Original Paper

Is the Nutritional Value of Fish Fillet Related to Fish Maturation or Fish Age? Integrated Analysis of Transcriptomics and Metabolomics in Blunt Snout Bream (*Megalobrama amblycephala*)

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Key Words

Megalobrama amblycephala • Flesh meat nutrition • Transcriptomics • Metabolomics • Omics association analysis

Abstract

Background/Aims: Fish is a protein-rich food and is increasingly favored by consumers. It has been well recognized that the flesh composition of fish is closely related to its maturation and growth stage, but few studies have explored these differences. Additionally, hormone residues in fish after artificial induction of reproduction also attract consumer concern. In this study, we attempt to address these concerns by using a combination of transcriptomics and metabolomics analyses to identify key regulated pathways, genes, and metabolites that may affect the flesh nutrition of one typical aquaculture species in China, blunt snout bream (*Megalobrama amblycephala*). **Methods:** The four groups of fish were used for transcriptomics and metabolomics analyses, including one-year-old immature (group I), two-year-old immature (group II), two-year-old mature (group III) and successfully spawned (group IV) female *M. amblycephala* after artificial induction of reproduction. **Results:** We identified a total of 1460 differential compounds and 1107 differentially expressed unigenes in muscle among the different groups. Differential metabolites related to fish age (group II vs group I, group III vs group I) were largely enriched in "Glycerophospholipid metabolism", "Linoleic acid metabolism", "α-Linolenic acid metabolism", and "Biosynthesis of unsaturated fatty acids". Between these two pairwise comparisons, metabolites that are beneficial to human health, such as docosapentaenoic acid, α-Linolenic acid, eicosapentaenoic acid, and docosahexaenoic

acid were found to be significantly decreased in two-year-old (group II, group III) compared with one-year-old (group I) *M. amblycephala*. Only one differential metabolite related to fish maturation, a triglyceride, was detected between groups III and II. Transcriptomics data showed that differently expressed genes (between group III vs group II, group III vs group I) related to maturation were highly enriched in "Cell adhesion molecules (CAMs)", "Sphingolipid metabolism" and "Phagosome". DEGs (between group II vs group I, group III vs group I) relating to fish age were enriched in the "cGMP-PKG signaling pathway", "FoxO signaling pathway", and "AMPK signaling pathway". The gene-metabolite interaction network showed pivotal genes, including fumarate hydratase and GNPAT, which played a major role in the regulation of glycerophospholipid metabolism. The nutritional components were also measured, which verified the metabolomics results. Moreover, the metabolomics results showed that after 24 hours of artificial hormone injection, the drug was completely metabolized. **Conclusion:** Integrated analysis demonstrated that the nutrition value of fish fillet was much more related to fish age compared with maturation status in *M. amblycephala* females.

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Introduction

With the development of the economy and people's living standards, higher food nutrition requirements are demanded. Fish, as a protein-rich food, is favored by an increasing number of people in recent years. Previous studies have indicated that the flesh nutrition is closely related to gonadal development, and puberty could have negative effects on the flesh composition of fish species [1, 2]. From the beginning of rapid growth of gonadal cells until the end of ovulation, the nutrients absorbed by fish *in vitro* are mainly consumed for the growth of gonadal cells (especially oocytes), and somatic cells might grow slowly. The content of protein and fat in muscle and liver show a continuous decline during this period, which would affect the quality of fish flesh [3]. Some studies have also shown that the nutrition of fish is relevant to its growth age. Xu et al. reported that with increasing age, the content of protein increases while moisture decreases in *Megalobrama hoffmanni*, and the fat content in two-year-old, 3-year-old and 4-year-old fish decline significantly compared with one-year-old fish [4]. However, whether the quality of fish meat is more related to fish age or fish maturation stage has not been addressed thus far.

In production, to conduct scaled production and obtain large batches of fry, people usually perform artificial propagation of mature individuals to trigger the release of gonadotropin (GtH) along the hypothalamic-pituitary-gonad (HPG) axis [5, 6]. However, whether these synthetic hormones with similar biological effects of endogenous hormones will form a residue in aquatic products and how long the injected hormone metabolites will be resolved in fish fillet have not been tested.

Metabolomics, as a burgeoning 'omics' research mode, can be used to detect all metabolites (molecules <1 kDa) in a cell or a tissue. As metabolites can reflect the changes in the internal environment of an organism and may reveal the function of genes directly, it has been applied to many fields, including drug discovery, disease research, and biomarker discovery, among others. In recent years, a number of studies related to muscle metabolomics have also been reported in mammals [7]. For example, Morales et al. identified new metabolites and quinolone conversion products in chicken muscle treated with enrofloxacin, as well as certain quinolone conversion products in chicken during the pH change from 1.5 to 8.0 [8]. Fazelzadeh et al. used muscle metabolomics to determine the effect of age and frailty on the metabolic signature of skeletal muscle tissue in humans, and the results indicated that primary differences in skeletal muscle metabolite levels between young and older tested specimens were associated with mitochondrial function, muscle fiber type, and tissue turnover [9]. Julia et al. elucidated the underlying biochemical processes of meat quality traits, and the potential key molecules related to meat quality traits-drip loss were identified [10]. Regarding fish species, a comprehensive metabolomics analysis has been widely applied to detect the metabolite differences in fish embryo [11], plasma [12, 13],

liver [13, 14], gonad [14-16] and skin mucus [17] under specific conditions or treatments. However, no metabolomics approach has been used to identify metabolites related to muscle composition, as well as those related to fish age and maturation.

It has been recognized that genomics provides information about what may occur and metabolomics about what will really happen. Researchers hope for the development of a comprehensive approach that not only incorporates the transcript level, but also the comparison of metabolites. The efficiency of RNA sequencing (RNA-Seq) will accelerate research of the mechanisms associated with changes in metabolites. Therefore, a concept of conjoint omics analysis has appeared. Recently, the integrated application of transcriptomics and metabolomics is becoming popular in the study of specific traits in plants and mammals. Qian et al. analyzed the mechanism of grape hyacinth (*Muscari*) color determination [18]. Jin et al. discovered that differences in gene expression and metabolite levels following cold stress treatment seemed to have contributed to the observed difference in the cold tolerance phenotype of two kinds of tobacco cultivars [19]. Chen et al. detected the biosynthesis of active compounds of *Isatis indigotica* based on transcriptome sequencing and metabolite profiling, and this database also offered candidate genes involved in the biosynthesis of effective metabolites in *I. indigotica* [20]. In fish species, Katsiadaki et al. investigated transcriptomic and metabolic changes in response to ethinyl-estradiol (EE2) exposure in three-spined stickleback (*Gasterosteus aculeatus*) [21]. As expected, transcription induction was observed for hepatic vitellogenins and choriogenins, as well as a series of other EE2-responsive genes. Xu et al. used a combination of transcriptomic and metabolomic methods to identify the key signaling pathways and genes affecting ovarian development of the estuarine tapertail anchovy (*Coilia nasus*) [16]. This kind of comprehensive analysis is increasingly applied to different research fields to explore the mechanism of specific traits.

The blunt snout bream (*Megalobrama amblycephala*), Cyprinidae, *Megalobrama* is a typic herbivorous cyprinid fish species with an annual production of 700, 000 tons in China [22]. In the present study, integrated application of transcriptomics and metabolomics was applied to investigate the comprehensive metabolic and gene expression profiles in muscle tissue of *M. amblycephala* from four groups, including one-year-old immature females, two-year-old immature females, two-year-old mature females and successfully spawned females after using artificial hormones. According to the results, we attempt to address the following three questions: 1) Are fish fillet compositions more related to fish age or fish maturation stage? 2) Do artificial hormonal drugs used for fish artificial reproduction form a residue in fish muscle after 24 hours of artificial spawning induction? 3) Are the changes in metabolite abundance consistent with variations in transcript levels?

Materials and Methods

Experimental design

Animals. A sufficient number of one-year-old and two-year-old *M. amblycephala* were obtained from the Tuanfeng Fish Breeding Base of Huazhong Agricultural University in May 2016. The fish were divided into two separate tanks by growth age for one week. All experimental procedures were performed under the conditions of the fish genetic and breeding laboratory in Huazhong Agriculture University (Wuhan, China). The fish were placed in well-aerated aquarium conditions in dechlorinated water and fed the same normal diet.

Sample groups. Samples were divided into four groups: one-year-old immature *M. amblycephala* females (group I), two-year-old immature *M. amblycephala* females (group II), two-year-old mature females before injection of hormones (group III) and two-year-old females after 24 hours of successful spawning by injection of oxytocin (group IV). The artificial oxytocin implementation procedure was as follows. For group IV, the fish were intraperitoneally injected with a luteinizing hormone-releasing hormone from the basal part of the pectoral fin (LRH-A2) (1 µg/kg fish body weight). After 10 h, the second injection was

administered (0.5 ml/kg body weight) (each ml contained LRH-A₂ 4 µg, human chorionic gonadotropin (HCG) 500 U, and domperidone (DOM) 5 mg of solution).

Sample collection. The gonads of fish were collected to identify the sex of one-year-old fish by traditional histological analysis, and muscle samples were only selected from the females (group I). All muscle samples were collected under the first spine of the dorsal fin. For the two-year-old *M. amblycephala*, muscle samples were first collected from individuals, and then gonads were collected to perform the histological analysis. Individuals with ovary development before pre-spawning phase were recognized as non-mature females (group II), while individuals with ovary development at the pre-spawning phase were recognized as mature females (group III). For group IV, the individuals that spawned normally during artificial reproduction were placed back in the tank, and then muscle samples were collected after 24 hours. For each group, muscle samples from 10 individuals were collected. After collection, muscle samples were immediately frozen in liquid nitrogen and then transferred to -80 °C for further study. Detailed information for the sample preparation and analysis are shown in Fig. 1.

Metabolomics analysis

Sample preparation. Muscle tissues (approximately 25 mg) from each individual were homogenized in 800 µl cold methanol and water (v/v = 1:1) using a Qiagen Tissue-Lyser (Retsch GmbH, Germany). Then, 400 µl supernatant was collected after centrifugation. The supernatant was removed, and the sediment in the tube was dried. The sediment was homogenized again in 800 µl cold dichloromethane and methanol (v/v = 3:1) and centrifuged, and then 200 µl supernatant were collected for QC (quality control) of the sample. The supernatant was evacuated under a vacuum, and 100 µl supernatant was re-collected after centrifugation for subsequent individual sample analysis.

Method development and validation. All samples were acquired using the liquid chromatograph-mass spectrometer (LC-MS) system according to the protocol supplied with the machine. First, all chromatographic separations were performed using an ultra-performance liquid chromatography (UPLC) system (Waters, USA). An ACQUITY UPLC BEH C18 column (100 mm*2.1 mm, 1.7 µm, Waters, USA) was used for reversed-phase separation. The column oven was maintained at 50 °C. The flow rate was 0.4 ml/min, and the mobile phase consisted of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile+0.1% formic acid). Gradient elution conditions were set as follows: 0~2 min, 100% phase A; 2~11 min, 0% to 100% B; 11~13 min, 100% B; 13~15 min, 100% A. The injection volume for each sample was 10 µl.

A high-resolution tandem mass spectrometer SYNAPT G2 XS QTOF (Waters, USA) was used to detect

Fig. 1. Diagram of muscle samples preparation and analysis.

metabolites eluted from the column. Q-TOF was operated in both positive and negative ion modes. For positive ion mode, the capillary and sampling cone voltages were set at 2 kV and 40 V, respectively. For negative ion mode, the capillary and sampling cone voltages were set at 1 kV and 40 V, respectively. The mass spectrometry data were acquired in Centroid MSE mode. The TOF mass ranged from 50 to 1200 Da, and the scan time was 0.2 s. For MS/MS detection, all precursors were fragmented using 20–40 eV, and the scan time was 0.2 s. During acquisition, the LE signal was acquired every 3 s to calibrate the mass accuracy.

The QC samples were used to provide robust quality assurance for each metabolic feature detected. The QC sample was prepared by pooling the same volume from each muscle sample and then prepared in the same way as the detection samples. Ten QC samples were run before the first muscle sample, and three QC samples were run at the last location to calibrate the drift in retention time of all analyses due to the matrix effect. In addition, a QC sample was inserted after each 10 tested muscle samples.

Data processing and statistical analysis. The raw data were collected using a mass spectrometer XevoG2-XS QTOF (Waters, USA). The raw data was imported into Progenesis QI (version 2.2): obtained retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. The ions with relative standard deviation greater than 30% were removed by filtration, and QC-RLSC (robust LOESS signal correction) was adopted to correct the data.

To screen out metabolites with statistical and biological significance, the pre-processed Ultra Performance Liquid Chromatography-mass spectrometer (UPLC-MS) data were imported into R language analysis for univariate analysis and multivariate statistics. The univariate analysis utilized the t test and fold change analysis (FC analysis). The p -value was obtained via the t test and corrected by the false discovery rate (FDR) to obtain the q -value. Subsequently, to discover potential metabolomics markers, the acquired data were processed by principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) of multivariate statistics. PCA is a dimensionality reduction method to observe the trend of separation between each stage [23]. The PLS-DA method utilized partial least-squares regression to establish the relationship between metabolite expression and sample size, which could further reflect the difference between the experimental samples. The variable importance in the projection (VIP) values reflected the importance terms in the PLS-DA model. The VIP value was calculated to measure the effect of each metabolite expression pattern on the classification of each sample, thus assisting the filtrated metabolites. In this research, the VIP values, fold-change and q -value were employed to analyze the differentially expressed metabolites. The filter conditions were as follows: (i) VIP value more than 1; (ii) fold change more than 2 or less than 0.5; (iii) q -value less than 0.05. The ions that met these three conditions were identified as differentiated ions. All putatively annotated metabolites were submitted to the Pathway Analysis (MetPA) module within MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/>) under accession number ID1239.

Transcriptome analysis

RNA extraction, library construction, and sequencing. Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's procedure for each muscle sample. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit supplied with the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 1.5 µg RNA per sample was used as input material for the RNA sample preparations. For each group, two Digital Gene Expression Profiling libraries were constructed with the equivalence RNA pool from five individuals for each library. Moreover, a reference transcriptome library was constructed by mixing equal volumes of RNA from the 4 groups. Sequencing libraries were generated using the 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. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. Following the adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments that were preferentially 150~200 bp in length, the library fragments were purified

with the AMPure XP system (Beckman Coulter, Beverly, USA). Subsequently, 3 μ l USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was then performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, the PCR products were purified (AMPure XP system) and library quality assessed on the Agilent Bioanalyzer 2100 system. All the raw RNA-Seq data were submitted to the NCBI databases (http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=run_browser) under accession number SRR6001849 for transcriptome data and SRR6002841 for DGE data.

De novo transcriptome assembly and annotation. Raw data (raw reads) in fastq format were first processed using in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, poly-N greater than 10% and low-quality reads from raw data. Transcriptome assembly was accomplished using Trinity with parameters of Kmer=25 [24]. First, short reads were assembled into contigs based on their overlap regions. The contigs were then assessed for sequence clusters to obtain unigenes with the sequence clustering software TGICL (V2.1) [25]. To obtain annotations, the unigenes were aligned by a series of protein databases using BLASTx (e-value <10⁻⁵): non-redundant protein (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and enKaryotic Ortholog Groups (KOG), and aligned by BLASTx to the nucleotide databases Nucleotide collection (Nt). Furthermore, biological function annotation was based on gene ontology (GO), while Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to label pathways.

Analysis of differentially expressed genes. The RSEM was used to quantify gene expression, and the quantitative results were converted to fragments per kilobase per million (FPKM) [26]. Differential expression analysis of four groups was screened using the NOISeq package [27]. We screened differentially expressed genes according to the following default criteria: fold change ≥ 2 and divergence probability ≥ 0.8 .

Pathway-based analysis could help to further understand the biological functions of genes. KEGG was used to perform pathway enrichment analysis of differentially expressed genes (DEGs). This analysis identified significantly enriched metabolic pathways or signal transduction pathways by DEGs compared with the whole genome background. After multiple testing corrections, pathways with a Q value ≤ 0.05 were defined as significantly enriched among the differentially expressed genes.

Quantitative real-time PCR (qRT-PCR) for mRNA expression

To validate the sequencing data, we screened genes in the nutrition regulatory pathway or genes with significant differences between the four groups based on the transcriptome results. Finally, 12 differentially expressed genes were selected for qRT-PCR analysis using the same RNA samples that were used for the transcriptome profiling. Primers were designed using Primer 5 software (Table S1), and the β -actin gene was selected as a control for RT-PCR analysis. The 20 μ l qRT-PCR was performed using SYBR Green PCR Master Mix (TaKaRa, Japan, RR820A). All the real-time reactions were performed in triplicate, and the relative expression levels were measured in terms of the threshold cycle value (Ct) and normalized using the equation $2^{-\Delta\Delta Ct}$. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for comparison of differences among the four groups.

Integrated analysis

In the combined analysis of transcriptomics and metabolomics, we preferred differential metabolites with a larger fragmentation score. Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.kegg.jp/>) was used for analysis of metabolic pathways. The Human Metabolome Database (HMDB, <http://www.hmdb.ca/>) was used to identify the function of each metabolite. The interaction between genes and metabolites was calculated as Pearson correlation coefficients, with a threshold value of 0.6 for inclusion in the network. Cytoscape was used to visualize the final interaction network [28].

Analysis of nutritional components

As indicated in the integrated analysis, the nutrition value of fish fillet was much more related to fish age than to maturational status. Thus, we selected fish in group I and II to analyze nutritional components of their muscle. The general nutrition components, amino acids and fatty acid in the two groups were measured and compared. Moisture, crude protein, crude fat and ash were detected separately according

to National Food Safety Standard methods GB5009-85-2016. An amino acid analyzer (HIT-ACHL-8900) was used to quantitate different amino acid contents. Quantitative analysis of fatty acids was performed by gas chromatography (YLSB076). Statistical analyses were conducted using SPSS 20.0, and significant differences were set at $P < 0.05$.

Results

Metabolite analysis

General metabolite analysis. To examine the biochemical basis of muscle in female *M. amblycephala* from different groups, the metabolic profiles were analyzed by UPLC-MS in positive (ESI+) and negative (ESI-) patterns. In total, 9487 variables (metabolite peaks) were selected for subsequent analyses (5727 peaks in ESI+ mode and 3760 peaks in ESI- mode).

All data were recorded for multiple statistical analyses, including PCA and PLS-DA. The PCA results illustrated that group II, group III and group IV were much closer than either to group I (Fig. 2). PLS-DA could reflect the differences among the different groups, and the results confirmed that the different groups were represented by different metabolic patterns (Fig. S1). For all supplemental material see www.karger.com/10.1159/000492837. Then, a large number of metabolites with statistical and biological significance were filtered out to elucidate the metabolic process of organisms and mechanistic changes.

Differential ions were identified by Progenesis Q1 (version2.2). The significance threshold was set at $VIP > 1$, $q < 0.05$ with a fold change > 2 or < 0.5 to confirm significant differences derived from the multivariate analysis. As a result, 469 differential ions in positive mode and 991 differential ions were detected in the four sample groups. There were 288, 10, 150, and 21 differential ions in positive mode and 559, 0, 431, and 1 differential ions in negative mode in group II vs group I, group III vs group II, group III vs group I, and group IV vs group III. The up- and down-regulated ions for each comparison group are shown in Fig. 3a. To identify metabolites, HMDB was used as the freely accessible database to elucidate the putative structures of the differential metabolites. In the four groups, 233 ions were identified by identification level 1 and 142 ions in identification level 2 were tested by in positive mode, while 351 ions in identification level 1 and 178 ions in identification level 2 were detected in negative mode. The details of the differential ions in different comparison groups are shown in Table 1. The Venn diagram displayed no overlapping differential ions for all comparison groups (Fig. 3b). However, between group III vs group I and group II vs group I, 469 overlapping differential ions were identified. In the following analysis, the analyzed

Fig. 2. PCA score scatter plots for the first two components of the four groups based on metabolomics data. (a) Positive ion mode; (b) negative ion mode.

Fig. 3. Statistics for all differential ions among the different groups. (a) The number of up/down-regulated compounds in each comparison group; (b) Venn diagram of differential compounds between pairwise comparisons.

Table 1. General information about metabolite analysis among different groups. ^aThe number of all differential m/z between two different groups, which including identified ions and unable identified ions; ^bThe number of identified ions by searching HMDB database associated with primary data (parent ions); ^cThe number of identified ions by searching fragmentation information available from HMDB database

Differential ions Comparison among groups	Detect mode	Total ion number ^a	Up- regulated number	Down- regulated number	Metabolites identification					
					Identification level 1 ions number ^b			Identification level 2 ions number ^c		
					Total	Up-regulated	Down-regulated	Total	Up-regulated	Down-regulated
Group II vs Group I	Positive ion	288	153	135	147	90	57	93	55	38
	Negative ion	559	198	361	204	82	122	107	46	61
Group III vs Group II	Positive ion	10	0	10	3	0	3	1	0	1
	Negative ion	0	0	0	0	0	0	0	0	0
Group IV vs Group III	Positive ion	21	0	21	16	0	16	5	0	5
	Negative ion	1	0	1	1	0	1	0	0	0
Group III vs Group I	Positive ion	150	55	95	67	25	42	43	16	27
	Negative ion	431	94	337	146	31	115	71	12	59

metabolites in the metabolic pathways tended to be selected from the results of metabolite identification level 2, which demonstrated more reliable results than level 1.

Metabolic pathway analysis could help researchers unravel the main biochemical and signal transduction pathways of annotated metabolites. Based on the KEGG results, 75 pathways, including “Steroid hormone biosynthesis”, “Arachidonic acid metabolism” and “Linoleic acid metabolism”, among others, were detected in muscle under negative patterns. Under positive patterns, 81 pathways, including “Steroid hormone biosynthesis”, “Arachidonic acid metabolism” and “Steroid biosynthesis”, among others, were discovered.

Differential metabolites related to fish maturation. Considering the ovary maturation stage, we attempted to identify differential metabolites in muscle tissue between immature females (group I and group II) and mature females (group III). As a result, representative differential metabolites identified between group I and III were mainly related to 7 pathways, including “Steroid hormone biosynthesis”, “Glycerophospholipid metabolism”, “Arachidonic acid metabolism”, “Linoleic acid metabolism”, “ α -Linolenic acid metabolism”, “Biosynthesis of unsaturated fatty acids”, and “GnRH signaling pathway” (Table 2). However, only one significantly different metabolite, triglyceride (M40), was detected between group II and group III (Table 2).

Of the metabolites with differential accumulation between group I and group III, many had previously been shown associated with meat nutrition, such as glycerophospholipid (M6, M13, M16-18, M25, M30, M37) and fatty acid (M24, M26, M28-29). Compared with

group I, derivatives of glycerolphospholipid, including phosphatidylethanolamine (PE) (M16, M37), phosphatidylcholine (PC) (M6, M17, M19, M25, M30) and phosphatidate (PA) (M13, M18), showed a significantly lower abundance in group III compared with group I. Fatty acids, including docosapentaenoic acid (M14), α -Linolenic acid (M24), eicosapentaenoic acid (M26), docosahexaenoic acid (M27) and dehydroepiandrosterone sulfate (M28), also showed a significantly lower abundance in group III (Fig. 4).

In addition to the above metabolites, steroid hormone (M9-M10, M36, M39) and diacylglycerol (M15, M33) also showed a significant differential abundance between group III and group I. Gonadotropin-releasing hormone (GnRH) was the main hypothalamic regulator of the reproductive system in teleost fish as in mammals [29]. It is well known that steroid hormones are key regulators of a series of physiological processes, including the maintenance of carbohydrate metabolism, sodium and fluid homeostasis, reproduction, and the development of secondary sex characteristics. In this study, steroid hormone (M36, M39)

Table 2. Differential metabolites identified among different groups. Potential biomarkers were identified by identification level 2 (Fragmentation score). Rt (min) retention time, the time of one component for its injection to form the peak. m/z:m/z mass-to-charge ratio, each detected ion with a specific value. Score the matching score by the identification level 1. “–” no significant different. The sample in the file named before “vs” was the treatment group and the sample after “vs” was the control group. “None” meant metabolite is not commented to a particular pathway. Pathway Ko00100, Steroid biosynthesis; Ko00140, Steroid hormone biosynthesis; Ko00260 Glycine, serine and threonine metabolism; Ko00500 Starch and sucrose metabolism; Ko00561 Glycerolipid metabolism; Ko00564 Glycerophospholipid metabolism; Ko00565 Ether lipid metabolism; Ko00590 Arachidonic acid metabolism; Ko00591 Linoleic acid metabolism; Ko00592 α -Linolenic acid metabolism; Ko00600 Sphingolipid metabolism; Ko00830 Retinol metabolism; Ko01040 Biosynthesis of unsaturated fatty acids; Ko04912, GnRH signaling pathway

No.	Metabolites	Rt (min)	m/z	Score	Fragmentation Score	Ratio of group II vs group I	Ratio of group III vs group I	Ratio of group III vs group II	Pathway
ESI+									
M1	PS(18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	9.40	818.54	38.3	16.50	8.13	—	—	Ko00260/Ko00564
M2	PC(18:3(9Z,12Z,15Z)/20:4(8Z,11Z,14Z,17Z))	10.09	804.55	41.1	14.90	5.34	—	—	Ko00564/Ko00590/Ko00591/Ko00592
M3	Glucosylceramide (d18:1/22:0)	10.65	784.66	37.0	0.94	4.55	—	—	Ko00600
M4	PE(24:0/15:0)	10.31	828.55	34.0	4.04	3.93	—	—	Ko00564
M5	7-Dehydrocholesterol	9.23	367.34	38.2	2.70	3.32	—	—	Ko00100
M6	LysoPC(P-16:0)	11.05	462.34	38.1	19.80	2.67	4.61	—	Ko00564
M7	3-O-Sulfogalactosylceramide (d18:1/20:0)	10.71	818.58	37.3	6.70	2.35	—	—	Ko00600
M8	PC(20:3(8Z,11Z,14Z)/P-16:0)	11.14	768.59	37.9	6.43	2.32	—	—	Ko00564/Ko00590/Ko00591/Ko00592
M9	Allopregnanolone	8.66	301.25	36.9	10.10	—	2.09	—	Ko00140
M10	22b-Hydroxycholesterol	9.67	425.34	41.7	41.60	2.31	1.96	—	Ko00140
M11	20a,22b-Dihydroxycholesterol	9.37	401.34	40.0	8.55	2.10	—	—	Ko00140
M12	Vitamin A	8.47	287.24	37.3	4.27	0.42	—	—	Ko00830
M13	LPA(0:0/18:1(9Z))	8.41	454.29	38.6	9.82	0.41	0.45	—	Ko00561/Ko00564/Ko04912
M14	Docosapentaenoic acid	8.93	353.25	37.2	0.17	0.35	0.36	—	Ko01040
M15	DG(16:1(9Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	10.76	637.48	41.2	25.90	0.31	0.32	—	Ko04912
M16	PE(15:0/22:2(13Z,16Z))	8.63	780.55	38.0	6.32	0.25	0.23	—	Ko00564
M17	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/P-18:1(9Z))	8.96	854.55	29.90	0.76	3.12	3.38	—	Ko00564/Ko00590/Ko00591/Ko00592
M18	LPA(16:0/0:0)	7.29	411.25	40.70	11.20	—	2.51	—	Ko00561/Ko04912
M19	LysoPC(0-18:0)	9.07	532.38	39.4	1.76	0.24	0.37	—	Ko00565
ESI-									
M20	PE(24:1(15Z)/20:4(8Z,11Z,14Z,17Z))	10.07	848.61	34.9	5.27	18.19	—	—	Ko00564
M21	PC(20:5(5Z,8Z,11Z,14Z,17Z)/P-18:0)	10.35	826.55	39.4	7.07	5.27	—	—	Ko00564/Ko00590/Ko00591/Ko00592
M22	9,10-Epoxyoctadecenoic acid	7.68	295.23	31.4	5.71	3.61	—	—	Ko00591
M23	Glucosylceramide (d18:1/20:0)	11.13	790.59	54.6	98.70	2.61	—	—	Ko00600
M24	α -linolenic acid	8.51	277.22	38.7	2.84	0.49	0.47	—	Ko00592/Ko01040
M25	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))	8.15	602.30	38.2	21.70	0.47	0.49	—	Ko00564
M26	Eicosapentaenoic acid	8.48	301.22	37.6	0.40	0.47	0.41	—	Ko01040
M27	TG(16:0/16:0/18:1(9Z))[iso3]	9.04	867.72	39.7	12.90	0.45	0.48	—	Ko00561
M28	Docosahexaenoic acid	8.67	327.23	38.1	1.57	0.41	0.39	—	Ko01040
M29	Dehydroepiandrosterone sulfate	8.85	367.16	36.8	0.45	0.37	0.30	—	Ko00140
M30	PC(22:4(7Z,10Z,13Z,16Z)/16:0)	8.66	808.58	34.7	4.88	0.37	0.34	—	Ko00564/Ko00590/Ko00591/Ko00592
M31	11,14,17-Eicosatrienoic acid	8.37	305.25	39.0	13.40	0.35	—	—	Ko01040
M32	Pregnanediol-3-glucuronide	9.17	531.27	42.7	21.60	0.35	0.50	—	Ko00500
M33	DG(18:1(9Z)/20:4(5Z,8Z,11Z,14Z)/0:0)	9.43	677.49	35.9	6.67	0.31	0.36	—	Ko04912
M34	Adrenic acid	8.54	331.27	40.7	17.10	0.20	—	—	Ko01040
M35	PA(P-16:0e/18:2(9Z,12Z))	8.67	655.47	38.3	3.74	0.18	0.18	—	Ko00565
M36	Pregnenolone	8.63	315.24	51.5	72.60	0.16	0.08	—	Ko00140
M37	PE(15:0/20:4(5Z,8Z,11Z,14Z))	9.17	760.47	37.1	5.14	0.13	0.19	—	Ko00564
M38	Lactosylceramide (d18:1/16:0)	8.47	896.58	36.5	7.84	0.10	0.12	—	Ko00600
M39	3a,21-Dihydroxy-5b-pregnane-11,20-dione	8.47	347.22	50.0	64.6	—	0.46	—	Ko00140
M40	Triglyceride	7.14	1044.90	29.5	0.67	—	—	0.29	None

showed a lower abundance in group III, while M9 and M10 had a higher abundance in group III. In the GnRH signaling pathway, derivatives of diacylglycerol (M15, M33) as upstream regulators of many downstream metabolites, such as arachidonic acid and PA, both showed a lower abundance in group III, which might indicate a decreasing level of GnRH metabolism.

Differential metabolites in muscle in relation to fish age.

In an attempt to generate a comprehensive picture of important metabolites in the response to fish age, we used the same method to screen out potential metabolites between two-year-old (groups II and III) and one-year-old (group I) fish. We found that enriched pathways of differential metabolites between group II and group I were similar to group III compared with group I, which were closely related to “Biosynthesis of unsaturated fatty acids”, “GnRH signaling pathway”, “Sphingolipid metabolism”, and “Steroid hormone biosynthesis”, among others. In comparison to group I, the abundances of 15 metabolites in muscle was reduced in group II, including M12-16, M19 and M24-38, whereas 21 other metabolites (M1-8, M10-11, M17, M20-23) showed an increasing trend in group II (Fig. 4). Among the glycerophospholipids, specially, M1-2 and M4 were significantly increased in group II with a fold change value greater than 3.9, while M25, M30, and M37 were significantly decreased. The 9, 10-epoxyoctadecenoic acid (M22), a peroxidation product of linoleic acid, showed an increasing trend in group II, while other fatty acids such as α -linolenic acid (M24), eicosapentaenoic acid (M26), docosahexaenoic acid (M28) and 11, 14, 17-eicosatrienoic acid and adrenic acid (M31) showed a declining abundance. Moreover, 22b-hydroxycholesterol (M10), and 20a, 22b-dihydroxycholesterol (M11), the intermediate products of steroid hormone, showed a higher abundance in group II, and the other three metabolites, including M29, M36, and M39, showed an opposite expression trend. After comparing the metabolites of group III vs group I, we found that they showed the same change trend as in the comparison of group II vs group I, which indicated that the representative metabolites in the fish muscle were more correlated with fish age.

Transcriptome analysis

Assembly and annotation of the reference transcriptome. To obtain a reference transcriptome for the muscle of *M. amblycephala*, a RNA-Seq library was constructed using total RNA of muscle samples from four groups. A total of 48, 479, 350 raw reads were generated using Illumina HiSeq2000. After quality control, high-quality data with a Q20 percentage of 97.9% and GC percentage of 49.08% were available for analysis (Table 3). After

Fig. 4. Heatmap of representative metabolites among the different groups.

assembly, a total of 53,699 unigenes were generated with an average length of 712 bp and an N50 of 1,205 bp. All the unigenes were successfully annotated through alignment to the reference database, with 11,263 (20.97%), 41,634 (77.53%), 22,856 (42.56%), 15,021 (27.97%) and 13,104 (24.40%) unigenes annotated using NCBI non-redundant protein sequences (NR), NCBI nucleotide sequences (NT), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups of proteins (COG), respectively.

Based on the NCBI NR database, the E-value distribution and homology percentage of the unigenes were determined, and most

of them showed strong homology to the available database sequences. Moreover, 53,699 (100%) unigenes were annotated to 5 top-hit species (*Danio rerio*, *Oncorhynchus mykiss*, *Astyanax mexicanus*, *Ctenopharyngodon idella*, *Clupe aharengus*), especially *D. rerio* (77.0%). A total of 18,731 *M. amblycephala* unigenes were classified into 3 GO categories ("biological process", "cellular component", and "molecular function"), an international standardized gene-function classification system (Fig. S2). In addition, 15,021 unigenes were mapped to 231 pathways. KEGG pathway analysis helped to gain a better understanding of the biological function of the genes, and it also was a carrier of the omics association analysis (Fig. S3).

Functional annotation and pathway assignment of DEGs. To identify functional genes in response to different ovary maturation stages and ages in *M. amblycephala*, 8 DGE (digital gene expression profiling) libraries were generated from 4 groups with two biological replicates for each group. The specific mapping results for the 8 libraries are shown in Table 4. The PCA plot was obtained from all the quantitative results of unigenes to show the similarity between groups (Fig. 5). The four different groups were clearly separated by the first two components, and samples from group I showed a looser clustering than the samples from the other groups, which indicated differences between one-year-old and two-year-old fish. The NOISeq method was used to screen DEGs between the two groups, and genes with a log-fold difference ($\log_2\text{Ratio}$) ≥ 2 and probability ≥ 0.8 were considered significantly differentially expressed. The numbers of up-regulated and down-regulated DEGs in the comparison of different groups are shown in Fig. 6a and b.

Functional classification of DEGs was accomplished using a GO analysis. These DEGs were assigned to three classes of GO (Fig. S4), which illustrated the distinct distribution of the main GO categories between different groups. KEGG pathway analysis helped to gain a better understanding of the biological function of the gene. It revealed that a total of 35, 46, 77 and 49 DEGs involved in 107, 104, 96 and 170 pathways were predicted in the

Table 3. Assembly and annotation results from transcriptome sequencing

Category	Number	Database	Number of unigenes
Total Raw Reads	48,479,350	NR	11,263
Total Clean Reads	47,053,752	NT	41,634
Q20 percentage	97.90%	Swiss-Prot	22,856
GC percentage	94.51%	KEGG	15,021
Total number of unigenes	53,699	COG	13,104
Total length (bp) of unigenes	38,261,082	GO	18,731
Mean length (bp) of unigenes	713	Total Number of annotated unigenes	53,699
N50 of unigenes (bp)	1205		

Table 4. Alignment results of eight digital gene expression profiling libraries mapping to the reference transcriptome

Samples	Total reads	Total mapped reads (%)	Unique match (%)	Multi-position match (%)	Total unmapped reads (%)
Group I-1	23,922,340	91.86	89.53	2.32	8.14
Group I-2	23,952,552	89.94	87.04	2.91	10.06
Group II-1	23,950,148	90.72	86.91	3.82	9.28
Group II-2	23,936,839	92.25	89.76	2.5	7.75
Group III-1	23,944,652	87.75	84.45	3.3	12.25
Group III-2	23,945,101	87.12	82.69	4.43	12.88
Group IV-1	23,946,054	88.27	83.64	4.63	11.73
Group IV-2	23,945,595	88.87	84.37	4.51	11.13

pairwise comparison of group II vs group I, group III vs group II, group III vs group I and group IV vs group III, respectively (Fig. 6c and d; Fig. S5-S6). The results showed that “Signal transduction” and “Cancers: Overview” were significantly enriched in the DEGs between group I and group II, while “Immune system” and “Transport and catabolism” were enriched between group II and group III and “Cancers: Overview”, “Signaling transduction” and “Infectious diseases: Bacterial” between group III and group I. To screen out representative genes, we only analyzed the genes that were enriched in the KEGG pathway.

Differential genes in muscle related to fish maturation

Considering the ovary maturation stage, we attempted to identify differentially expressed genes between immature (group I and group II) and mature (group III) females. A total of 18 DEGs were found in both group III vs group II and group III vs group I. Based on the annotation, disease-related genes were identified, such as CD8A, NLRP3, iduronate 2-sulfatase, SULF, 5-oxoprolinase, sphingolipid metabolism-related gene ENPP7, and fatty acid-related gene ZDHHC. KEGG enrichment analysis of these DEGs revealed a high enrichment for “Cell adhesion molecules (CAMs)”, “Sphingolipid metabolism” and “Phagosome”, which suggested that these genes might be affected in muscle corresponding to gonadal maturation (Fig. 7).

Additionally, 27 DEGs were specifically identified in group III vs group II, which were enriched in “Hippo signaling pathway-fly” and “metabolic pathway”. Based on the annotation, some representative DEGs that had been reported to be related to meat nutrients were identified, such as PFKFB3 (c18742_g2), TTN (c22294_g1), and ZDHHC (c20449_g1), all of which had higher expression in group III.

Moreover, 59 DEGs were specifically identified in group III vs group I, which were mainly enriched in signal transduction pathways, including the “cGMP-PKG signaling pathway”, “FoxO signaling pathway”, “mTOR signaling pathway” and “PI3K-Akt signaling pathway”. After identifying DEGs by KEGG, we found some DEGs involved in the nutrition metabolism pathway in

Fig. 5. PCA results for the first two components of the four groups based on the transcriptomics data.

Fig. 6. Statistics for all differentially expressed genes (DEGs) among the different groups. (a) The number of up/down-regulated DEGs among comparison groups; (b) Venn diagram of DEGs between pairwise comparison groups; (c) The number of up/down-regulated DEGs enriched in the KEGG pathway in each comparison group; (d) Venn diagram of DEGs enriched in the KEGG pathway between pairwise comparisons.

Fig. 7. Heatmap of DEGs between group III vs group II and group III vs group I. Red text denotes mutual DEGs between different group comparisons, while black text denotes special DEGs between different group comparisons.

group III vs group I, such as acetyl-CoA C-acetyltransferase (NAD⁺, c42839_g1) and aldehyde dehydrogenase (c53053_g1), which are related to fatty acid degradation [30]. In addition, in this comparison group, ACOT1/2/4 (c22947_g2), which plays important cellular roles in fatty acid metabolism [31, 32], showed elevated expression in group III. Moreover, some DEGs involved in maturation were also identified. CEL (c51633_g1), an enzyme that can regulate cholesterol production from cholesterol ester, showed a lower expression level in group III compared with group I. This change might affect the content of cholesterol and subsequently the role of downstream pathways, including steroid hormone biosynthesis, thereby affecting gonadal development. CALM (c14137_g1), a gene involved in the GnRH

signaling pathway, displayed a lower expression level in group III, which might influence gonadotropin gene expression based on the KEGG pathway.

Differential genes in muscle related to fish age. Considering fish age, we attempted to identify differentially expressed genes between one-year-old (group I) and two-year-old (group II and group III) fish (Fig. 8). As reported previously, AMPK signaling controls an integrated signaling network involved in the regulation of healthspan and lifespan, e.g., via the FoxO and mTOR signaling pathways [33]. Moreover, Yan et al. showed that amino acid metabolism,

Fig. 8. Heatmap of DEGs between group III vs group I and group II vs group I. Red text denotes mutual DEGs between different group comparisons, while black text denotes special DEGs between different group comparisons.

saccharometabolism, lipid metabolism, nucleoside metabolism, inositol metabolism and the tricarboxylic acid cycle merit mention in relation to age [34]. Based on the annotation, a total of 18 genes were differentially expressed between both group II vs group I and group III vs group I. These genes were enriched for the “cGMP-PKG signaling pathway”, “FoxO signaling pathway”, and “AMPK signaling pathway”. Among these common DEGs, we found immunity-related genes, such as IRS1, MKPs, GABRG, CD4, F13A1, MHC, ARX and ENDOD1, the glycerophospholipid metabolism-related gene GNPAT, and arboxypeptidase B and fumC. Salminen et al. confirmed that IRS plays a regulatory role in the activity of AMPK signaling in upstream signaling pathways, which was found to be significantly decreased in two-year-old fish [34]. In these pairwise comparisons, fumC, which was located in the TCA cycle, showed a tendency to decrease in two-year-old fish.

Excluding 18 mutual genes, 59 DEGs were only identified in group III vs group I, which was enriched in pathways related to “Sphingolipid metabolism”, “Vascular smooth muscle contraction”, and “Tight junction”. Moreover, 17 DEGs were only identified in group II vs group I, which were enriched in the “PI3K-Akt signaling pathway” and “Cell adhesion molecules (CAMs). As a nutrition

Fig. 9. Comparison of expression levels for the 14 detected differentially expressed mRNAs by RNA-Seq (black column) and qRT-PCR (red column). All data are shown as the mean $\pm$ SE. The different letters for the qRT-PCR results denote a significant difference between groups ($P < 0.05$).

Fig. 10. Visualization of enriched metabolites and gene profiles. The red line indicates that the Pearson correlation coefficient between the two groups was greater than 0.6, showing a strong correlation. The gray line indicates that the Pearson correlation coefficient between the two was less than 0.6. The circle filled with green denotes the gene that played a key regulatory role.

value-responsive gene, SETD7 (c6136_g1) was significantly increased in group I. In addition, PFKFB3, which was found in group III vs group II and is related to meat nutrition, was also found in group II vs group I, with a decreasing tendency in group II. The annotation results and expression of these genes are shown in Fig. 8. Between group II and group I, only one DEG was annotated as GNPAT (c52107_g1) in glycerophospholipid metabolism, and it was located at the most upstream position to regulate the entire pathway.

Fig. 11. Heatmap of DEGs between group IV vs group III.

Quantitative analysis of mRNA expression. The qRT-PCR results for 12 mRNAs revealed similar changes in expression to those detected using the RNA-seq data (Fig. 9). Among these genes, most, including EDA, ENPP7, F13A1, GNPAT, MAPK7, MTHFD, MYH, NLRP3, and TTN, showed the highest expression levels in group III. The ENDOD1 and ZDHHC genes showed the highest expression in group II, and PFKFB3 had the highest expression in group IV.

Interaction network analysis between regulated genes and metabolites

Interaction networks can be used to help understand the functional relationship between genes and metabolites and to aid in identifying new regulatory elements [35]. Here, we mapped all the differential metabolites and genes to the metabolic pathway and then searched for both enriched pathways. The interaction networks were built according to both enriched pathways. From the clusters shown in Fig. 10, several pathways were detected, including “Glycerophospholipid metabolism”, “Adipocytokine signaling pathway”, “Fatty acid biosynthesis”, “Sphingolipid metabolism”, and “GnRH signaling pathway”. The interaction relationships illustrated that fumarate hydratase (c992_g1) and GNPAT (52107_g1) were two genes that might play a major role in the regulation of glycerophospholipid metabolism. ACOT1/2/4 (22947_g2) was the pivotal gene in fatty acid metabolism and steroid biosynthesis. The results also suggested that HIBCH (42839_g1), ENPP7 (c1837_g1) and ALDH (c53053_g1), which acted as major regulatory genes, might play some role in not only the biosynthesis of unsaturated fatty acids but also primary bile acid biosynthesis.

Analysis of artificial hormone residue

Due to LC-MS advantages of selectivity, high sensitivity, high throughput and simultaneous determination of multiple targets, we also wanted to determine whether the artificial hormonal drugs used for artificial fish reproduction would form a residue in fish fillet after 24 hours of artificial spawning induction. After screening all the differential metabolites based on HMDB in group IV vs group III, several pathways, including “Fatty acid degradation”, “Steroid hormone biosynthesis” and “Primary bile acid biosynthesis”, were enriched by the metabolites (Table 5). Detailed information on these metabolites between group IV vs group III is provided in Table 4. The L-palmitoylcarnitine (M42) exhibited a promotion role in the oxidation of fatty acid. Elaidic acid (M44) was the final breakdown product of fatty acid metabolism. The other two factors, including tetrahydrocorticosterone (M40) and 3a,7a,12a-

Table 5. Differential metabolites identified among different groups before and after artificial spawning. Potential biomarkers were identified by identification level 2 (Score). Rt (min) retention time, the time of one component for its injection to form the peak. m/z:m/z mass-to-charge ratio, each detected ion with a specific value. The sample in the file named before “vs” was the treatment group and the sample after “vs” was the control group. Pathway Ko00071 Fatty acid degradation; Ko00120 Primary bile acid biosynthesis; Ko00140, Steroid hormone biosynthesis; Ko04080, Neuroactive ligand-receptor interaction

No.	Metabolites	Rt (min)	m/z	Score	Fragmentation Score	Ratio of group IV vs group III	Pathway
ESI+							
M40	Tetrahydrocorticosterone	6.56	368.28	37.60	0.00	0.16	Ko00140
M41	2-Arachidonylglycerol	6.94	396.31	38.30	0.00	0.21	Ko04080
M42	L-Palmitoylcarnitine	7.42	400.34	39.00	0.00	0.14	Ko00071
M43	3a,7a,12a-Trihydroxy-5b-cholestan-26-al	7.61	452.38	38.20	0.00	0.19	Ko00120
ESI-							
M44	Elaidic acid	7.51	281.25	36.30	0.00	0.12	Ko00061

Table 6. The content of general nutrition components compared between one-year-old and two-year-old *M. amblycephala* groups. All data were shown as mean $\pm$ SE

Dry weight	One-year-old group	Two-year-old group
Moisture (%)	78.94 $\pm$ 0.02	79.22 $\pm$ 0.91
Crude fat (%)	9.85 $\pm$ 0.26	9.30 $\pm$ 0.47
Crude protein (%)	90.89 $\pm$ 2.35	87.71 $\pm$ 2.45
Ash (%)	6.05 $\pm$ 0.06	5.81 $\pm$ 0.18

Trihydroxy-5b-cholestan-26-al (M43), were intermediate metabolites of corticosterone and bile acid, respectively. Moreover, 2-arachidonylglycerol (M41) acted as an endogenous ligand that could rapidly enhance the metabolism of phospholipids. All these metabolites had significantly higher abundances in group III. Additionally, a certain number of metabolites that were not annotated to metabolic pathways were also analyzed for a source. After detecting all the metabolites between group IV vs group III, they were found to be endogenous

or derived from food, and no metabolites were associated with artificial hormonal residues.

Based on the transcriptomic data, 88 up-regulated and 180 down-regulated DEGs were identified between group IV vs group III. KEGG enrichment analysis for these DEGs revealed that “Cell adhesion molecules (CAMs)”, “T cell receptor signaling pathway”, and “Primary immunodeficiency” were highly enriched, which suggested that the injection of artificial hormones might result in autoimmune-type reactions (Fig. 11). Based on the annotation, CD4 (c24591.g1), CD8A (c30245.g1; c41521.g1), and ICOS (c18875.g2), which belong to primary immunodeficiency, were differentially expressed between group IV vs group III. In addition to these DEGs, fabD (c42839.g1), the important modulatory factor in “Fatty acid metabolism”, “Fatty acid biosynthesis”, “Fat digestion and absorption”, and “Fatty acid degradation”, was also detected.

Comparison of nutritional components

To verify the omics results, we compared the fish fillet nutritional components between the one- and two-year-old fish groups. The results showed that the contents of crude fat, crude protein and ash were higher in one-year-old than in two-year-old fish fillet (Table 6). Regarding the contents of amino acids, there were no significant differences between the one- and two-year-old fish groups (Table 7, $P < 0.05$). However, for fatty acids, the contents of tridecanoic acid, pentadecanoic acid, palmitic acid, heptadecanoic acid, stearic acid, oleic acid, arachidonic acid, and eicosapentaenoic acid were all higher in the one-year-old compared with the two-year-old fish group (Table 8).

Discussion

Meat nutrients are a source of human dietary fat and high-value animal protein, which is increasingly important worldwide [36]. In recent years, the nutrient content of meat and its influential factors have been increasingly studied. In the present study, an integrated application of transcriptomics and metabolomics was first applied to analyze the nutritional value of fish fillet in relation to different ovarian stages and ages.

In *M. amblycephala*, differential metabolites in muscle related to fish maturation were analyzed between the maturation (group III) and non-maturation (group II and I) groups, as well as between the one-year old (group I) and two-year old (group II and III) groups. A certain number of differential metabolites were identified between group I and II and between group I and III. However, only one differential metabolite was detected between group II and III. Moreover, the tendencies of abundance changes in the same metabolites between group II and I were quite similar to those between group III and I. These results indicated that metabolite abundance in fish muscle is more related to fish age and not maturation stage. Based on the data from the two comparison groups related to age, the following key pathways reflected the muscle nutrition changes in metabolite abundance: "Glycerophospholipid metabolism", "Linoleic acid metabolism", "α-Linolenic acid metabolism", and "Biosynthesis of unsaturated fatty acids".

Glycerophospholipids are the most abundant phospholipid in organisms, characterized by a glycerol backbone with one primary hydroxyl group esterified to phosphoric acid. Previous research has indicated that phospholipids play an important role in the early growth and developmental stages of [37]. In addition, because phospholipids are important structures of lipoproteins, they play a vital function in the fish lipid metabolism process [38]. In the early stages of fish development, phospholipids are an important source of energy and essential fatty acids [39]. A previous study had demonstrated that glycerophospholipids play a crucial role both in the structure and function of all animal

Table 7. The content of amino acids compared between one-year-old and two-year-old *M. amblycephala* groups. All data were shown as mean ± SE. "Δ" represented the essential amino acids; "●" represented non-essential amino acids; "▲" represented fresh flavor amino acids

Amino acid (mg/100 mg)	One-year-old group	Two-year-old group
▲ Aspartic acid	1.81±0.12	1.83±0.16
Δ Threonine	0.77±0.05	0.78±0.07
● Serine	0.71±0.05	0.72±0.07
▲ Glutamate	2.59±0.17	2.56±0.23
● Glycine	0.85±0.06	0.89±0.09
▲ Alanine	1.01±0.07	1.02±0.10
Δ Valine	0.80±0.05	0.82±0.07
Δ Methionine	0.53±0.04	0.55±0.05
Δ Isoleucine	0.75±0.06	0.76±0.07
Δ Leucine	1.41±0.10	1.43±0.12
● Tyrosine	0.68±0.06	0.68±0.06
Δ Phenylalanine	0.79±0.06	0.80±0.06
Δ Lysine	1.66±0.10	1.69±0.13
● Histidine	0.48±0.05	0.47±0.05
● Arginine	0.99±0.06	1.02±0.09
● Proline	0.65±0.06	0.68±0.06
Total amount of amino acids	16.50±1.17	16.77±1.46

Table 8. The content of fatty acids compared between one-year-old and two-year-old *M. amblycephala* groups. All data were shown as mean ± SE. "●" represents saturated fatty acids (SFA); "○" represents monounsaturated fatty acids (MUFA); "⊙" represents polyunsaturated fatty acids (PUFA). The different letters mean significant difference between two groups ($P>0.05$)

Fatty acid (mg/100g)	One-year-old group	Two-year-old group
● Tridecanoic acid	2.66±0.53	2.19±0.37
● Myristic acid	2.45±0.52	2.69±0.37
● Pentadecanoic acid	15.23±3.89	14.89±6.8
● Palmitic acid	152.11±24.56	139.30±29.10
● Heptadecanoic acid	2.03±0.39	1.70±0.38
● Stearic acid	49.87±7.12	49.17±12.56
○ Oleic acid	161.77±20.10	149.19±22.96
⊙ Linoleic acid	102.22±12.12	126.7±19.25
⊙ γ-Linolenic acid	6.97±0.96 ^b	9.97±0.81 ^a
⊙ Arachidonic acid	112.41±15.47	77.39±16.28
⊙ Eicosapentaenoic acid	27.53±5.95	23.03±4.55
● Behenic acid	10.42±1.03 ^b	18.17±2.21 ^a
⊙ Docosahexaenoic acid	73.64±13.44 ^b	127.07±28.85 ^a

cells [38], and they also participate in cell membrane recognition and signal transduction of proteins. For humans, the intake of phospholipids from fish or other food can promote fat metabolism, prevent fatty liver, lower serum cholesterol and improve blood circulation [40]. PC (also known as lecithin) shoulders the metabolism of cells, energy metabolism, information transmission and other functions [41]. In summary, glycerolphospholipids do not have a direct nutritional effect; however, their role is to provide the basic material for normal growth of the body. In the presence of phospholipids, the content of PE (also known as cephalin) is similar to lecithin, and it is widely used in health food. However, as derivatives of PC and PE, M6, M17, M30, M16, and M37 showed a different degree of increasing or decreasing tendency both in group III vs group I and group II vs group I. In addition, PA (M13), a constituent of the cell membrane, was up-regulated in group I compared with the other two groups. Taken together, these results suggest that some changes occur in fish muscle that sustain life activities.

Fatty acids are important constituents of cell membranes and the largest reserve in body [42]. However, fatty acids, not only in adipose tissue but also in muscle, contribute significantly to meat quality and the nutritional value of meat [43]. Moreover, the “Linoleic acid metabolism”, “ α -Linolenic acid metabolism”, and “Biosynthesis of unsaturated fatty acids” pathways play major roles in fatty acid metabolism. In our study, the differential metabolites, α -linolenic acid (ALA, M24), docosahexaenoic acid (DHA, M28), eicosapentaenoic acid (EPA, M26), and docosapentaenoic acid (DPA, M29), belonging to the n-3 series of unsaturated fatty acids were identified. Previous studies have shown that to ensure normal growth and development, fish larvae must assimilate essential fatty acid directly from their environment [44]. The essential fatty acids in seawater bony fish are mainly EPA and DHA [45-48]. In freshwater fish, α -linolenic acid and linoleic acid, as essential fatty acids, play important roles in physiological and biochemical functions [49]. In humans, essential fatty acids such as EPA and DHA cannot be synthesized in the body but are required for maintenance of optimal health. Consequently, people can only obtain these fatty acids through food such as fish. Studies have shown that dietary DHA can reduce blood triglyceride levels and have essential functions in the brain [50]. In addition, EPA might be beneficial for patients with acute lung injury [51], and dietary enrichment with EPA has been shown to protect against thrombosis [52]. Moreover, ALA is the basic material constituting cell membranes and biological enzymes, and it plays a decisive role in human health. Although humans can convert ALA to EPA and DHA, the conversion rate is quite low. Therefore, people must eat many foods that contain ALA [53]. Based on the metabolomics data in this study, we could observe that the content of n-3 series of unsaturated fatty acid was higher in one-year-old than two-year-old fish. Therefore, we inferred that the four n-3 series of unsaturated fatty acids, DPA, DHA, EPA and ALA, might play roles in promoting the growth and development of juvenile fish. The content analysis of fatty acids also indicated that the one-year-old fish group had higher values than the two-year-old fish group. All these results demonstrated that the one-year-old fish had more nutritive fatty acids than the two-year-old fish, which would be beneficial to humans as a food source.

In general, the PCA plot obtained from all the quantitative results of unigenes supported our metabolomics results. Both data sets indicated that group I showed a looser clustering than the samples from the other groups. Based on the KEGG metabolic pathway, we analyzed the DEGs from the transcriptome data. The common DEGs in muscle related to fish maturation were mainly enriched in signal transduction pathways. ENPP7, a common DEG (c10770_g1, c1837_g1, c41141_g1), is a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family, which plays a role in sphingolipid metabolism. As reported previously, it can hydrolyze sphingomyelin to generate ceramide, which is a potent antiproliferative and proapoptotic molecule, and it influences sphingomyelin levels and thus affects cholesterol absorption [54]. Sphingolipid molecules can regulate gonadal cell apoptosis, and sphingolipid messengers can adjust the actions of progesterone and then regulate the maturity of *X. laevis* oocytes [55]. With the increases in ENPP7 expression in group III, we speculated that it might play a role in the sexual maturity of *M. amblycephala*.

Regarding the specific DEGs identified in group III vs group II, PFKFB3 has been previously reported to be associated with amino acid and fatty acid contents and nutrient metabolism in muscle [56]. TTN is the major myofibrillar component of vertebrate and invertebrate striated muscle [57]. Moreover, we found ZDHHC, which has been shown to affect fatty acid content [58]. Both TTN and ZDHHC were up-regulated in group II to group III. In addition, Acots has been shown to play important cellular roles in mammalian fatty acid metabolism through the modulation of cellular concentrations of activated fatty acyl-CoAs [31]. With the increasing Acots in group III, the contents of fatty acids might show a corresponding change.

In another aspect, the annotation of common DEGs in muscle related to fish age were enriched in signal transduction pathways, including the “cGMP-PKG signaling pathway”, “FoxO signaling pathway”, and “AMPK signaling pathway”. A previous study has shown that AMPK signaling controls an integrated signaling network and is involved in the regulation of health span and life span, e.g., via the FoxO and mTOR signaling pathways [33]. IRS1 (c45450_g1, c1260_g1, c31602_g1, c43417_g1, c45450_g1, c47038_g1), the main annotated gene, is a key regulatory gene in the “Regulation of lipolysis in adipocytes” and “Insulin signaling pathway”. Specific DEGs such as SETD7 (c6136_g1) decreased in group II compared with group I, which have been known significantly associated with marbling patterns and intramuscular fat in pigs [59]. In addition, PFKFB3 was also found in group II vs group I, and it was significantly decreased in group II. Overall, these phenomena represent possible internal causes of the metabolite changes.

The gene-metabolite interaction networks coordinate gene and metabolite expression enriched in common pathways, providing a variety of applications in biology [60]. The constructed network can demonstrate signaling pathways [61], transcriptional regulatory network [62] and functional genes and metabolites. In this study, the interaction networks showed that the clusters mainly assembled in “Glycerophospholipid metabolism”, “Adipocytokine signaling pathway”, “Fatty acid biosynthesis”, “Sphingolipid metabolism”, and “GnRH signaling pathway”. Interestingly, our analysis allowed us to identify genes, including fumarate hydratase (c992_g1) and GNPAT (52107_g1), as promising regulatory candidates in glycerphospholipid metabolism, as well as ACOT1/2/4 (22947_g2) in steroid biosynthesis. These interactions might provide assistance for further research.

In previous studies, researchers have used metabolomics to detect the residues of hormones (such estrogen, androgen, and progesterone, among others) in fish meat [5, 63]. Xie et al. developed a method for the determination of five hormone residues (estradiol, ethinylestradiol, norethisterone, diethylstilbestrol and norgestrel) in meat of *Pampus sinensis* by HPLC [63]. The average recoveries for all five hormone residues ranged from 60.1% to 89%. In addition, this method was applied in marketing samples (*P. sinensis*, *Carassius auratus*, *Ctenopharyngodon idellus*), and no hormones were detected in the samples. Real sample tests showed that the metabolomics method was rapid, sensitive and reliable for the simultaneous determination of hormones in fish samples. These studies provide an experimental foundation for our research. In our study, after screening the differential metabolites in muscle before and after artificial induction, we did not find any metabolites associated with the injected artificial hormones. Therefore, we deemed that after 24 hours of artificial induction, the hormones were completely metabolized and did not leave any corresponding residues in fish muscle.

In general, the muscle metabolite profiles of the four groups were clearly separated, as well as the profiles of the one-year-old *M. amblycephala*. Based on the metabolomics data, we could speculate that the nutritional value of fish fillets was much more related to fish age than to fish maturation stage in *M. amblycephala* females. The results of the transcriptome analysis could explain the findings of the metabolomics to some extent, but the specific modalities must be confirmed by further studies. Additionally, use of the metabolomics method to detect fishery drug residues could elucidate protective measures for human health, and our study indicated that artificial hormones could be completely metabolized 24 hours after injection.

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Disclosure Statement

The authors declare to have no competing interests.

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