NMR tube for analysis.

Table 2. Primers for relative quantitative real-time PCR

Gene	Primer sequence (5'–3')
β-Actin	F: GACCTGACAGACTACCTCATG
	R: AGTTGAAGGTGGTCTCGTGGA
col1a1	F: CAACAGCCGCTTCACATACA
	R: ACAGACGGGTCCAACTTCAA
col6a1	F: GGATACGTGTTGCTCCTTCG
	R: TTTCACCCTTTTCTCCTCTGC
col5a2	F: TGTGACGAAATCCAGTGCG
	R: GGTGTTGGTGGTGCTTGTATC
timp2	F: GTGATTAGAGCAAAGGTGGTGG
	R: CATTGGCATCCAGGGTAACA
gatm	F: TCTCATTCAAGGACCCCAAC
	R: GAATCAGAGGTGTCGGAGGTT
sdh	F: CACCAACATTAGCAAACCAACT
	R: CATTCATACAGCCCGTCCA
bhmt	F: GAGATGGCACCTGTCAAGAAG
	R: CCAGAGCAAACACGAAACCT
pfkm	F: TTGGTGGATTTGAGGCTTATG
	R: TAGAGGTGATGGTGTTGAGGG
scd1	F: GGTACTTCTGGGGTGAATCCT
	R: ATGGCACTGAAAGCAACTAATG
p4ha1	F: CCATACATTGTCCGCTACCTT
	R: GCCTTAATCGTGGCTTTGC

^{1}H-NMR spectroscopy. Spectra were collected using a Bruker AV III 600 MHz spectrometer equipped with an inverse cryoprobe operating at a proton NMR frequency of 600·13 MHz. The first increment of a 2D-1H, ^{1}H-NOESY pulse sequence was used for the acquisition of ^{1}H-NMR data and for suppressing the solvent signal. Spectra were collected at 25°C, with a total of 180 scans over a period of about 20 min.

Quantification of the metabolites. The collected free induction decay signal was automatically zero-filled and fourier-transformed in processing module in Chenomx NMR Suite 7.7 (Chenomx Inc.). The data were then carefully phased and corrected to baseline in the Chenomx Processor. All the spectra were referenced for the quantification to the internal standard. The quantification was performed by comparing the integral of a known reference signal (DSS-d6) with the signals derived from a library of compounds containing chemical shifts. The identifiable metabolites were chosen for quantification by matching to the Chenomx Library. All concentrations of metabolites were collected and normalised by weight across all parallel samples before being used in the later multivariable analysis.

Data analysis

The chemometric analysis for metabolites was performed in the R software environment using the PLS package[23]. Plots were made using the ggplot2 package[24]. The variable importance in

the projection (VIP) values and corresponding loadings for partial least squares (PLS) models were applied to identify the variable. If VIP is greater than 1, this variable was considered a very important variable for the model and selected as candidate biomarker variables[25]. It was also considered to be significant.

For gene expression determination, reads per kilobase of gene per million mapped reads (RPKM) was performed as a standard calculation, which quantifies gene expression from RNA sequencing data by normalising for total read length and the number of sequencing reads[26]. The gene expression abundance between the two samples was calculated using the MA-plot-based method with the random sampling model in the DEGseq package[27]. The false discovery rate (FDR) was used to determine the *P* value threshold. We have used stringent criteria to identify differentially expressed genes (DEG) at FDR < 0·001, $|\log_2$ fold change$| > 1$, *q* value (*P* value was adjusted using Benjamini) < 0·001 and RPKM > 20 for at least one sample. A search for enriched gene ontology (GO) classes and KEGG pathways of DEG was performed. An enrichment score threshold of *q* value < 0·05 was selected for statistical significance.

Results

Gene expression profiling by RNA sequence

According to the screening criteria ($q < 0{\cdot}001$, RPKM > 20, $\log_2$ (fold change) > 1), a total of 334 genes were identified as DEG, of which 112 genes were not annotated in the B-D group and H-D group. Meanwhile, 298 genes and thirty-six of these DEG were significantly up-regulated and down-regulated, respectively, in the H-D group. The results of gene expression validation from qRT-PCR are shown in Fig. 1. The expression levels of ten genes selected were up-regulated in the B-D diet compared with the H-D diet, which showed a high consistent trend from the results of transcriptomic assay.

The GO and KEGG classified analysis of DEG is shown in Table 3. These genes participate in the pathway of antigen processing and presentation, PPAR signalling pathway, protein digestion and absorption. Meanwhile, enrichment of DEG was observed in extracellular matrix–receptor interaction, extracellular region part, extracellular region, macromolecular complex, organelle part and structural molecule activity. Genes in the group that was supplemented with Hyp showed sizeable and mainly increased expression. Examples are given in Table 4. A $\log_2$ (fold change) value greater than 1 indicated an up-regulation in the H-D group compared with the B-D group, and the ones less than −1 indicated a down-regulation in the H-D group compared with the B-D group. In comparison with the B-D group, gene expression of *betaine-homocysteine S-methyltransferase* (*bhmt*) involved in glycine, serine and threonine metabolism, cysteine and methionine metabolism in the H-D group was up-regulated. Other up-regulation of genes involved in amino acid metabolism including *glycine amidinotransferase* (*gatm*), *glycyl-tRNA synthetase* (*gars*) and *aminoacylase 1* (*acy1*) were observed in the H-D group. Expression levels of *aconitate hydratase* (*aco*), *ATP-dependent 6-phosphofructokinase, muscle type-like* (*pfkm*), *fructose-bisphosphate aldolase A* (*aldoa*), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and *succinate dehydrogenase* (*sdh*) involved in glucose degradation of carbohydrate catabolism were increased in the H-D group. Several other genes, which were involved in collagen gene expression, biosynthesis and degradation, were up-regulated in the H-D group. These genes included *collagen α-1(I) chain-like* (*col1a1*), *collagen α-2(V) chain-like* (*col5a2*), *collagen, type VI, α* (*col6a*), *protein disulphide-isomerase A1* (*pidia1*), *prolyl 4-hydroxylase subunit α-1 isoform X2* (*p4ha1*), *lysyl oxidase* (*lox*) and *tissue inhibitor of metalloproteinase 2* (*timp2*). The gene expression of *col1a1* showed the greatest up-regulation ($\log_2$ (fold change) = 5·18). Up-regulated genes involved in lipid metabolism included *binding protein* (*fabp3*), *fatty acid-binding protein 2a* (*fabp2*), *stearoyl-CoA desaturase* (*Δ-9 desaturase*) (*scd1*), *lipoprotein lipase* (*lpl*), *diacylglycerol O-acyltransferase 2* (*dgat*) and *long-chain fatty acid transport protein 1-like* (*fatp1*). These genes also participated in the PPAR signalling pathway. The genes involved in energy metabolism and immune system were observed. Muscle fibre-related genes including in *myosin heavy chain* (*mhy*), *myosin light polypeptide 6* (*myl6*) and *calpain-3* (*capn3*) were up-regulated.

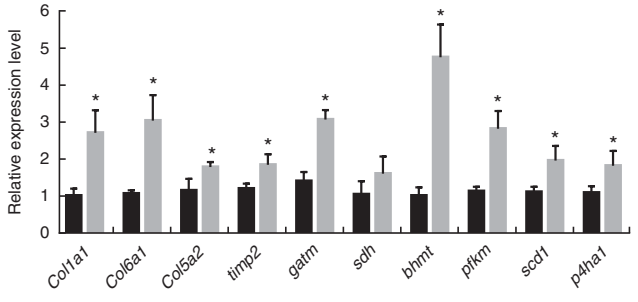

Fig. 1. Quantitative real-time PCR of selected genes from the transcriptomic profile. The gene expression level is the relative average $2^{-\Delta\Delta Ct}$ as normalised to the housekeeping gene *β-actin*. Values are means with standard errors represented by vertical bars, and statistical analysis was done by unpaired *t* test (*$P < 0{\cdot}05$). (■) B-D, basal diet; (▨) H-D, Hyp supplementation diet.

Table 3. Enrichment terms of gene ontology (GO) and the KEGG pathway of differentially expressed genes in muscle of large yellow croaker fed dietary hydroxyproline supplementation

Term	GO/KEGG number	Genes	*q* Value
Antigen processing and presentation	ko04612	9	0·000000
Cardiac muscle contraction	ko04260	7	0·000162
PPAR signalling pathway	ko03320	6	0·000746
Adrenergic signalling in cardiomyocytes	ko04261	6	0·021327
Protein digestion and absorption	ko04974	4	0·048195
Intestinal immune network for IgA production	ko04672	2	0·048195
Extracellular matrix–receptor interaction	ko04512	4	0·048195
Extracellular region part	GO:0044421	13	0·022017
Extracellular region	GO:0005576	22	0·006266
Macromolecular complex	GO:0032991	52	0·000037
Organelle part	GO:0044422	64	0·006529
Cellular component biogenesis	GO:0044085	18	0·006529
Establishment of localisation	GO:0051234	40	0·007237
Localisation	GO:0051179	41	0·038536
Structural molecule activity	GO:0005198	26	0·000000

Table 4. Differentially expressed genes in muscle of larger yellow croaker fed dietary hydroxyproline supplementation

Gene	Description	$\log_2$ (fold change) H-D/B-D
Aat	ADP/ATP translocase 2 like	1·79
Aco	Aconitate hydratase, mitochondrial	1·32
Acsl1	Long-chain fatty acid–CoA ligase 1 isoform X2	2·52
Acta1	Actin, *α* skeletal muscle	1·36
Acy1	Aminoacylase 1	1·17
Aldoa	Fructose-bisphosphate aldolase A	2·35
Arf1	ADP-ribosylation factor 1 like	1·25
Atp2a1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 like	2·00
Atp6	ATP synthase F0 subunit 6	1·27
B2m	*β*-2-microglobulin precursor	1·46
Bhmt	Betaine-homocysteine *S*-methyltransferase 1	2·52
CA2	Carbonic anhydrase 2	1·75
Capn3	Calpain-3	2·10
Cd74	Major histocompatibility complex class II antigen-associated invariant chain	2·24
Cd99l2	CD99 antigen-like protein 2 like	1·04
Cfl2	Cofilin-2	1·76
Col1a1	Type 1 collagen *α* 1	5·18
Col5a2	Collagen *α*-2(V) chain like	2·69
Col6a1	Collagen, type VI, *α* 1	2·61
Cyb5r3	NADH-cytochrome b5 reductase 3 like	1·08
Cytb	Cytochrome b	1·02
Dcn	Decorin	2·12
Dgat2	Diacylglycerol *O*-acyltransferase 2	4·70
Fabp2	Fatty acid-binding protein 2a	1·29
Fabp3	Muscle fatty acid-binding protein 3	1·50
Fatp1	Long-chain fatty acid transport protein 1 like	1·03
Fbxo32	F-box protein 32 like	−1·48
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1·25
Gars	Glycyl-tRNA synthetase	1·28
Gatm	Glycine amidinotransferase	1·11
Gst	Glutathione *S*-transferase	1·23
Hsp90b	Heat-shock protein 90-*β*	1·52
Hspa1-8	Heat-shock 70-kDa protein 1/8	1·07
Loxl2a	Lysyl oxidase homolog 2A like	1·55
Loxl2b	Lysyl oxidase homolog 2B like	2·23
Loxl3	Lysyl oxidase homolog 3 isoform X2	1·55
Lpl	Lipoprotein lipase	2·49
Mhc1	Major histocompatibility complex, class I	2·03
Mhc2	Major histocompatibility complex, class II	2·20
Mhy	Myosin heavy chain	2·22
Myl4	Myosin light chain 4 like	2·05
Myl6	Myosin light polypeptide 6	2·40
Ndufa4	NADH dehydrogenase (ubiquinone) 1 *α* Subcomplex subunit 4	1·43
P4ha1	Prolyl 4-hydroxylase subunit *α*-1 isoform X2	1·68
Pcolce2	Procollagen C-endopeptidase enhancer 2 precursor	1·94
Pdia1	Protein disulphide-isomerase A1	1·27
Pdia3	Protein disulphide isomerase family A, member 3	1·46
Pfkm	ATP-dependent 6-phosphofructokinase, muscle type like	1·32
Scd1	Stearoyl-CoA desaturase (Δ-9 desaturase)	3·83
Sdh	Succinate dehydrogenase	1·03
Smyhc2	Slow myosin heavy chain 2	2·26
Ssr1	Translocon-associated protein subunit *α*	1·48
Timp2	Tissue inhibitor of metalloproteinase 2	3·12
Tkt	Transketolase	2·12
Tnni1	Troponin I, slow skeletal muscle like	1·73
Tpm1	Tropomyosin *α*-1 chain-like isoform X2	1·49
TTN	Titin	1·59

B-D, basal diet; H-D, Hyp supplementation diet.

Metabolite profiling by ^{1}H-NMR spectroscopy

A typical ^{1}H-NMR spectrum in the present study is shown in Fig. 2. A total of sixty-two metabolites were identified in the spectra for the muscle samples of large yellow croaker. Amino acids and their derivatives, organic acids, sugars and nucleic acid components were detected. A partial least squares discriminant analysis (PLS-DA) classification model was constructed to evaluate the metabolic differences of muscle tissues between the two groups (Fig. 3). The score plot (Fig. 3(a)) shows that the B-D group and the H-D group are clearly distinct from each other. The corresponding loading plot (Fig. 3(b)) helps the visual selection of biomarkers. It indicates that the further the metabolites get away from the core, the more the contributions can be distinguished from the two groups. The metabolites with VIP above 1 (Table 5) are significant variables that contribute to distinguishing the two groups. The concentration of metabolites in the B-D diet including *trans*-4-hydroxy-L-proline, glucose-6-phosphate, trimethylamine *N*-oxide (TMAO), taurine and inosinic acid (inosine monophosphate (IMP)) were up-regulated compared with those in the group of H-D, whereas the metabolites including betaine, lactate, creatine, histamine and serine were down-regulated. These biomarker metabolites were mainly related to the pathways of collagen metabolism, amino acid metabolism, glucose metabolism and fish muscle flavour.

Discussion

In the previous study, Hyp supplementation of 0·6 % in diet increased the muscle texture parameters (hardness, adhesiveness, springiness and chewiness), pH and lipid-holding capacity compared with the control group, which indicated an improvement of flesh quality[22]. The present study explores the possible underlying mechanism.

Higher collagen content is closely related to firmer fillet and higher muscle pH in sea bass, *Dicentrarchus labrax* L.[28]. A previous study on large yellow croaker also found that dietary supplementation of Hyp significantly increased collagen content in muscle[22]. In the present study, the expression of three collagen genes (*col1a1*, *col5a2* and *col6a*) observed from the transcriptomics was up-regulated in the H-D group. Five types of collagen (types I, III, IV, V and VI) were found in the muscle of *Gadus morhua*[29]. Type I collagen is the most abundant and studied collagen, whereas the content of type V collagen is relatively less, but more reported in fish connective tissue related with flesh quality[30]. A coordinated significant variation of expression was observed in genes involved in the metabolism of collagen. Four collagen biosynthesis-related genes including *pidia1*, *pcolce2*, *p4ha1* and *lox* observed from the transcriptomics was up-regulated in the Hyp supplementation group. The *p4ha1* plays a central role in collagen synthesis. It catalyses the formation of 4-Hyp in collagens and other proteins with collagen-like sequences by the hydroxylation of proline residues in peptide linkages[31]. The *lox* is essential to promote cross-linking and plays a critical role in the biogenesis of connective tissue matrices, which catalyse the oxidative deamination of the *ε*-amino group of lysines and hydroxylysines in collagen and elastin[32]. Additionally, the gene of tissue inhibitor

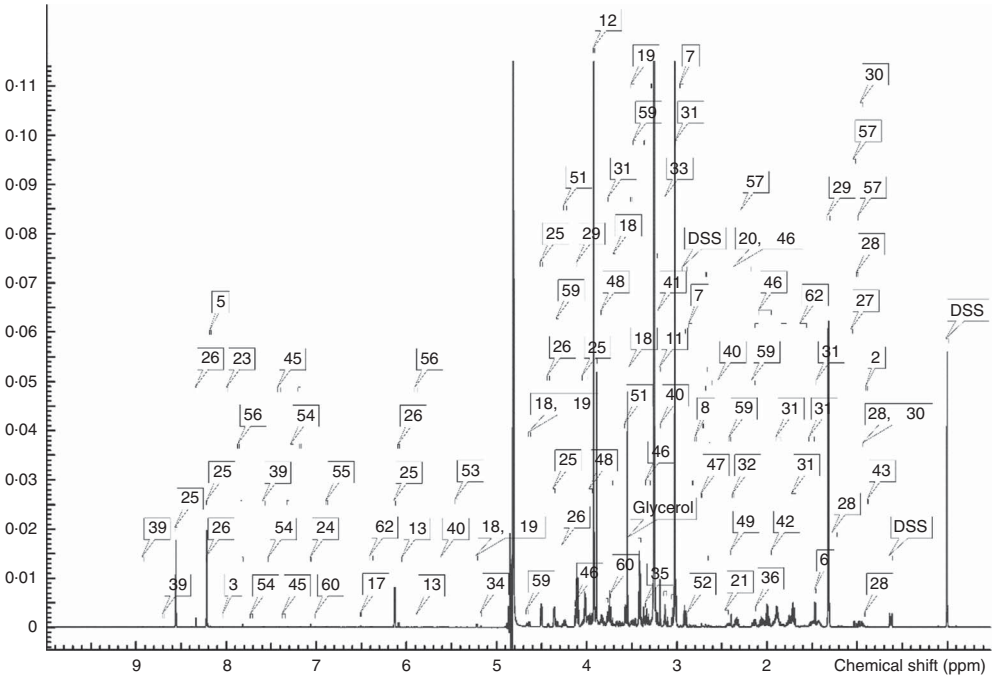

Fig. 2. A typical ^{1}H-NMR spectrum obtained from muscle samples of large yellow croaker fed diet with hydroxyproline supplementation. 1, 2-aminobutyrate; 2, 2-hydroxybutyrate; 3, 3-methylxanthine; 4, acetate; 5, adenine; 6, alanine; 7, asparagine; 8, aspartate; 9, betaine; 10, carnitine; 11, choline; 12, creatine; 13, cytidine; 14, dimethylamine; 15, ethanol; 16, formate; 17, fumarate; 18, glucose; 19, glucose-6-phosphate; 20, glutamate; 21, glutamine; 22, glycine; 23, guanosine; 24, histamine; 25, inosine monophosphate; 26, inosine; 27, isobutyrate; 28, isoleucine; 29, lactate; 30, leucine; 31, lysine; 32, malate; 33, malonate; 34, mannose; 35, methanol; 36, methionine; 37, *N*,*N*-dimethylglycine; 38, *N*-methylhydantoin; 39, niacinamide; 40, *O*-acetylcarnitine; 41, *O*-phosphocholine; 42, ornithine; 43, pantothenate; 44, trimethylamine *N*-oxide; 45, phenylalanine; 46, proline; 47, sarcosine; 48, serine; 49, succinate; 50, taurine; 51, threonine; 52, trimethylamine; 53, glucose-1-phosphate; 54, tryptophan; 55, tyrosine; 56, uridine; 57, valine; 58, sn-glycero-3-phosphocholine; 59, *trans*-4-hydroxy-L-proline; 60, *π*-methylhistidine; 61, 3-aminoisobutyrate; 62, citrulline; ppm, Parts per million.

of metalloproteinase has the ability to inhibit matrix metalloproteinases (MMP)[33]. In the present study, the gene expression of the *timp2* observed from the transcriptomics was significantly up-regulated by the supplementation of Hyp in diet. It is hypothesised that the MMP play important roles in the degradation of collagens, thus causing the postmortem softening of fish muscle. For example, MMP-2 is a major proteinase responsible for the degradation of type I and V collagens in common carp (*C. carpio*), thus resulting in the softening of fish muscle during chilled storage[34]. The results of the present study suggest that dietary Hyp regulated collagen turnover in muscle partly through increasing the ability of collagen biosynthesis and suppressing the degradation of collagen in the muscle of large yellow croaker, which is in agreement with the previous study[22]. A similar study was observed in rat, where an oral intake of L-hydroxyproline had a stimulatory effect on collagen metabolism and turnover[35].

Apart from collagen metabolism, dietary Hyp could play a role in taurine-related amino acid metabolism. The intake of certain nutrients can affect gene expression of *bhmt* in salmonid, such as methionine[36]. In the present study, the expression of *bhmt* observed from the transcriptomics showed up-regulation by dietary Hyp supplementation. The *bhmt* is a cytosolic enzyme that catalyses the conversion of betaine and homocysteine to dimethylglycine and methionine, respectively[37]. Methionine is a precursor for taurine biosynthesis; its degradation leads to the synthesis of taurine[38]. This mechanism is consolidated by the increased concentration of taurine observed from the metabolomics. It suggests that Hyp supplementation in fish diet could affect taurine metabolism leading to the increased concentration of taurine. Taurine plays an important role in multiple physiological processes such as the regulation of antioxidation[39] and acting as a pH buffer for regulation of mitochondrial metabolism[40]. It was also reported in mammals that taurine was involved in the control of glucose homoeostasis by regulating the expression of genes that are required for glucose-stimulated insulin secretion and that it enhances peripheral insulin sensitivity[41]. The present study showed that an increased concentration of glucose-6-phosphate observed from the metabolomics coincided with an up-regulated gene expression of glycolysis-related genes including *pfkm*, *aldoa* and *gapdh* observed from the transcriptomics in the Hyp-supplemented group, which indicated a better utilisation of carbohydrates. It is well known that teleost fish show persistent hyperglycaemia and are generally considered to be glucose intolerant[42]. The results suggest a tendency towards better utilisation of glucose when fish are fed dietary Hyp, and provide evidence that the regulation of glucose by Hyp supplementation could be partly due to the increased taurine content. The enhancement of glycolysis involved in the aerobic metabolism correlates with a firm fillet. A previous study observed that fish fed dietary Hyp supplementation had a firmer fillet[22]. The present study showed that several gene expression increased observed from the trancriptomics, such as *sdh* (a citric acid cycle enzyme) and *aco*.

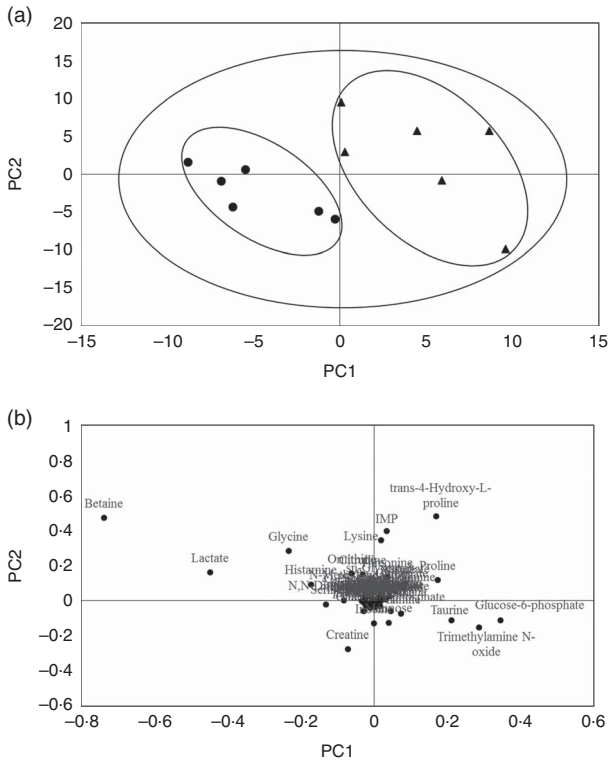

Fig. 3. The partial least squares discriminant analysis (PLS-DA) model in muscle of large yellow croaker fed dietary hydroxyproline supplementation. The components score plot in muscle of large yellow croaker fed a diet with no Hyp supplementation compared with those fed a diet with Hyp supplementation, the ellipses indicate clusters of the treatment groups in the experiment (a). Loading plot that explained the further the metabolites get away from the core, the more the contributions distinguishing two groups (b). (●) B-D, basal diet; (▲) H-D, Hyp supplementation diet.

Table 5. Variable importance in projection (VIP) scores of muscle metabolites of large yellow croaker fed dietary hydroxyproline supplementation*

Metabolite	VIP	H-D/B-D
Betaine	4·32	Down
Trans-4-hydroxy-L-proline	3·32	Up
Lactate	2·76	Down
Glucose-6-phosphate	1·96	Up
Trimethylamine *N*-oxide	1·63	Up
Taurine	1·39	Up
Creatine	1·30	Down
IMP (inosinic acid)	1·29	Up
Histamine	1·20	Down
Serine	1·00	Down

B-D, basal diet; H-D, Hyp supplementation diet; IMP, inosine monophosphate.
* It shows the VIP > 1 metabolites, which contributed to a distinction between control group and Hyp supplementation group.

These genes are vital regulators of energy metabolism regarding mitochondrial functions. This agrees with the study of Atlantic salmon that showed a higher mitochondrial metabolism with firmer fillets[12,13]. In the present study, the increased carbohydrate metabolism together with mitochondrial processes indicated an enhanced cellular aerobic respiration in the Hyp supplementation group, which could be attributed to a good energy utilisation with a firmer fillet.

The PPAR signal pathway, which is related with the lipid metabolism, was significantly enriched as observed from the transcriptomics in the present study. In this pathway, *lpl* was up-regulated. The *lpl* is a key enzyme in lipid metabolism that hydrolyses TAG to provide free fatty acids for cells and affects the maturation of circulating lipoproteins[43]. It is also known as an activator (and/or ligands) of the various PPAR[44]. The *fabp3* is known to function as a transport protein for mitochondrial *β*-oxidation in muscle tissues[45]. It increased in the H-D group in the present study. The *fatp1* and acyl-CoA *acsl1* were also improved in the H-D group and, respectively, facilitated the transport of fatty acids across the cell membrane. Meanwhile, their esterification prevented their efflux and facilitated fatty acid uptake[46]. In addition, *scd1*, which plays an important role in lipogenesis, was also up-regulated. It is the rate-limiting enzyme that catalysed the conversion of saturated long-chain fatty acids into MUFA[47]. The results indicated that fish fed dietary Hyp supplementation enhanced the lipid metabolism. The up-regulated gene of lipid metabolism owing to Hyp supplementation indicates that the consumption of fat as fuel provided cellular energy, which was verified by the expression of genes involved in energy metabolism. Positive correlations with firmness were shown for genes involved in different aspects of lipid metabolism[12]. This was further confirmed with the firmer muscle texture in Hyp supplementation group in the previous study[22].

IMP is a breakdown/degradation product of ATP. It is an important taste enhancer and used as an index of freshness quality in a wide variety of fish[48,49]. Several researches have also reported that the decrease or disappearance of IMP is correlated with the loss of fresh fish flavour in most species[50,51]. The high level of IMP in the H-D group observed from the metabolomics in the present study indicated a good flesh freshness in muscle of large yellow croaker. The level of trimethylamine (TMA) is strongly correlated to the sensory quality of cod[52], and can be also used as an objective index to predict the fish quality in freshness[53]. Its concentration increases with the decrease of the fish freshness[54]. The TMA is secreted by the decomposition of TMAO, and its concentration increased after the fish died[55]. An inhibition of conversion from TMAO to TMA will have a better fish freshness. In the present study, although no change was found for TMA, the concentration of TMAO observed from the metabolomics increased. It suggests a tendency towards a good flesh freshness when fish is fed a diet with supplementation of Hyp.

In conclusion, combining analysis of transcriptomics and metabolomics revealed that the increased collagen content in muscle of large yellow croaker fed a diet with supplementation of Hyp was partly due to the enhancement of synthesis and the diminished degradation of collagen. The up-regulation of glycolysis coinciding with the mitochondrial processes suggests better utilisation of carbohydrates and an enhanced cellular aerobic respiration in the Hyp supplementation group. This could lead to a good energy utilisation with a firm fillet of large yellow croaker. The enhancement PPAR signalling pathway was significantly enriched by Hyp supplementation and it had stimulatory effects on improving flesh quality of large yellow croaker. Meanwhile, dietary Hyp could improve fish freshness by inhibiting conversion from TMAO to TMA.

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Z. W. completed the experiment and prepared the manuscript. H. Z. and Y. Z. analysed the samples. W. Z. designed the experiment and revised the manuscript. K. M. designed the experiment.

The authors declare that there are no conflicts of interest.

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