

## Article

# Dietary Methionine Regulates Hepatic Autophagy and Apoptosis via m<sup>6</sup>A Methylation in Juvenile *Megalobrama amblycephala*

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## Abstract

This study investigated the epigenetic mechanisms through which graded levels of dietary methionine (Met) regulates growth, muscle quality, and health in juvenile *Megalobrama amblycephala* fed an all-plant-protein diet. Dietary Met supplementation improved growth performance in a dose-dependent manner and enhanced muscle nutritional quality, particularly protein content and amino acid composition. Optimal Met intake also strengthened hepatic antioxidant defenses, stabilized mitochondrial function, and modulated plasma metabolite profiles, including metabolites associated with antioxidant, anti-inflammatory, and antimicrobial activity. Epigenetic analysis revealed that dietary Met influenced hepatic N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) RNA methylation and the expression of genes involved in autophagy and apoptosis, suggesting that these molecular pathways contribute to the observed physiological benefits. Collectively, these findings indicate that appropriate dietary Met of 10.1 g/kg not only supports growth and nutrient utilization but also promotes metabolic and cellular homeostasis through epigenetic regulation. This work provides novel insights into the nutritional and molecular strategies for improving the health and performance of juvenile *M. amblycephala* under plant-based feeding regimes, with potential implications for sustainable aquaculture practices.

**Keywords:** *Megalobrama amblycephala*; all-plant-protein diet; methionine; m<sup>6</sup>A methylation; *wtap*/*fto*



Academic Editor: Evangelos Zoidis

Received: 16 September 2025

Revised: 23 October 2025

Accepted: 24 October 2025

Published: 3 November 2025

**Citation:** Sun, Q.; Qian, L.; Xue, C.; Ren, Q.; Jiang, W.; Lin, Y.; Lu, S.; Gu, Z.; Miao, L. Dietary Methionine Regulates Hepatic Autophagy and Apoptosis via m<sup>6</sup>A Methylation in Juvenile *Megalobrama amblycephala*.

*Antioxidants* **2025**, *14*, 1327. <https://doi.org/10.3390/antiox14111327>

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## 1. Introduction

In recent years, the growing significant challenges to the sustainable development of aquaculture and the feed industry have led to an increasing interest in developing alternative non-grain protein sources (e.g., plant proteins, insects, fungi, microalgae, and single-cell proteins from bacteria) to replace fish meal [1]. Among these, plant proteins have become the dominant protein source in aquafeeds due to their wide availability and cost-effectiveness [2]. However, The application of plant proteins in aquafeeds is often limited by anti-nutritional factors and deficiencies in essential amino acids (EAA), which restrict their effectiveness as complete fish meal replacements [3,4]. Supplementing plant-based diets with these limiting amino acids, tailored to the species' nutritional requirements, can substantially improve protein utilization [5–7].

Methionine (Met), a vital sulfur-containing EAA for aquatic species and the first limiting amino acid in plant protein-based diets, is crucial in promoting somatic growth and improving feed conversion efficiency. Beyond its fundamental function as a precursor for protein synthesis, Met is intricately involved in various metabolic processes, including immune regulation and antioxidant defense mechanisms [8–10]. Moreover, it is a primary methyl group donor, contributing significantly to biochemical reactions and epigenetic modifications. In aquatic animals, Met is metabolized through four main pathways: (1) it functions directly as a substrate for protein translation [10]; (2) it is converted into S-adenosylmet (SAM), a universal methyl donor involved in the methylation of proteins, DNA, and RNA, and can be remethylated to regenerate Met [11]; (3) upon activation to SAM, it contributes to polyamine biosynthesis, leading to the formation of spermidine and spermine [12]; (4) through transmethylation its intermediate product homocysteine (Hcy) is further metabolized to produce glutathione (GSH) and taurine, both of which show strong antioxidant properties [13]. These metabolic pathways underscore the key role of Met in protein synthesis, methyl donor (SAM), and antioxidant precursors (GSH/taurine) in aquatic animals.

N<sup>6</sup>-methyladenosine (m<sup>6</sup>A), the most abundant and evolutionarily conserved internal modification in eukaryotic mRNA, is a methylation at the nitrogen-6 position of adenosine and is dynamically regulated by methyltransferases (writers, e.g., methyltransferase like 3 (*mettl3*) and methyltransferase like 14 (*mettl14*)), demethylases (erasers, e.g., fat mass and obesity-associated protein (*fto*) and alkylation repair homolog 5 (*alkbh5*)), and m<sup>6</sup>A-binding proteins (readers, e.g., YTH domain family member (*ythdf1/2/3*) and embryonic lethal abnormal vision-like protein1 (*elavl1*)) [14,15]. This epitranscriptomic mark is regulated dynamically and reversibly by a specialized enzymatic machinery comprising m<sup>6</sup>A modification regulates various post-transcriptional processes, including pre-mRNA splicing, 3'-end processing, translational regulation, nuclear export, mRNA degradation, and the biogenesis of non-coding RNAs. Accordingly, m<sup>6</sup>A has regulated diverse physiological and pathological pathways, such as tumorigenesis, immune homeostasis, and metabolic disorders (obesity) [16]. Met, as an important methyl group donor, is converted to SAM and participates directly in regulating m<sup>6</sup>A methylation in vivo. In mammals, supplementation with lysine and Met in low-protein diets has been shown to modulate lipid metabolism and reduce fat deposition in lambs by downregulating the levels of methyltransferases (*mettl3*, *mettl14*) and demethylases (*fto*, *alkbh5*) [17]. Similarly, selenomethionine (SeMet) has been reported to alleviate hepatic oxidative stress and inflammation in laying hens by reducing *mettl3*-mediated m<sup>6</sup>A methylation of nuclear factor erythroid-2-related factor 2 (*nrf2*), thus enhancing liver antioxidant and anti-inflammatory capacity [18].

Blunt snout bream (*Megalobrama amblycephala*), commonly known as Wuchang bream, is a widely favored freshwater herbivorous fish species, with considerable potential and high economic value for aquaculture development due to its low production cost, rapid growth, high survival rate and high proportion of edible flesh. *M. amblycephala* is naturally distributed in the Yangtze River, China and is primarily cultured in freshwater polyculture systems across the country [19]. Existing literature indicates that species-specific characteristics, developmental stage, and rearing conditions influence the dietary Met requirement in fish. For example, juvenile *M. amblycephala* (3.34 ± 0.03 g) have been reported to require 8.4–8.5 g/kg Met when fed low-fishmeal diets containing 5% fishmeal [20], whereas fingerlings (101.80 ± 1.30 g) under similar dietary conditions show a lower requirement of 7.4–7.6 g/kg [21]. However, under the current demand for sustainable aquaculture, the Met requirement and its functional effects in juvenile *M. amblycephala* fed fishmeal-free, all-plant-protein diets remain unclear. Notably, no study has yet linked methionine supplementation to epigenetic (m<sup>6</sup>A) regulation in fish fed all-plant diets. Therefore, this study

examined the optimal dietary Met demand for juvenile *M. amblycephala* under a completely plant-based diet and evaluated the potential epigenetic mechanisms by which Met (as a methyl donor) regulates nutrient metabolism and health in this species.

## 2. Materials and Methods

### 2.1. Ethics Approval and Consent to Participate

All experimental protocols, methods, and feeding procedures were approved by the Institutional Animal Care and Use Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. All animal handling and experimental procedures were conducted in accordance with the approved ethical guidelines.

### 2.2. Fish and Diets

This study evaluated the effects of graded levels of DL-Met supplementation in plant protein-based diets on juvenile *M. amblycephala* (Huahai No. 1 strain). Six isonitrogenous and isoenergetic diets were formulated with increasing DL-Met levels, resulting in measured dietary Met concentrations of 4.4 g/kg (CON), 7.0 g/kg (MET<sub>1</sub>), 8.6 g/kg (MET<sub>2</sub>), 10.1 g/kg (MET<sub>3</sub>), 12.5 g/kg (MET<sub>4</sub>), and 14.2 g/kg (MET<sub>5</sub>), respectively. The detailed composition of the basal diet and corresponding amino acid profiles are provided in Tables 1 and A1.

**Table 1.** Amino acid composition of experimental feed.

| g/100 g         | CON  | MET <sub>1</sub> | MET <sub>2</sub> | MET <sub>3</sub> | MET <sub>4</sub> | MET <sub>5</sub> |
|-----------------|------|------------------|------------------|------------------|------------------|------------------|
| * Methionine    | 0.44 | 0.70             | 0.86             | 1.01             | 1.25             | 1.42             |
| * Histidine     | 1.00 | 1.00             | 1.07             | 1.06             | 1.07             | 1.02             |
| * Threonine     | 1.24 | 1.25             | 1.28             | 1.25             | 1.26             | 1.24             |
| * Valine        | 1.94 | 2.00             | 1.98             | 1.90             | 1.98             | 1.99             |
| * Phenylalanine | 2.04 | 2.12             | 2.08             | 2.04             | 2.05             | 2.06             |
| * Arginine      | 3.84 | 4.02             | 3.95             | 3.91             | 3.91             | 3.93             |
| * Isoleucine    | 1.48 | 1.54             | 1.54             | 1.50             | 1.51             | 1.53             |
| * Leucine       | 2.53 | 2.63             | 2.60             | 2.54             | 2.57             | 2.58             |
| * Lysine        | 2.39 | 2.48             | 2.48             | 2.39             | 2.49             | 2.42             |
| Aspartic acid   | 4.21 | 4.44             | 4.42             | 4.28             | 4.30             | 4.37             |
| Glutamic acid   | 8.42 | 8.81             | 8.69             | 8.46             | 8.55             | 8.58             |
| Serine          | 1.54 | 1.58             | 1.56             | 1.56             | 1.56             | 1.52             |
| Glycine         | 1.68 | 1.71             | 1.73             | 1.69             | 1.72             | 1.70             |
| Alanine         | 1.60 | 1.67             | 1.66             | 1.62             | 1.64             | 1.63             |
| Taurine         | 0.05 | 0.05             | 0.07             | 0.07             | 0.07             | 0.06             |
| Tyrosine        | 1.04 | 1.06             | 1.03             | 1.01             | 1.02             | 1.00             |
| Cysteine        | 0.17 | 0.16             | 0.14             | 0.15             | 0.15             | 0.15             |
| Proline         | 1.93 | 2.02             | 1.97             | 1.80             | 1.75             | 1.90             |

Note: The data in the table were measured average values ( $n = 3$ ). \* essential amino acids. dietary CON, an experimental diet with the DL-Met concentrations of 4.4 g/kg; MET<sub>1</sub>, an experimental diet with the DL-Met concentrations of 7.0 g/kg; MET<sub>2</sub>, an experimental diet with the DL-Met concentrations of 8.6 g/kg; MET<sub>3</sub>, an experimental diet with the DL-Met concentrations of 10.1 g/kg; MET<sub>4</sub>, an experimental diet with the DL-Met concentrations of 12.5 g/kg; MET<sub>5</sub>, an experimental diet with the DL-Met concentrations of 14.2 g/kg.

### 2.3. Feeding Trial and Management

Approximately 480 healthy juvenile *M. amblycephala* with uniform body weight (initial mean weight:  $12.34 \pm 0.07$  g) were randomly allocated into six experimental groups, each comprising four replicates. The feeding trial was conducted under a controlled indoor temperature-regulated recirculating aquaculture system at the Nanquan Facility of the Freshwater Fisheries Research Center, affiliated with the Chinese Academy of Fishery Sciences (Wuxi, China). After a one-week acclimatization phase in indoor glass tanks (water capacity: 300 L), the formal 10-week feeding trial was initiated. Fish were hand-fed

to apparent satiation thrice daily (07:00, 12:00, and 17:00), allowing clear observation of the fish's feeding behavior and activity through the transparent glass walls. When the fish ceased to respond actively to the feed, feeding was stopped to ensure that all pellets were completely consumed without residue. One-third of the tank water was replaced bi-daily to maintain optimal water conditions. The water quality during the feeding trial was explained in Appendix B.1.

#### 2.4. Sample Collection

After 24 h of fasting, all fish were anesthetized using 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222) at the concentration of 100 mg/L for sampling procedures. Growth performance and feed conversion ratio were recorded based on total biomass and fish count/tank. Blood samples were obtained from three fish per tank via caudal vein puncture using 1.0 mL syringes pre-treated with sterilized sodium heparin. Plasma and liver tissues were collected and processed for biochemical, metabolomic, and molecular analyses. Dorsal muscle samples were collected post skin removal (both flanks) for nutritional components and hydrolyzed amino acids determination. Moreover, three other fish were selected from each tank for immediate liver dissection, and mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) levels were measured. The sample collection procedure was explained in Appendix B.2. Equal amounts of liver tissue from three fish originating from the same rearing glass tank were pooled, and four pooled liver samples from each treatment group were used for determining hepatic m<sup>6</sup>A levels, SAM contents and m<sup>6</sup>A methylation sequencing.

#### 2.5. Laboratory Analysis

##### 2.5.1. Growth Performance Calculation

Final body weight (FBW, g) =  $W_t/N$ ;

Weight gain rate (WGR, %) =  $100 \times (W_t - W_0)/W_0$ ;

Specific growth rate (SGR, % day<sup>-1</sup>) =  $100 \times (\ln W_t - \ln W_0)/t$ ;

Feed conversion ratio (FCR) =  $F/(W_t - W_0)$ .

Note:  $W_0$  is the total weight of each tank of fish at the beginning of the culture test (g);  $W_t$  is the total weight of each tank of fish at the end of the feeding trial (g);  $t$  is the feeding days (d);  $F$  is the total intake of feed (dry basis, g);  $N$  is the number of fish per tank at the end of the culture test.

##### 2.5.2. Assessment of Plasma Biochemical and Hepatic Antioxidant Indexes

Plasma concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and glucose (GLU) were quantified using a fully automated biochemical analyzer (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) in combination with commercially available assay kits (Shanghai Zhicheng Biotechnology Co., Ltd., Shanghai, China). The supernatants of 10% liver homogenates were collected for determining enzymatic activity of catalase (CAT) and levels of glutathione (GSH), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) via specific commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

##### 2.5.3. Determination of Hepatic MMP and ROS Production

Mitochondria were isolated, and the MMP and intracellular ROS levels were quantified using the JC-1 assay and the DCFH-DA method using commercially available kits, respectively, by the supplied instructions (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China). Fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) was employed for visualization. Intracellular ROS oxidizes the non-fluorescent DCFH to its fluorescent form (DCF), indicating oxidative stress. For MMP analysis, high membrane

potential leads to JC-1 aggregation within the mitochondrial matrix, emitting red fluorescence. However, reduced membrane potential prevents JC-1 aggregation, resulting in green fluorescence due to monomeric forms. Fluorescence intensity was quantified via Image J software (v1.8.0), and results were expressed accordingly.

#### 2.5.4. Determination of Muscle Nutritional Components

Muscle nutrient components including dry matter content, crude protein content, crude fat content, and ash content were determined with reference to the methods specified in the national standard of the atmospheric pressure drying method, Kjeldahl nitrogen fixation, Soxhlet extraction, and the calcination method of 560 °C.

#### 2.5.5. Determination of Hydrolyzed Amino Acids (HAA) in Muscle and FAA in Plasma

The amino acid composition in muscle (including taurine) were quantified after acid hydrolysis using an amino acid analyzer of Agilent 1100 (Agilent, Santa Clara, CA, USA). High-performance liquid chromatography (HPLC) was employed to measure FAA composition and content in plasma using a Waters 2695 HPLC system (Waters, Milford, MA, USA). The sample treatment procedure was explained in Appendix B.3.

#### 2.5.6. LC-MS-Based Untargeted Metabolomics Analysis of Plasma

Plasma metabolites were analyzed using LC-MS. using a Vanquish UHPLC system coupled with a mass spectrometer (Q Exactive™ HF-X, ThermoFisher Scientific, Waltham, MA, USA). The plasma metabolomics analysis procedure was explained in Appendix B.4.

#### 2.5.7. Analysis of Hepatic m<sup>6</sup>A Level and SAM Content

The EpiQuik™ m<sup>6</sup>A RNA Methylation Quantification Kit (Colorimetric; Beijing Chunye Technology Co., Ltd., Beijing, China) was used to quantify N6-methyladenosine (m<sup>6</sup>A) modifications in hepatic RNA. Liver SAM content was quantified using a fish SAM ELISA kit (Shanghai ChaoRui Biotechnology Co., Ltd., Shanghai, China). Absorbance was measured at 450 nm using a microplate reader, and SAM concentrations were calculated accordingly. The hepatic m<sup>6</sup>A level and SAM content analysis procedures were explained in Appendix B.5.

#### 2.5.8. M<sup>6</sup>A Methylation Sequencing

Trizol (Invitrogen, CA, USA) was used to extract and purify RNA from liver tissue. The Epicentre Ribo-Zero Gold Kit (Illumina, San Diego, CA, USA) was used to remove the rRNA of total RNA that met the conditions, and then the RNA was fragmented using the magnesium ion interruption Kit (NEBNext® Magnesium RNA Fragmentation Module, Ipswich, MA, USA) under high-temperature conditions. Finally, m<sup>6</sup>A-seq and RNA-seq libraries were constructed and sequenced according to the reported method [22].

#### 2.5.9. Hepatic Gene Expression Analysis

Gene expression levels in liver tissue were determined via qRT-PCR analysis [23]. Target gene transcripts were quantified using a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers were designed using the NCBI Primer Blast tool (Table A2) and synthesised by Shanghai BioEngineering Co., Ltd (Shanghai, China). *β-actin* was selected as the housekeeping gene, and relative transcription levels were analyzed using the 2<sup>−ΔΔCT</sup> method.

### 2.6. Statistical Analyses

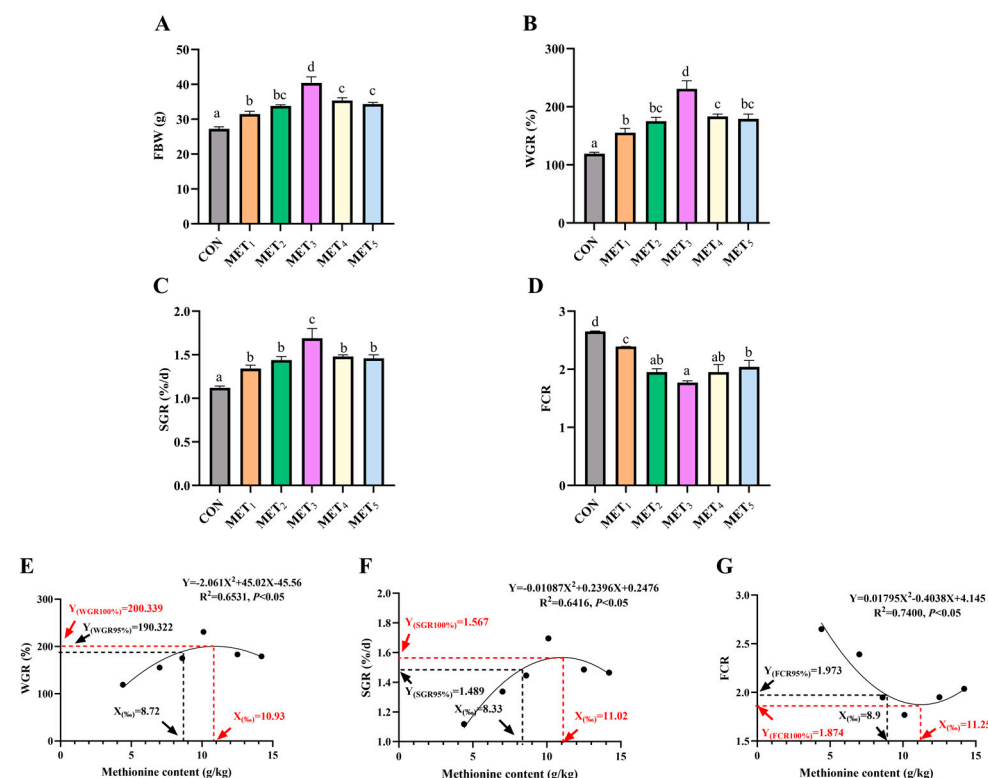
Data was statistically analyzed via SPSS 27.0 (SPSS Inc., Chicago, IL, USA). Experimental data were examined for normality using the Shapiro–Wilk test, confirming adherence to

the assumptions of normal distribution and homogeneity of variance. Substantial variations among groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test, with a significance threshold set at  $p < 0.05$ . For comparison between the CON and MET<sub>3</sub> groups, an independent samples  $t$ -test was conducted. A  $p$ -value  $< 0.05$  was depicted as statistically significant, while  $p < 0.01$  was interpreted as highly significant. General Linear Model (Univariate) was conducted to estimate effect size (the Partial Eta Squared,  $\eta^2$ ). Data are shown as mean  $\pm$  SEM.

### 3. Results

#### 3.1. Growth Performance and Feed Efficiency in Response to Dietary Met Supplementation

Dietary Met supplementation induced quadratic changes in growth performance, with FBW, WGR, and SGR increasing and then decreasing, while FCR exhibited the opposite trend. All Met-supplemented groups showed significantly higher FBW than CON, with MET<sub>3</sub> achieving the highest value ( $p < 0.05$ ,  $\eta^2 = 0.866$ ). Similarly, WGR ( $\eta^2 = 0.855$ ) and SGR ( $\eta^2 = 0.766$ ) were elevated in all Met groups, peaking in MET<sub>3</sub> ( $p < 0.05$ ). FCR decreased significantly in all Met groups compared with CON, with MET<sub>3</sub> showing the lowest value ( $p < 0.05$ ,  $\eta^2 = 0.837$ , Figure 1A–D). Quadratic regression analysis (Figure 1E–G) revealed that the optimal dietary Met inclusion level was approximately 11 g/kg. Specifically, the levels for maximizing weight gain rate (WGR) and specific growth rate (SGR) and minimizing feed conversion ratio (FCR) were 10.93 g/kg (corresponding to a maximum WGR of 200.34%), 11.02 g/kg (maximum SGR of 1.567%/d), and 11.25 g/kg (minimum FCR of 1.874), respectively.



**Figure 1.** Effects of methionine supplementation in all-plant-protein diet on growth performance and feed utilization of juvenile *M. amblycephala*. (A) final body weight; (B) weight gain rate; (C) specific growth rate; (D) feed conversion ratio; (E–G) quadratic regression fitting curve of optimal methionine content in all-plant-protein diet (WGR, SGR, FCR). The various superscripts represent remarkable differences across six groups ( $p < 0.05$ ). Effect size classification based on Partial Eta Squared ( $\eta^2$ ): Small effect:  $0.01 \leq \eta^2 < 0.06$ ; Medium effect:  $0.06 \leq \eta^2 < 0.14$ ; Strong effect:  $\eta^2 \geq 0.14$ .

### 3.2. Plasma Biochemical Indicators and Hepatic Antioxidant Profile Against Dietary Met Supplementation

Progressive addition of Met in the all-plant-protein diet was associated with a general decline in plasma ALT and LDH activities. ALT levels were remarkably reduced in the MET<sub>2</sub>, MET<sub>3</sub>, and MET<sub>5</sub> groups compared to the CON and MET<sub>1</sub> groups ( $p < 0.05$ ,  $\eta^2 = 0.311$ , Figure 2A). Similarly, LDH activities in MET<sub>2</sub> through MET<sub>5</sub> were substantially lower than those in the CON group, while MET<sub>2</sub>, MET<sub>3</sub>, and MET<sub>4</sub> showed lower values than MET<sub>1</sub> ( $p < 0.05$ ,  $\eta^2 = 0.442$ , Figure 2C). AST activity depicted a biphasic pattern, initially decreasing and increasing with elevated Met supplementation. AST levels in the MET<sub>1-4</sub> groups were statistically distinct from those in the CON and MET<sub>5</sub> groups, with the MET<sub>5</sub> group showing elevated levels relative to the CON group ( $p < 0.05$ ,  $\eta^2 = 0.624$ , Figure 2B). Plasma GLU concentrations showed a transient elevation followed by a decline as Met levels increased. The MET<sub>2</sub> group represented the highest GLU value, which was significantly higher than those in the CON, MET<sub>1</sub>, MET<sub>4</sub>, and MET<sub>5</sub> groups. The MET<sub>3</sub> group also displayed higher GLU levels than both the CON and MET<sub>5</sub> groups ( $p < 0.05$ ,  $\eta^2 = 0.442$ , Figure 2D).

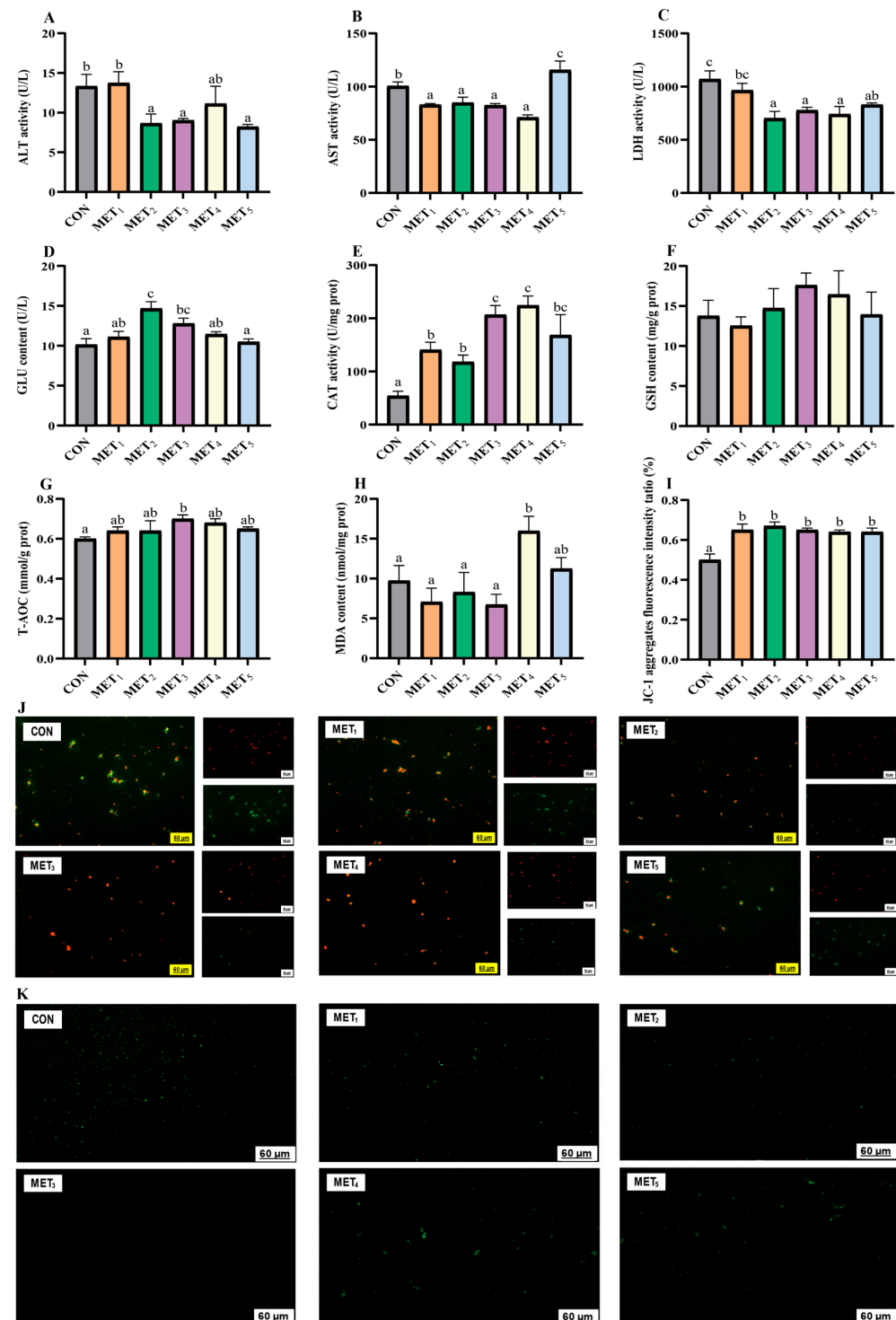
Liver antioxidant capacity was modulated by dietary Met levels, showing a biphasic response. Both CAT activity and T-AOC increased with moderate Met supplementation and declined at higher levels. Both MET<sub>3</sub> and MET<sub>4</sub> groups showed the highest CAT activity, showing significant variations relative to the CON, MET<sub>1</sub>, and MET<sub>2</sub> groups, while the MET<sub>5</sub> group also depicted significantly elevated CAT activity relative to the CON group ( $p < 0.05$ ,  $\eta^2 = 0.482$ , Figure 2E). T-AOC was maximized in the MET<sub>3</sub> group, which presented considerably higher values than the CON group ( $p < 0.05$ ;  $\eta^2 = 0.171$ , Figure 2G). MDA levels in the MET<sub>4</sub> group were remarkably elevated in comparison to CON and MET<sub>1-3</sub> groups ( $p < 0.05$ ,  $\eta^2 = 0.272$ , Figure 2H), indicating increased lipid peroxidation. No significant differences were observed in hepatic GSH levels among the experimental groups ( $p > 0.05$ ,  $\eta^2 = 0.067$ , Figure 2F), suggesting that GSH content remained stable despite variations in Met intake. The MMP levels were positively correlated with the red fluorescence/red-to-green fluorescence intensity ratio (Figure 2I,J), and compared to the CON group, the MMP levels in the Met addition groups were considerably higher ( $p < 0.05$ ). The ROS levels in the liver were positively correlated with the green fluorescence intensity, as shown in Figure 2K. With increasing Met addition, the fluorescence intensity of ROS initially decreased and then increased.

### 3.3. Changes in Muscle Nutritional Components and HAA Profiles in Response to Dietary Met Supplementation

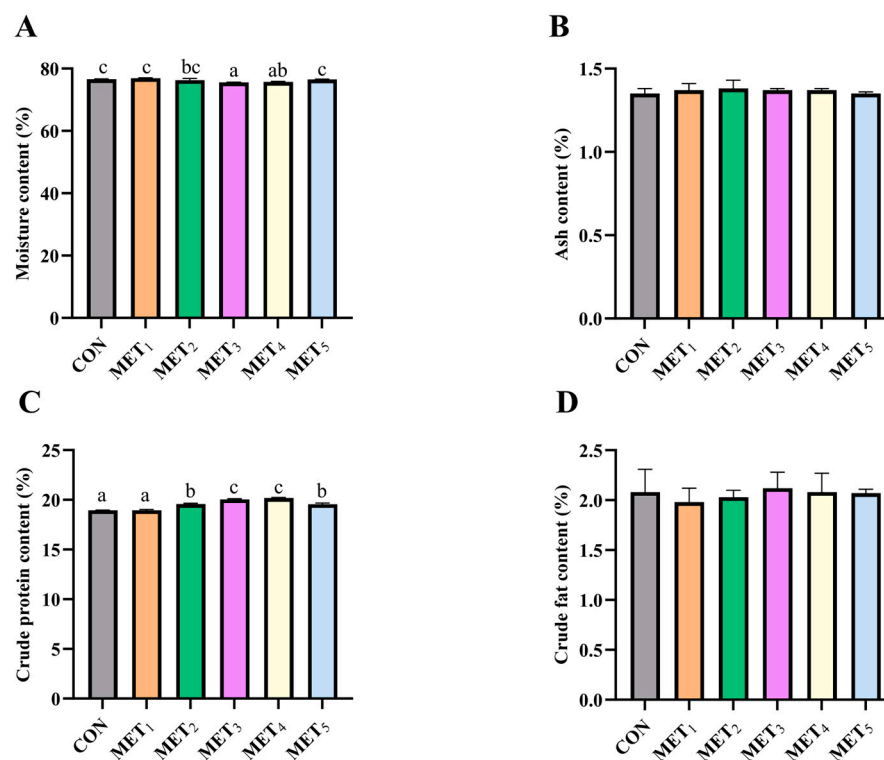
Variations in dietary Met levels exerted a differential influence on muscle composition in juvenile *M. amblycephala*. As Met concentration increased, a non-linear trend was observed in moisture and crude protein content. Muscle moisture content showed an initial decline followed by an increase. Both MET<sub>3</sub> and MET<sub>4</sub> groups showed lower moisture levels than the CON, MET<sub>1</sub>, and MET<sub>5</sub> groups, with the MET<sub>3</sub> group also showing a significant reduction relative to MET<sub>2</sub> group ( $p < 0.05$ ; Figure 3A). Crude protein content increased at moderate Met levels, initially rising and then declining with higher Met inclusion, with the MET<sub>3</sub> and MET<sub>4</sub> groups exhibiting the highest values ( $p < 0.05$ ; Figure 3C).

As presented in Table 2, the hydrolyzed Met content in muscle increased with rising dietary Met levels and reached a stable phase. The MET<sub>2-5</sub> groups showed higher Met levels compared to the CON group ( $p < 0.05$ ). Among the non-essential amino acids (NEAA), hydrolyzed serine, taurine, and cysteine concentrations initially increased and decreased with higher dietary Met levels. Serine levels were elevated in the MET<sub>3-5</sub> groups relative to the CON and MET<sub>1</sub> groups ( $p < 0.05$ ). Taurine concentration reached its highest level in the MET<sub>4</sub> group and differed from the values observed in the CON, MET<sub>1</sub>, MET<sub>2</sub>,

and MET<sub>5</sub> groups ( $p < 0.05$ ). Taurine levels in the MET<sub>3</sub> and MET<sub>5</sub> groups were also higher than that in the CON, MET<sub>1</sub>, and MET<sub>2</sub> groups ( $p < 0.05$ ). Cysteine content was higher in the MET<sub>4</sub> group and exceeded the levels in the CON and MET<sub>1</sub> groups ( $p < 0.05$ ).



**Figure 2.** Effects of methionine supplementation in all-plant-protein diet on plasma biochemical index and hepatic reactive oxygen species of juvenile *M. amblycephala*. (A) alanine aminotransferase; (B) aspartate aminotransferase; (C) lactate dehydrogenase; (D) glucose; (E) catalase; (F) glutathione; (G) total antioxidant capacity; (H) malondialdehyde; (I,J) hepatic mitochondrial membrane potential ((I) ratio of red fluorescence intensity to red and green fluorescence intensity; (J) the red fluorescence is JC-1 aggregates, the green fluorescence is JC-1 monomer); (K) hepatic reactive oxygen species in mitochondria. The various superscripts represent remarkable differences across six groups ( $p < 0.05$ ).



**Figure 3.** Effects of methionine supplementation in all-plant-protein diet on muscle nutritional components of juvenile *M. amblycephala*. (A) moisture content in muscle; (B) crude ash content in muscle; (C) crude protein content in muscle; (D) crude fat content in muscle. The various superscripts represent remarkable differences across six groups ( $p < 0.05$ ).

**Table 2.** Content of hydrolyzed amino acids in muscle of juvenile *M. amblycephala* (wet weight).

| g/100 g                        | CON                      | MET <sub>1</sub>          | MET <sub>2</sub>          | MET <sub>3</sub>          | MET <sub>4</sub>         | MET <sub>5</sub>          | $\eta^2$ |
|--------------------------------|--------------------------|---------------------------|---------------------------|---------------------------|--------------------------|---------------------------|----------|
| Essential amino acids          |                          |                           |                           |                           |                          |                           |          |
| Methionine                     | 0.20 ± 0.10 <sup>a</sup> | 0.34 ± 0.00 <sup>ab</sup> | 0.45 ± 0.01 <sup>b</sup>  | 0.41 ± 0.00 <sup>b</sup>  | 0.44 ± 0.06 <sup>b</sup> | 0.43 ± 0.07 <sup>b</sup>  | 0.549    |
| Histidine                      | 0.66 ± 0.01              | 0.66 ± 0.01               | 0.65 ± 0.01               | 0.65 ± 0.01               | 0.66 ± 0.02              | 0.65 ± 0.01               | 0.189    |
| Threonine                      | 0.76 ± 0.02              | 0.76 ± 0.01               | 0.76 ± 0.02               | 0.77 ± 0.01               | 0.77 ± 0.01              | 0.77 ± 0.02               | 0.071    |
| Valine                         | 1.02 ± 0.00              | 1.02 ± 0.01               | 0.99 ± 0.04               | 0.97 ± 0.01               | 1.00 ± 0.01              | 0.99 ± 0.02               | 0.296    |
| Phenylalanine                  | 0.86 ± 0.02              | 0.88 ± 0.01               | 0.86 ± 0.02               | 0.88 ± 0.01               | 0.88 ± 0.02              | 0.88 ± 0.02               | 0.180    |
| Arginine                       | 1.16 ± 0.06              | 1.17 ± 0.03               | 1.15 ± 0.01               | 1.13 ± 0.01               | 1.15 ± 0.04              | 1.19 ± 0.03               | 0.140    |
| Isoleucine                     | 0.90 ± 0.03              | 0.91 ± 0.01               | 0.91 ± 0.02               | 0.92 ± 0.01               | 0.92 ± 0.02              | 0.91 ± 0.02               | 0.060    |
| Leucine                        | 1.51 ± 0.04              | 1.55 ± 0.03               | 1.53 ± 0.04               | 1.55 ± 0.02               | 1.56 ± 0.03              | 1.55 ± 0.03               | 0.141    |
| Lysine                         | 1.72 ± 0.06              | 1.77 ± 0.02               | 1.74 ± 0.05               | 1.80 ± 0.03               | 1.79 ± 0.04              | 1.80 ± 0.05               | 0.191    |
| Nonessential amino acids       |                          |                           |                           |                           |                          |                           |          |
| Aspartic acid                  | 2.25 ± 0.06              | 2.29 ± 0.04               | 2.26 ± 0.04               | 2.34 ± 0.01               | 2.28 ± 0.05              | 2.28 ± 0.06               | 0.161    |
| Glutamic acid                  | 3.12 ± 0.11              | 3.16 ± 0.06               | 3.13 ± 0.08               | 3.18 ± 0.01               | 3.20 ± 0.08              | 3.18 ± 0.09               | 0.071    |
| Serine                         | 0.65 ± 0.02 <sup>a</sup> | 0.66 ± 0.01 <sup>a</sup>  | 0.68 ± 0.01 <sup>ab</sup> | 0.70 ± 0.01 <sup>b</sup>  | 0.71 ± 0.01 <sup>b</sup> | 0.70 ± 0.01 <sup>b</sup>  | 0.625    |
| Glycine                        | 0.95 ± 0.03              | 0.94 ± 0.03               | 0.97 ± 0.01               | 0.95 ± 0.03               | 0.91 ± 0.03              | 1.01 ± 0.05               | 0.301    |
| Alanine                        | 1.15 ± 0.04              | 1.15 ± 0.03               | 1.15 ± 0.01               | 1.15 ± 0.01               | 1.16 ± 0.02              | 1.18 ± 0.02               | 0.067    |
| Taurine                        | 0.30 ± 0.01 <sup>a</sup> | 0.30 ± 0.01 <sup>a</sup>  | 0.31 ± 0.00 <sup>a</sup>  | 0.36 ± 0.01 <sup>bc</sup> | 0.37 ± 0.01 <sup>c</sup> | 0.35 ± 0.01 <sup>b</sup>  | 0.876    |
| Tyrosine                       | 0.47 ± 0.04              | 0.49 ± 0.02               | 0.62 ± 0.01               | 0.60 ± 0.03               | 0.62 ± 0.04              | 0.61 ± 0.03               | 0.680    |
| Cysteine                       | 0.04 ± 0.00 <sup>a</sup> | 0.05 ± 0.00 <sup>a</sup>  | 0.06 ± 0.01 <sup>ab</sup> | 0.05 ± 0.01 <sup>ab</sup> | 0.07 ± 0.01 <sup>b</sup> | 0.06 ± 0.01 <sup>ab</sup> | 0.531    |
| Proline                        | 0.61 ± 0.03              | 0.62 ± 0.02               | 0.65 ± 0.02               | 0.61 ± 0.02               | 0.64 ± 0.02              | 0.67 ± 0.02               | 0.365    |
| Total amino acids              | 18.34 ± 0.66             | 18.74 ± 0.26              | 18.87 ± 0.35              | 19.02 ± 0.13              | 19.14 ± 0.43             | 19.22 ± 0.45              | 0.200    |
| Total essential amino acids    | 7.63 ± 0.27              | 7.90 ± 0.09               | 7.89 ± 0.20               | 7.95 ± 0.10               | 8.03 ± 0.18              | 7.97 ± 0.22               | 0.147    |
| Total nonessential amino acids | 10.70 ± 0.40             | 10.84 ± 0.18              | 10.98 ± 0.15              | 11.06 ± 0.03              | 11.12 ± 0.26             | 11.25 ± 0.25              | 0.270    |

Note: the various superscripts represent remarkable differences across six groups ( $p < 0.05$ ).  $\eta^2$ , Partial Eta Squared. A higher partial eta-squared indicates a larger magnitude of difference or effect, and the commonly cited thresholds for small, medium, and large effects are approximately 0.01, 0.06, and 0.14.

### 3.4. Changes in Plasma FAA Profiles in Response to Dietary Met Supplementation

As presented in Table 3, no differences were observed in plasma free Met levels among the groups. However, the levels of histidine, threonine, and arginine were affected by

dietary Met content. Dietary Met supplementation induced distinct alterations in the plasma concentrations of FAAs in juvenile *M. amblycephala*. A progressive increase in plasma taurine levels was observed with increasing Met levels, with the MET<sub>4</sub> and MET<sub>5</sub> groups showing significantly elevated concentrations relative to the CON and MET<sub>1</sub> groups ( $p < 0.05$ ). Plasma serine levels demonstrated a biphasic trend, increasing initially and declining. The MET<sub>2</sub> and MET<sub>3</sub> groups displayed the highest serine concentrations, which were statistically greater than those in the CON, MET<sub>1</sub>, and MET<sub>5</sub> groups ( $p < 0.05$ ), while the MET<sub>4</sub> group also showed elevated levels relative to CON and MET<sub>1</sub> ( $p < 0.05$ ).

In addition, the levels of plasma free histidine and free threonine were increased across all Met-supplemented groups ( $p < 0.05$ ), while the arginine level in the MET<sub>3</sub> group was significantly higher than in MET<sub>4</sub> and MET<sub>5</sub> ( $p < 0.05$ ). Glycine content was significantly higher in the MET<sub>1</sub> group than in the CON, MET<sub>2</sub>, and MET<sub>3</sub> groups ( $p < 0.05$ ). Aspartic acid concentration was significantly reduced in the MET<sub>5</sub> group compared to all other treatments ( $p < 0.05$ ). In case of glutamic acid, plasma concentrations were significantly elevated in the MET<sub>3</sub> and MET<sub>4</sub> groups in comparison to other groups ( $p < 0.05$ ). The total FAA levels in all Met-treated groups (MET<sub>1–5</sub>) were significantly elevated in contrast to the CON group ( $p < 0.05$ ). A comparable trend was observed for plasma EAA, where MET<sub>1–4</sub> groups demonstrated increased concentrations relative to the CON and MET<sub>5</sub> groups ( $p < 0.05$ ).

**Table 3.** Content of free amino acids in plasma of juvenile *M. amblycephala*.

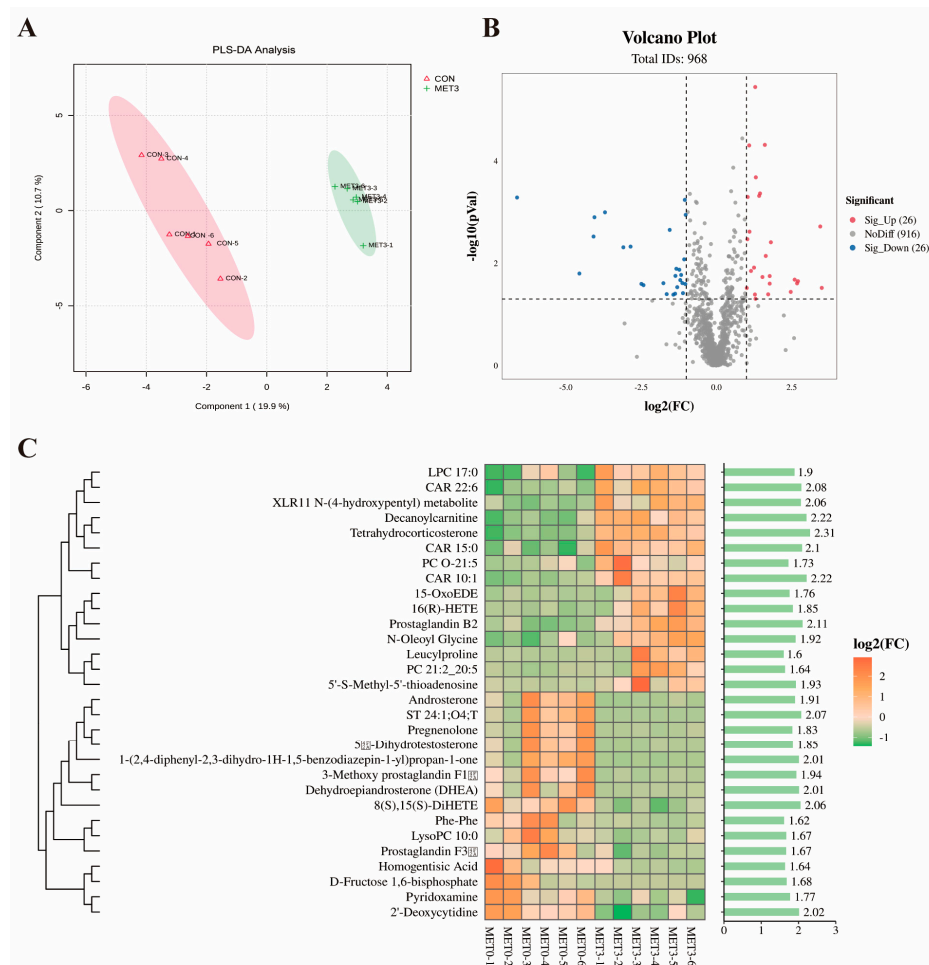
| g/L                            | CON                        | MET <sub>1</sub>            | MET <sub>2</sub>             | MET <sub>3</sub>            | MET <sub>4</sub>            | MET <sub>5</sub>             | $\eta^2$ |
|--------------------------------|----------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|------------------------------|----------|
| Essential amino acids          |                            |                             |                              |                             |                             |                              |          |
| Methionine                     | 21.00 ± 2.00               | 21.33 ± 2.40                | 20.33 ± 2.02                 | 18.00 ± 1.73                | 20.00 ± 2.00                | 15.00 ± 1.53                 | 0.382    |
| Histidine                      | 89.67 ± 4.10 <sup>a</sup>  | 111.67 ± 0.67 <sup>b</sup>  | 115.00 ± 0.00 <sup>b</sup>   | 113.00 ± 6.35 <sup>b</sup>  | 118.33 ± 1.45 <sup>b</sup>  | 97.00 ± 6.93 <sup>a</sup>    | 0.751    |
| Threonine                      | 25.50 ± 0.29 <sup>a</sup>  | 36.00 ± 2.31 <sup>c</sup>   | 29.67 ± 0.33 <sup>b</sup>    | 30.67 ± 0.88 <sup>b</sup>   | 31.00 ± 0.00 <sup>b</sup>   | 32.67 ± 0.88 <sup>bc</sup>   | 0.809    |
| Valine                         | 35.67 ± 2.73               | 33.67 ± 1.20                | 36.33 ± 2.60                 | 31.33 ± 0.88                | 33.00 ± 0.58                | 32.67 ± 0.67                 | 0.342    |
| Phenylalanine                  | 19.00 ± 0.58               | 21.33 ± 1.33                | 21.50 ± 0.86                 | 19.33 ± 0.33                | 21.00 ± 0.00                | 20.00 ± 0.58                 | 0.463    |
| Arginine                       | 52.67 ± 1.76 <sup>ab</sup> | 50.67 ± 3.18 <sup>ab</sup>  | 52.67 ± 4.41 <sup>ab</sup>   | 57.67 ± 3.18 <sup>b</sup>   | 42.00 ± 2.08 <sup>a</sup>   | 44.33 ± 3.93 <sup>a</sup>    | 0.575    |
| Isoleucine                     | 18.00 ± 0.58               | 17.33 ± 0.33                | 18.67 ± 0.88                 | 17.67 ± 0.33                | 18.33 ± 0.67                | 19.33 ± 0.67                 | 0.368    |
| Leucine                        | 32.67 ± 1.20               | 32.33 ± 0.88                | 36.33 ± 2.91                 | 31.67 ± 1.33                | 37.00 ± 2.08                | 35.00 ± 0.58                 | 0.425    |
| Lysine                         | 40.33 ± 0.33               | 42.00 ± 1.53                | 43.33 ± 0.33                 | 42.33 ± 0.33                | 37.67 ± 0.88                | 38.00 ± 4.00                 | 0.417    |
| Nonessential amino acids       |                            |                             |                              |                             |                             |                              |          |
| Aspartic acid                  | 24.67 ± 1.20 <sup>b</sup>  | 24.00 ± 1.53 <sup>b</sup>   | 21.00 ± 0.58 <sup>b</sup>    | 23.67 ± 0.88 <sup>b</sup>   | 24.00 ± 0.58 <sup>b</sup>   | 17.00 ± 1.53 <sup>a</sup>    | 0.740    |
| Glutamic acid                  | 52.67 ± 0.88 <sup>c</sup>  | 47.67 ± 1.76 <sup>bc</sup>  | 26.67 ± 3.18 <sup>a</sup>    | 63.00 ± 0.58 <sup>d</sup>   | 62.50 ± 0.87 <sup>d</sup>   | 41.50 ± 5.48 <sup>b</sup>    | 0.913    |
| Serine                         | 3.67 ± 0.88 <sup>a</sup>   | 5.33 ± 1.86 <sup>a</sup>    | 13.67 ± 1.45 <sup>c</sup>    | 16.33 ± 2.73 <sup>c</sup>   | 11.33 ± 0.67 <sup>bc</sup>  | 7.00 ± 0.58 <sup>ab</sup>    | 0.811    |
| Glycine                        | 17.66 ± 0.88 <sup>ab</sup> | 21.33 ± 0.33 <sup>c</sup>   | 16.33 ± 0.3 <sup>a</sup>     | 16.33 ± 1.86 <sup>a</sup>   | 18.33 ± 0.33 <sup>abc</sup> | 20.00 ± 1.15 <sup>bc</sup>   | 0.632    |
| Alanine                        | 43.00 ± 2.65               | 46.00 ± 1.00                | 41.67 ± 2.40                 | 38.67 ± 2.03                | 38.67 ± 1.33                | 44.33 ± 1.45                 | 0.507    |
| Taurine                        | 156.33 ± 2.02 <sup>a</sup> | 165.00 ± 6.93 <sup>a</sup>  | 168.67 ± 11.35 <sup>ab</sup> | 174.33 ± 1.20 <sup>ab</sup> | 189.00 ± 7.94 <sup>b</sup>  | 190.00 ± 3.00 <sup>b</sup>   | 0.640    |
| Tyrosine                       | 30.00 ± 2.65               | 34.00 ± 0.58                | 33.67 ± 2.33                 | 31.67 ± 1.86                | 34.00 ± 1.73                | 31.33 ± 1.20                 | 0.255    |
| Cysteine                       | 4.00 ± 1.00                | 4.67 ± 1.76                 | 2.67 ± 1.76                  | 4.67 ± 0.33                 | 4.67 ± 1.33                 | 4.33 ± 0.33                  | 0.142    |
| Proline                        | 9.00 ± 0.58                | 13.00 ± 3.78                | 12.00 ± 2.52                 | 11.33 ± 1.45                | 11.33 ± 0.33                | 12.00 ± 1.00                 | 0.157    |
| Total amino acids              | 682.17 ± 2.89 <sup>a</sup> | 737.00 ± 9.29 <sup>bc</sup> | 720.67 ± 10.74 <sup>b</sup>  | 753.00 ± 5.03 <sup>c</sup>  | 762.17 ± 11.97 <sup>c</sup> | 709.83 ± 8.92 <sup>b</sup>   | 0.673    |
| Total essential amino acids    | 290.50 ± 5.48 <sup>a</sup> | 325.33 ± 3.84 <sup>b</sup>  | 331.67 ± 8.41 <sup>b</sup>   | 315.33 ± 4.26 <sup>b</sup>  | 326.33 ± 5.24 <sup>b</sup>  | 298.00 ± 5.00 <sup>a</sup>   | 0.835    |
| Total nonessential amino acids | 391.67 ± 2.60 <sup>a</sup> | 411.67 ± 6.89 <sup>ab</sup> | 389.00 ± 9.87 <sup>a</sup>   | 437.67 ± 4.33 <sup>b</sup>  | 435.83 ± 8.66 <sup>b</sup>  | 411.82 ± 13.18 <sup>ab</sup> | 0.610    |

Note: The various superscripts represent remarkable differences across six groups ( $p < 0.05$ ).  $\eta^2$ , Partial Eta Squared. A higher partial eta-squared indicates a larger magnitude of difference or effect, and the commonly cited thresholds for small, medium, and large effects are approximately 0.01, 0.06, and 0.14.

### 3.5. Plasma Metabolomic Analysis

Figure 4A shows that partial least squares discriminant analysis (PLS-DA) revealed distinct clustering and separation of plasma metabolites between the CON and MET<sub>3</sub> groups, indicating clear metabolic differences. As shown in the volcano plot (Figure 4B), approximately 52 differential metabolites were detected in the MET<sub>3</sub> group relative to the CON group, with 26 metabolites showing increased and 26 revealing decreased levels.

Based on the absolute value of  $\log_2(\text{MET}_3/\text{CON})$ , the Heatmap analysis of differential metabolites identified 30 differential metabolites between the  $\text{MET}_3$  and CON groups, including the top 14 metabolites with significantly increased levels and the top 16 metabolites with significantly decreased levels. As shown in Figure 4C, the metabolites with significantly increased levels in the  $\text{MET}_3$  group's plasma included tetrahydrocorticosterone, 16(R)-hydroxyeicosatetraenoic acid (16(R)-HETE), prostaglandin B2, leucylproline, and 5'-S-methyl-5'-thioadenosine (MTA) ( $p < 0.05$ ), while the metabolites with significantly decreased levels included androsterone, dehydroepiandrosterone (DHEA), lysophosphatidylcholine (LysoPC), prostaglandin F3 $\beta$ , and homogentisic acid ( $p < 0.05$ ).

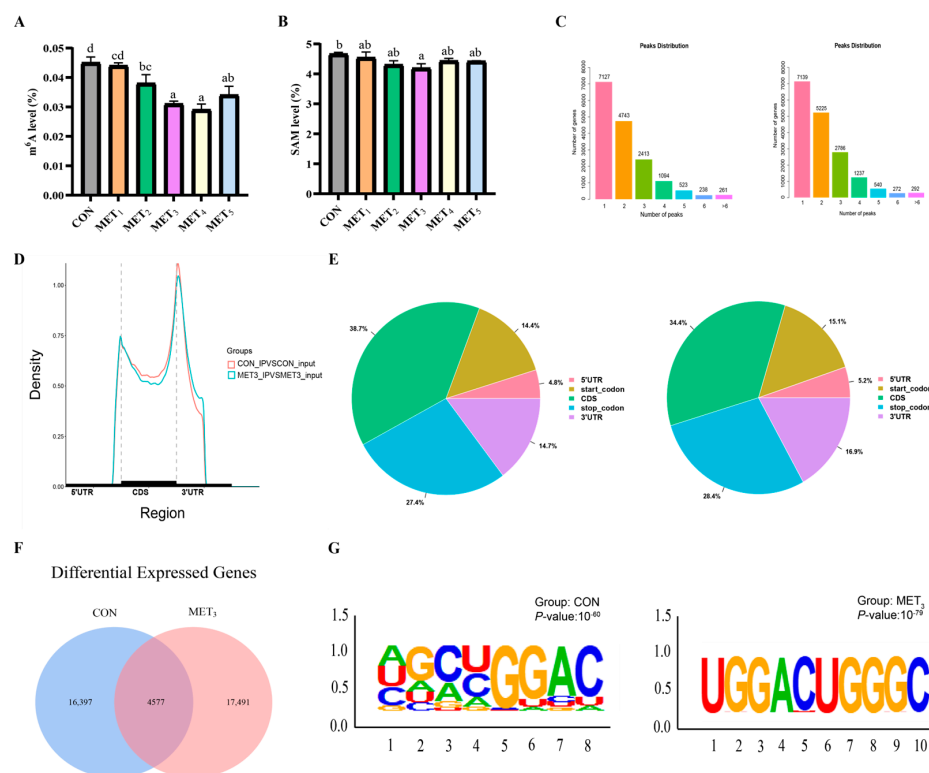


**Figure 4.** Plasma metabolomic analysis between CON and  $\text{MET}_3$  groups. (A) partial least squares discriminant (PLS-DA) analysis: pink represents CON group and green represents  $\text{MET}_3$  group; (B) volcanic map: red indicates significant increase, blue indicates significant decrease, and gray indicates no significant difference; (C) differential metabolite heatmap and VIP bar graphs: the higher the VIP value, the greater the contribution of metabolites to sample classification.

### 3.6. Hepatic $m^6\text{A}$ Methylation Analysis

An upward adjustment in dietary Met levels was associated with a non-linear pattern in hepatic  $m^6\text{A}$  methylation and SAM content in juvenile *M. amblycephala*, characterized by an initial decline followed by an elevation. The  $m^6\text{A}$  levels in the  $\text{MET}_3$  and  $\text{MET}_4$  groups were lower than those in the CON,  $\text{MET}_1$ , and  $\text{MET}_2$  groups, while the  $\text{MET}_5$  group showed reduced levels relative to the CON and  $\text{MET}_1$  groups. Furthermore, the  $\text{MET}_2$  group revealed decreased  $m^6\text{A}$  levels in comparison to the CON group ( $p < 0.05$ ; Figure 5A). Hepatic SAM content in the  $\text{MET}_3$  group was also lower than that in the CON group ( $p < 0.05$ ; Figure 5B). The gene-wide distribution of  $m^6\text{A}$  peaks in the CON group

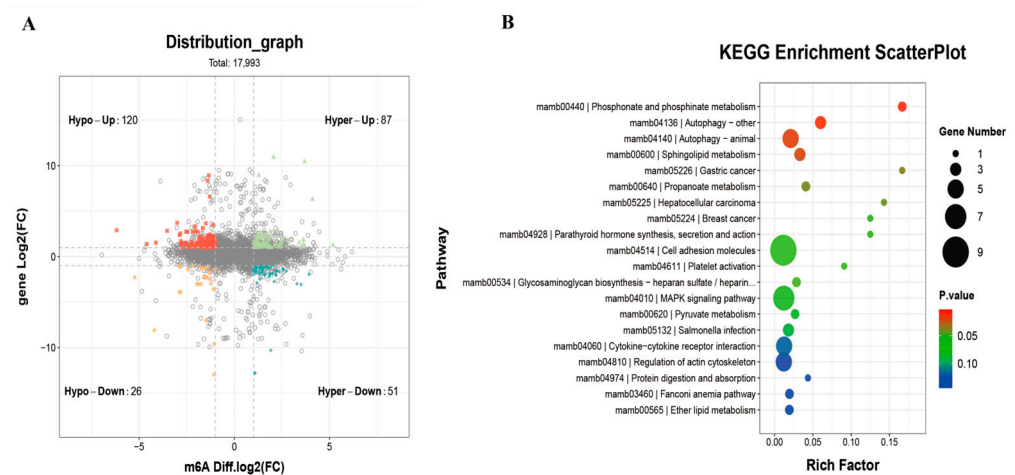
revealed that 43% of genes harbored a single m<sup>6</sup>A modification site, and 87% possessed between one and three sites. A comparable distribution pattern was identified in the MET<sub>3</sub> group, where 40% of genes contained a single site and 86% contained one to three sites (Figure 5C). Enrichment analysis of m<sup>6</sup>A peak locations demonstrated a preferential accumulation at the 3' UTR initiation sites and the 5' UTR terminal sites. The coding sequence (CDS) regions displayed a relatively lower peak density. Compared to the CON group, a reduction in m<sup>6</sup>A peak density at the 3' UTR start region was observed in the MET<sub>3</sub> group (Figure 5D). The proportional distribution of m<sup>6</sup>A methylation sites in the liver of the CON group was as follows: 38.7% in the CDS region, 27.4% in the terminator region, 14.4% in the start codon region, 14.7% in the 3' UTR, and 4.8% in the 5' UTR. In the MET<sub>3</sub> group, the proportions were 34.4%, 28.4%, 15.1%, 16.9%, and 5.2%, respectively (Figure 5E). Approximately 20,974 and 22,068 m<sup>6</sup>A methylation sites were identified in the CON and MET<sub>3</sub> groups, respectively, with 4577 sites shared between both groups, as illustrated in Figure 5F. Motif analysis revealed the presence of the common m<sup>6</sup>A motif GGAC in both the CON and MET<sub>3</sub> groups, consistent with the typical m<sup>6</sup>A modification pattern (Figure 5G).



**Figure 5.** Hepatic m<sup>6</sup>A methylation analysis between CON and MET<sub>3</sub> groups. For MeRIP-Seq, m<sup>6</sup>A peaks were identified with exomePeak2 (v1.12.0;  $p < 1 \times 10^{-5}$ ) and annotated by ANNOVAR (Ver. 20240617). Significant peaks met  $|\log_2FC| > 1$  and  $FDR < 0.05$ . Motifs were discovered with HOMER (v4.11) and visualized via ggseqlogo/ggplot2. Differential peaks were analyzed with DiffBind (v2.8.0). For RNA-Seq, transcripts were assembled with StringTie (v1.3.4), quantified with RSEM (v1.2.19), and DEGs identified with DESeq2 (v1.20.0;  $|\log_2FC| \geq 2$ ,  $FDR < 0.05$ ). (A) m<sup>6</sup>A level, the various superscripts represent remarkable differences across six groups ( $p < 0.05$ ); (B) S-adenosyl methionine content, the various superscripts represent remarkable differences across six groups ( $p < 0.05$ ); (C) the number of m<sup>6</sup>A modification sites of CON (left) and MET<sub>3</sub> (right) group genes; (D) differences in the cumulative distribution of m<sup>6</sup>A peaks in the CON and MET<sub>3</sub> groups; (E) distribution position of m<sup>6</sup>A peaks in CON (left) and MET<sub>3</sub> (right) groups; (F) Venn diagram of m<sup>6</sup>A modification sites in mRNAs of CON and MET<sub>3</sub> groups; (G) analysis and comparison of m<sup>6</sup>A motif enrichment in mRNAs of CON and MET<sub>3</sub> groups.

### 3.7. Combined Analysis of m<sup>6</sup>A-Seq and RNA-Seq

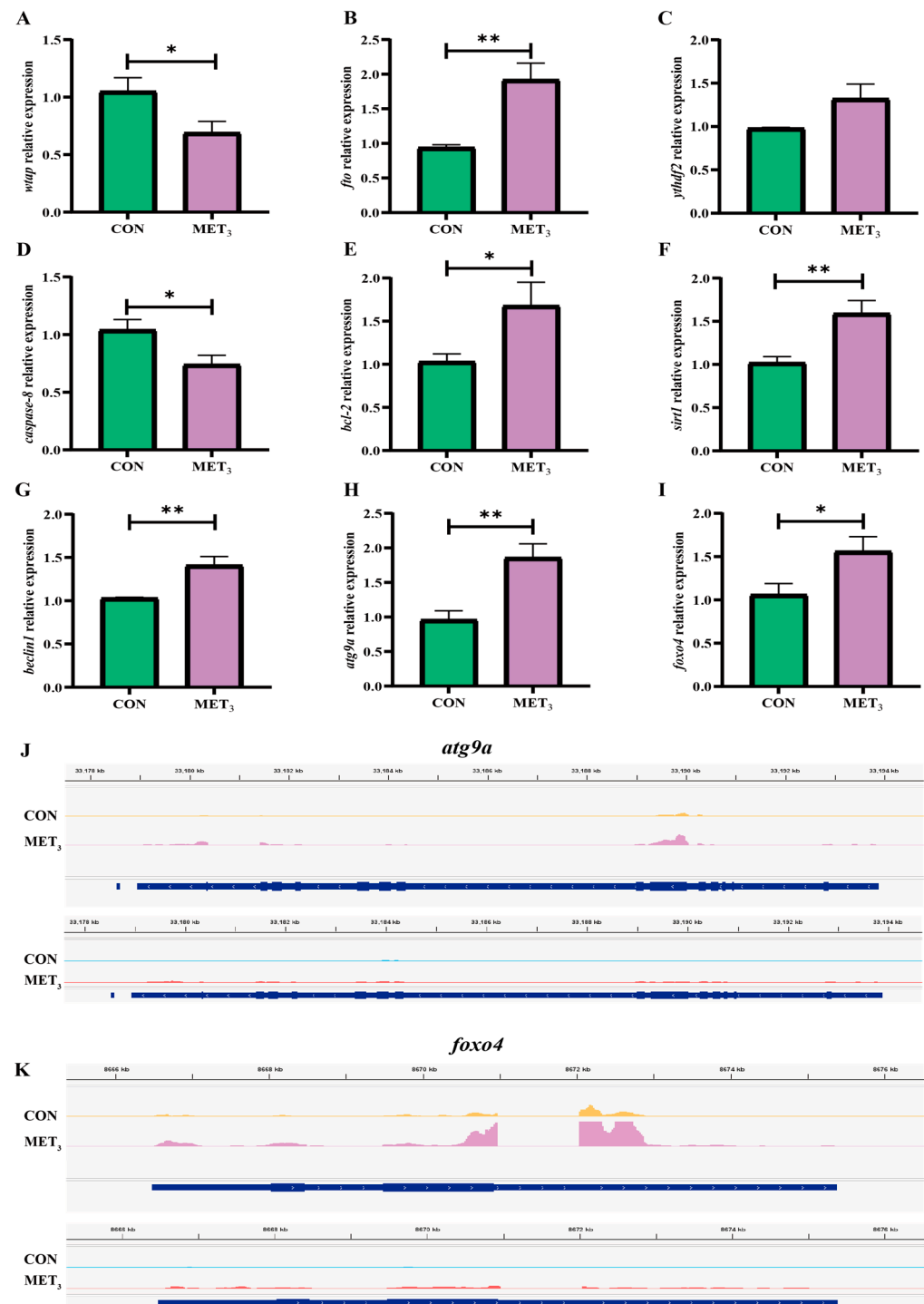
A combined analysis of differentially modified m<sup>6</sup>A methylation genes and differentially expressed mRNAs between the CON and MET<sub>3</sub> groups revealed 284 genes with similar changes in both m<sup>6</sup>A methylation and transcriptional expression (Figure 6A). These genes were classified into four regulatory categories: a subset of 87 genes, including *casp3b*, *foxo4*, and *atg9a*, showed upregulation of m<sup>6</sup>A methylation and mRNA expression (Hyper-up); 51 genes, such as *cep112*, *apba1b*, and *eva1ba*, showed increased m<sup>6</sup>A methylation along with reduced mRNA expression (Hyper-down); 120 genes, including *csdc2b*, *marchf2*, and *bach1b*, demonstrated decreased m<sup>6</sup>A methylation alongside elevated mRNA expression (Hypo-up); and 26 genes, such as *mapta*, *cadpsb*, and *cracdlb*, displayed downregulation of both m<sup>6</sup>A methylation and mRNA expression (Hypo-down). KEGG pathway enrichment analysis (Figure 6B) indicated that these overlapping genes were primarily enriched in pathways related to autophagy (Autophagy-Other; Autophagy-Animal), sphingolipid metabolism, hepatocellular carcinoma, and MAPK signaling.



**Figure 6.** Association analysis between differential m<sup>6</sup>A methylation modified genes and differential RNA transcription and expression genes. Functional enrichment of peak-related genes and DEGs was performed using GO.db (v3.14.0) and KEGG (Release 101; adjusted  $p < 0.05$ ). Differential expression and m<sup>6</sup>A peaks were further analyzed with edgeR (v4.4.1;  $|\log_2FC| > 1$ ,  $p < 0.05$ ). (A) four quadrant diagram of association analysis between differential m<sup>6</sup>A methylation modified genes and differential RNA transcription and expression genes; (B) KEGG richness analysis of differential expressing genes by joint m<sup>6</sup>A-seq and RNA-seq analysis.

### 3.8. Expression of Regulatory Genes Related to Liver Methylation and Inflammatory Injury

Compared to the CON group, the expression of the methyltransferase *watp* in the liver of juveniles in the MET<sub>3</sub> group was significantly downregulated ( $p < 0.05$ , Figure 7A), while the expression of the demethylase *fto* was upregulated ( $p < 0.01$ , Figure 7B). There were no substantial variations in the expression of *ythdf2* ( $p > 0.05$ , Figure 7C). In comparison to the CON group, the expression of *caspase-8* in the liver of juveniles in the MET<sub>3</sub> group was significantly downregulated ( $p < 0.05$ , Figure 7D). However, the expressions of *bcl-2* and *foxo4* were upregulated ( $p < 0.05$ , Figure 7E,I), and the expressions of *sirt1*, *beclin1*, and *atg9a* were extremely upregulated ( $p < 0.01$ , Figure 7F–H). IGV visualization analysis showed the m<sup>6</sup>A peak expression patterns of *atg9a* and *foxo4*. High methylation occurred in the 3' UTR region of *atg9a* and *foxo4*, resulting in changes in gene expression. The expression of *atg9a* and *foxo4* in the MET<sub>3</sub> group was substantially higher than in the CON group (Figure 7J,K).



**Figure 7.** Expression of regulatory genes related to liver methylation and inflammatory injury. (A) wilms tumor1-associated protein; (B) fat mass and obesity-associated protein; (C) YTH domain family member2; (D) cysteine aspartate-specific protease 8; (E) B-cell lymphoma-2; (F) silent information regulator 1; (G) benzyl chloride 1; (H) autophagy-related protein 9a; (I) forkhead box protein O4; (J,K) peak abundance of m<sup>6</sup>A (IP, yellow and peak) and expression (Input, blue and red) in *atg9a* and *foxo4* in liver of CON and MET<sub>3</sub> groups. \* means the significant difference between the two groups (Independent *t*-test, *p* < 0.05), and \*\* means the extremely significant difference between the two groups (Independent *t*-test, *p* < 0.01).

## 4. Discussion

### 4.1. Effect of Different Met Levels in Plant-Based Diets on Growth Performance and Antioxidant Capacity

Amino acids are essential for protein synthesis and play a critical role in the growth and development of aquatic species. Appropriate dietary methionine (Met) enhances growth in juvenile *M. amblycephala* under low fishmeal diets, whereas excessive supplementation may inhibit growth [24], a pattern also observed in grouper (*Epinephelus coioides*) [25], grass carp (*Ctenopharyngodon Idella*) [26], and black sea bream (*Sparus macrocephalus*) [27]. Consistent with these findings, Met supplementation in plant-based diets improved growth and reduced feed conversion ratio in this study. Previous studies reported that juvenile *M. amblycephala* of two sizes groups ( $3.34 \pm 0.03$  g and  $101.80 \pm 1.30$  g) require 8.4–8.5 g/kg Met or 7.4–7.6 g/kg in low fishmeal diets [20,21]. Based on secondary regression analysis, the estimated dietary Met requirement for achieving optimal WGR, SGR, and minimum FCR in juvenile *M. amblycephala* fed all-plant-protein diets was approximately 11 g/kg. These values were higher than typical levels observed in fishmeal-based diets, reflecting the relatively low Met content of plant-derived protein sources. Plasma ALT, AST, and LDH are important indicators of liver health. Higher levels of ALT, AST, and LDH suggest that the liver may be experiencing oxidative stress, leading to functional impairment [23]. Studies have reported that Met can alleviate the increase in ALT and AST levels in the plasma of ricefield eel (*Monopterus albus*) caused by feeding low-protein diets and improve liver damage induced by low-protein diets [28]. The present study suggested that appropriate Met supplementation (8.6 g/kg and 10.1 g/kg) enhanced hepatic function and improved antioxidant capacity in fish fed plant-based protein diets, as indicated by reduced plasma ALT, AST, and LDH levels.

ROS continuously accumulate in mitochondria under oxidative stress, leading to cell damage [29]. They are primarily generated by mitochondrial complex I and III, with the production sites affected by MMP [30]. MMP plays an essential role in cellular homeostasis as it drives the ATP production in organelles [31]. Aberrant MMP, whether elevated or reduced, has been associated with mitochondrial dysfunction, promoting cellular apoptosis and necrosis [32]. Met restriction has been demonstrated to induce pronounced mitochondrial impairment in human osteosarcoma cells, characterized by a clear reduction in MMP and elevation in ROS generation [33]. In this study, Met supplementation increased the red fluorescence and red-to-green fluorescence ratio in the mitochondrial matrix of juvenile *M. amblycephala* livers, indicating that MMP became more stable. Similarly, fluorescence detection of ROS in the liver showed that Met supplementation in plant-based diets reduced the liver's ROS content, especially when the Met level was 10.1 g/kg, which alleviated oxidative stress damage and maintained MMP stability in juvenile *M. amblycephala*. The findings were consistent with hepatic antioxidant enzyme analysis, indicating that dietary Met supplementation in plant-based formulations enhanced liver antioxidant enzyme activities in juvenile *M. amblycephala*. Importantly, 10.1 g/kg supplementation levels were associated with elevated CAT activity and T-AOC. Similarly, Met supplementation has been reported to reduce hepatic oxidative stress in large yellow croaker (*Larimichthys crocea*) subjected to high-fat diets by decreasing MDA concentrations and enhancing T-AOC, thus highlighting hepatic antioxidant defenses [34]. These findings revealed that methionine enhances antioxidant capacity by influencing the SAM/SAH levels and regulating the expressions and activities of antioxidant genes. Simultaneously, elevated methionine promotes the trans-sulfuration pathway, increasing cysteine and glutathione (GSH) levels. Together, these mechanisms link methionine nutrition to epigenetic regulation, mitochondrial performance, and redox homeostasis. Furthermore, this study observed that when the Met content in plant-based diets was high ( $\geq 12.5$  g/kg), a large amount of MDA was generated in the

liver. This phenomenon has been reported in both mammals and aquatic animals, where excessive Met causes damage to rats, increasing ROS content in liver mitochondria and damaging mitochondrial DNA [35]; excessive Met in yellow catfish (*Pelteobagrus fulvidraco*) diets also induces cell apoptosis [36].

#### 4.2. Effects of Different Met Levels in Plant-Based Protein Diets on Muscle Nutrient Composition and Blood Metabolite Profiles of Juvenile *M. amblycephala*

Met functions as the initiating amino acid during protein translation and serves as a rate-limiting factor in protein biosynthesis [37]. Adequate Met supplementation in plant-based protein diets has been shown to enhance muscle crude protein content in species such as hybrid crucian carp (*Carassius auratus gibelio*) and Nile tilapia (*Oreochromis niloticus*) [5,38]. In this study, it was similarly found that when the Met content in plant-based protein diets ranged from 8.6 g/kg to 14.2 g/kg, the protein content in the muscles of juvenile *M. amblycephala* substantially increased, suggesting that Met is involved in promoting muscle protein synthesis in juvenile *M. amblycephala*. Further examination of muscle amino acid composition demonstrated a progressive elevation in Met, taurine, cysteine, and serine concentrations with increasing dietary Met levels. Comparable trends have been reported in other teleost species; for instance, dietary Met supplementation elevated the intramuscular concentrations of free Met, cysteine, and serine in *C. idella* [26]. In studies of *C. auratus gibelio*, supplementing Met in low-fishmeal diets improved cysteine deposition in juvenile fish [39]. Furthermore, dietary Met concentration significantly influenced taurine accumulation in *C. carpio* muscle, showing a positive correlation with increased Met levels [11]. This effect may be attributed to the metabolic interconnection among sulfur-containing amino acids, as both taurine and cysteine are involved in the metabolic pathways of Met within the organism. Moreover, Met generates homocysteine through transmethylation, and in the homocysteine metabolic pathway, serine, through remethylation, provides methyl groups for Met synthesis and condenses with homocysteine to form cystathionine [40].

The FAA content in plasma is an important indicator reflecting the protein status of the animal body, as well as amino acid metabolism and consumption [9]. In the present study, elevated dietary Met levels facilitated the accumulation of free taurine in plasma. This observation suggests that the supplemented Met may have undergone metabolic conversion to taurine via the trans-sulfuration pathway in juvenile *M. amblycephala*. Similarly, supplementing Met in the plant-based protein diet of *O. niloticus* also increased the plasma taurine content, although the change was not statistically significant [41]. The Met level affects the content of most FAAs in plasma in the diet [42]. In this study, supplementing Met in the plant-based protein diet also caused changes in the plasma levels of histidine, threonine, and other amino acids in juveniles. This may be related to the excess or disproportionate intake of Met, which affects the utilization of other amino acids [43,44]. Moreover, this study found that appropriate Met supplementation could increase the total content of FAA, EAA, and NEAA in the plasma of juvenile *M. amblycephala*. However, the plasma Met content did not increase with the higher level of Met in the diet. Considering the observed elevation in hydrolyzed Met levels in muscle tissue, it is suggested that Met metabolism varies between blood and muscle. Met is primarily used for protein synthesis within muscle tissue and is metabolized into taurine through the trans-sulfuration pathway in the bloodstream and muscle.

Metabolites are small molecular compounds produced or consumed through metabolic processes in organisms, playing vital roles in energy supply, metabolic regulation, immune regulation, and other functions. 16(R)-HETE is a metabolite of the arachidonic acid metabolic pathway cytochrome P450 (CYP450), involved in blood pressure regulation, lipid synthesis, and inflammatory responses [45]. Studies have found that 16 (R)-HETE can inhibit CYP1A2 activity and reduce cardiac toxicity [46]. Prostaglandin B2, a bioactive lipid

mediator derived from arachidonic acid, inhibits the accumulation of cyclic AMP in hepatocytes stimulated by glucagon and has anti-inflammatory effects [47]. Tetrahydrocortisol is a corticosteroid closely related to glucose and lipid metabolism, with anti-inflammatory and immune-regulatory effects [48]. Previous studies have shown that tetrahydrocortisol can alleviate acute skin inflammation in mice [49]. In the present study, plasma concentrations of 16(R)-HETE, prostaglandin B2, and tetrahydrocortisol were substantially elevated in the MET<sub>3</sub> group of juvenile *M. amblycephala* relative to the CON group. These findings suggest that Met supplementation may enhance anti-inflammatory responses and immunological function in juvenile *M. amblycephala* consuming a plant-based protein diet, thus contributing to improved growth performance. Furthermore, an evident increase in plasma levels of MTA was observed in the MET<sub>3</sub> group, along with an evident reduction in LysoPC concentrations. MTA is a metabolite produced through the polyamine pathway from SAM and can regulate cell proliferation, differentiation, and apoptosis processes [50]. MTA is also a protein methyltransferase inhibitor, regulating the transcription and activation of relevant genes by inhibiting H3K4 methylation [51]. LysoPC is a lipid mediator with regulatory functions in epigenetics, including regulating cell apoptosis and signal transduction. Studies have shown that LysoPC can induce cysteine protease-dependent cell death in mouse liver cells and trigger endoplasmic reticulum stress [52]. Therefore, it is predicted that supplementing an appropriate amount of Met in the plant-based protein diet improves resistance to oxidative damage in juvenile *M. amblycephala* by modulating MTA and LysoPC metabolism and involvement in the methylation pathway.

#### 4.3. Met Regulates Hepatic Autophagy and Apoptosis in Juvenile *M. amblycephala* by Affecting Liver m<sup>6</sup>A Methylation Levels

M<sup>6</sup>A methylation is a key regulatory mechanism in RNA biology, influencing protein synthesis and enabling rapid modulation of dynamic cellular processes [53]. Liver is the primary organ responsible for Met metabolism, and evidence indicates that up to 85% of m<sup>6</sup>A methylation events and approximately 48% of Met metabolism occur in hepatic tissue [54]. The abundance of m<sup>6</sup>A reflects the overall methylation status within the examined tissue. SAM, a central metabolite in hepatic Met metabolism, is a crucial methyl donor in the m<sup>6</sup>A modification pathway [55]. This study found that Met decreased the total m<sup>6</sup>A levels and SAM content in the liver of juvenile *M. amblycephala*, which was different from the findings on Salmon alevins. These differences may result from variations in methionine requirements and physiological functions between marine and freshwater fish, or from the different types of methionine supplements (L-Met vs. DL-Met) used in the diets [56,57]. Met mainly participates in four physiological metabolic processes: protein synthesis, methylation, polyamine production, and trans-sulfuration. Combined with the increased muscle protein and plasma taurine content in juvenile *M. amblycephala*, it is speculated that with appropriate Met supplementation, its primary physiological function may involve protein metabolism and maintaining tissue health homeostasis, decreasing overall liver methylation.

This study further revealed that the m<sup>6</sup>A peak in the liver of the MET<sub>3</sub> group was remarkably reduced in the CDS region and the promoter of the 3' UTR, suggesting that the demethylation of Met likely occurs in the central region of the gene's 3' UTR. This could improve mRNA stability and translation efficiency or alleviate miRNA-mediated suppression of physiological function regulation. Therefore, Met possibly exerts regulatory effects by performing more refined and selective 'demethylation' of key genes. Methyltransferases catalyze the methylation activity of m<sup>6</sup>A modifications, while demethylases catalyze the demethylation of RNA substrates with m<sup>6</sup>A modifications [18]. Research shows that *wtap* can promote endoplasmic reticulum stress and apoptosis in cardiomyocytes by down-

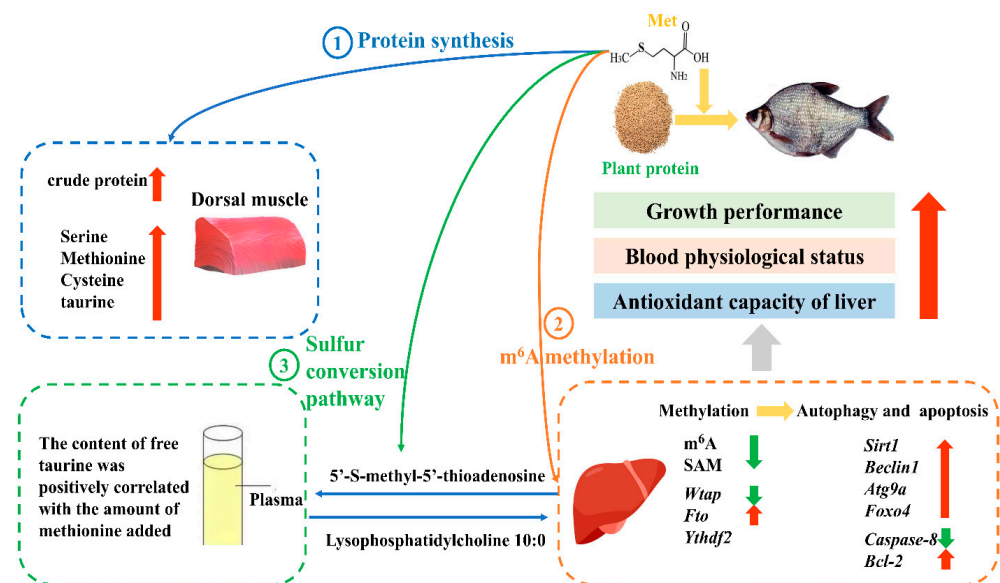
regulating the m<sup>6</sup>A modification of activating transcription factor4 (*atf4*) mRNA, causing myocardial damage [58]. In hepatic ischemia–reperfusion injury (IRI), *fto* expression is significantly downregulated; however, upregulation of *fto* has been shown to reduce liver IRI by attenuating apoptosis and autophagy [59]. In this study, Met considerably reduced the m<sup>6</sup>A methylation levels in the liver of juvenile *M. amblycephala*, along with a significant decrease in *wtap* and an increase in *fto*, indicating a synergistic interaction between *wtap* and *fto* in the health impairment of juvenile *M. amblycephala*. Similar studies have also found that [Cu(tpy-tpy)Br<sub>2</sub>]Br reduces SAM and S-adenosyl homocysteine levels in liver cells by inhibiting the Met cycle, thus inducing the senescence of cancer cells in the liver [60].

Autophagy represents a highly conserved catabolic mechanism in eukaryotic cells, responsible for the selective degradation and recycling of damaged intracellular components. This process is pivotal in maintaining cellular energy homeostasis and facilitating adaptive responses under conditions of nutritional deprivation [61]. Sirtuins (Sirts), a class of nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent deacetylases, are crucially involved in the regulation of cell propagation, programmed cell death, and autophagic activity [39]. *Sirt1* is the most studied and widely distributed member of the Sirt family and is closely related to autophagy, and the Foxo pathway also involves key physiological processes such as autophagy, differentiation, and oxidative stress. In cancer research, it has been found that *foxo4* can inhibit cancer cell proliferation, regulate the cell cycle, and promote autophagy and apoptosis [62]. The Atg ubiquitin-like conjugation system is involved in the autophagy initiation, extension, and maturation stages. *Atg9a*, an essential integral membrane protein, functions as a central regulatory node in autophagosome membrane biogenesis and is crucially involved in mediating membrane remodeling required for autophagosome closure, thus facilitating the terminal stages of autophagy [63]. Moreover, *beclin1* is a specific gene involved in autophagy, which positively regulates the occurrence of autophagy [64]. There is a close relationship between genes regulating autophagy. *Sirt1* can upregulate *foxo4* expression by regulating the Foxo pathway, thus improving the organism's ability to resist oxidative stress [65]. *Sirt1* induces BCL2 interacting protein 3 (*bnip3*) expression by deacetylating *foxo3*, thus triggering autophagy by promoting the dissociation of the anti-apoptotic gene *bcl-2* and the *beclin1* complex [66]. The Foxo pathway can also upregulate the activation of Atg genes through transcription.

Cellular autophagy and apoptosis are basic regulatory mechanisms by which organisms respond to internal and external stressors. Apoptosis, a tightly controlled form of programmed cell death, is used to preserve cellular homeostasis and eliminate damaged or unwanted cells in a regulated manner. *Caspase-8*, a member of the cysteine protease family, is considered an apoptosis promoter. Once activated, it can induce cell apoptosis [67]. Evidence indicates that autophagy may be initiated as a cellular adaptive response preceding apoptosis, particularly under growth factor deprivation or exposure to cytotoxic agents, to reduce cellular damage and delay the onset of programmed cell death [68]. Supplementing with Met can reduce the apoptosis signal of *aip56* in the head kidney leukocytes of European bass, showing lower cysteine aspartate-specific protease 3 (*caspase-3*) activity [69]. Curcumin, when used as a methyl donor in cardiomyocytes of diabetic cardiomyopathy mice, has been demonstrated to induce autophagy through activation of the AMPK signaling pathway. This activation facilitates the dissociation of the *bcl-2-beclin1* complex, thus attenuating apoptotic processes [70]. In the present study, dietary Met supplementation elicited activation of key autophagy-related *sirt1*, *atg9a*, and *foxo4* transcription factors in juvenile *M. amblycephala* maintained on a plant-based diet. This intervention was associated with an elevated expression of the *bcl-2* and a reduction in *caspase-8* expression. These results indicate that Met may suppress apoptosis by enhancing autophagic mechanisms, thus improving the growth impairment of juveniles caused by feeding a plant-based diet.

## 5. Conclusions

This study concluded that an optimal dietary Met level of 10.1 g/kg enhances growth rate, improves hematological parameters, and strengthens hepatic antioxidant potential in juvenile *M. amblycephala*. This supplementation modulates the profiles of muscle hydrolyzed amino acids and plasma free amino acids, elevates plasma metabolites associated with antioxidant, anti-inflammatory, and antibacterial activities, and promotes hepatic autophagy through the *wtap/fto* demethylation pathway. These effects collectively contribute to reduced hepatocellular apoptosis and improved overall fish health (Figure 8). This study proposes a potential regulatory mechanism linking methionine, m<sup>6</sup>A methylation, and autophagy/apoptosis. However, functional validation experiments (e.g., gene knockdown or inhibitor treatments) were not conducted to substantiate the causal relationships. Future investigations should incorporate functional assays to validate the roles of key regulatory factors and to further clarify the causal pathways through which methionine modulates m<sup>6</sup>A methylation and the associated autophagy and apoptosis processes.



**Figure 8.** The epigenetic mechanisms of dietary methionine regulating growth and health in juvenile *Megalobrama amblycephala*. The red arrows represent the significant increase in indicators, while the green arrows represent the significant decrease in indicators.

**Author Contributions:** Conceptualization, L.M.; methodology, Q.S., W.J., Y.L. and Z.G.; software, Q.S. and W.J.; validation, Q.S., L.Q., C.X. and Q.R.; formal analysis, Q.S.; investigation, Q.S., L.Q., C.X. and Q.R.; resources, L.M.; data curation, Q.S. and L.M.; writing—original draft preparation, Q.S.; writing—review and editing, S.L., W.J., Y.L., Z.G. and L.M.; visualization, Q.S.; supervision, L.M.; project administration, L.M.; funding acquisition, L.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by the China Agriculture Research System (CARS-45) and Science and Technology Innovation Team (2023TD63).

**Institutional Review Board Statement:** The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (ethical protocol code 2022113015, approved on 1 March 2023). All animal handling and experimental procedures were conducted in accordance with the approved ethical guidelines.

**Data Availability Statement:** The authors confirm that the data supporting the findings of this study are available within the manuscript and table.

**Acknowledgments:** We would like to express our sincere gratitude to Zexia Gao for her kind assistance in providing the experimental juvenile fish. We would like to thank the post-graduate students of the Fish Disease and Nutrition Department, Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences (CAFS), China, for their help throughout the research period.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|                   |                                                    |
|-------------------|----------------------------------------------------|
| 16(R)-HETE        | 16(R)-hydroxyeicosatetraenoic acid                 |
| 8(S),15(S)-DiHETE | 8(S),15(S)-dihydroxyeicosatetraenoic acid          |
| Alkbh5            | Alkylation repair homolog 5                        |
| ALT               | Alanine aminotransferase                           |
| AST               | Aspartate aminotransferase                         |
| Atf4              | Activating transcription factor4                   |
| Atg9a             | Autophagy-related protein 9a                       |
| Bcl-2             | B-cell lymphoma-2                                  |
| Beclin1           | Benzyl chloride 1                                  |
| Bnip3             | BCL2interacting protein3                           |
| Caspase-3         | Cysteine aspartate-specific protease 3             |
| Caspase-8         | Cysteine aspartate-specific protease 8             |
| CAT               | Catalase                                           |
| CDS               | Coding sequence                                    |
| CYP               | Arachidonic acid metabolic pathway cytochrome P450 |
| DHEA              | Dehydroepiandrosterone                             |
| EAA               | Essential amino acid                               |
| Elavl1            | Embryonic lethal abnormal vision-like protein1     |
| FAA               | Free amino acid                                    |
| FBW               | Final body weight                                  |
| FCR               | Feed conversion ratio                              |
| Foxo3             | forkhead box protein O 3                           |
| Foxo4             | forkhead box protein O 4                           |
| Fto               | Fat mass and obesity-associated protein            |
| GLU               | Glucose                                            |
| GSH               | Glutathione                                        |
| HAA               | Hydrolyzed amino acid                              |
| HPLC              | High-performance liquid chromatography             |
| IRI               | Ischemia–reperfusion injury                        |
| LDH               | Lactate dehydrogenase                              |
| LysoPC            | Lysophosphatidylcholine                            |
| m <sup>6</sup> A  | N-methyladenosine                                  |
| MDA               | Malondialdehyde                                    |
| Met               | Methionine                                         |
| Mettl14           | Methyltransferase like 14                          |
| Mettl3            | Methyltransferase like 3                           |
| MMP               | Mitochondrial membrane potential                   |
| MTA               | 5'-S-methyl-5'-thioadenosine                       |
| NAD <sup>+</sup>  | Nicotinamide adenine dinucleotide                  |
| NEAA              | Non-essential amino acids                          |
| Nrf2              | Nuclear factor erythroid-2-related factor2         |

|        |                                             |
|--------|---------------------------------------------|
| PLS-DA | Partial least squares discriminant analysis |
| ROS    | Reactive oxygen species                     |
| SAM    | S-adenosylmet                               |
| SeMet  | Selenomethionine                            |
| SGR    | Specific growth rate                        |
| Sirt1  | Sirtuins silent information regulator 1     |
| T-AOC  | Total antioxidant capacity                  |
| WGR    | Weight gain rate                            |
| Wtap   | Wilms tumor1-associated protein             |
| Ythdf  | YTH domain family member                    |

## Appendix A

**Table A1.** Experimental feed formulation and nutritional composition (% dry basis).

| Ingredient                     | CON    | MET <sub>1</sub> | MET <sub>2</sub> | MET <sub>3</sub> | MET <sub>4</sub> | MET <sub>5</sub> |
|--------------------------------|--------|------------------|------------------|------------------|------------------|------------------|
| Soybean meal <sup>1</sup>      | 20.00  | 20.00            | 20.00            | 20.00            | 20.00            | 20.00            |
| Rapeseed meal <sup>1</sup>     | 20.00  | 20.00            | 20.00            | 20.00            | 20.00            | 20.00            |
| Cottonseed meal <sup>1</sup>   | 19.00  | 19.00            | 19.00            | 19.00            | 19.00            | 19.00            |
| Cottonseed protein concentrate | 15.00  | 15.00            | 15.00            | 15.00            | 15.00            | 15.00            |
| Wheat flour <sup>1</sup>       | 14.00  | 13.75            | 13.50            | 13.25            | 13.00            | 12.75            |
| Rice bran <sup>1</sup>         | 1.80   | 1.80             | 1.80             | 1.80             | 1.80             | 1.80             |
| Soybean oil                    | 6.50   | 6.50             | 6.50             | 6.50             | 6.50             | 6.50             |
| Calcium dihydrogen phosphate   | 1.00   | 1.00             | 1.00             | 1.00             | 1.00             | 1.00             |
| Mineral premix <sup>2</sup>    | 1.00   | 1.00             | 1.00             | 1.00             | 1.00             | 1.00             |
| Choline chloride               | 0.30   | 0.30             | 0.30             | 0.30             | 0.30             | 0.30             |
| Bentonite                      | 0.20   | 0.20             | 0.20             | 0.20             | 0.20             | 0.20             |
| DMPT                           | 0.50   | 0.50             | 0.50             | 0.50             | 0.50             | 0.50             |
| lysine                         | 0.70   | 0.70             | 0.70             | 0.70             | 0.70             | 0.70             |
| Methionine                     | 0.00   | 0.25             | 0.50             | 0.75             | 1.00             | 1.25             |
| Total                          | 100.00 | 100.00           | 100.00           | 100.00           | 100.00           | 100.00           |
| Nutritional ingredient         |        |                  |                  |                  |                  |                  |
| Crude fat                      | 8.62   | 8.43             | 8.88             | 8.60             | 8.56             | 8.89             |
| Crude protein                  | 36.43  | 36.36            | 36.95            | 36.36            | 36.39            | 36.92            |
| Gross energy                   | 17.06  | 17.02            | 16.97            | 16.93            | 16.88            | 16.84            |

Note: <sup>1</sup> Provided by Beijing Dabeinong Technology Group Co., Ltd. (Huaian, China). Soybean meal: crude protein 46.7%, crude fat 4.25%; Rapeseed meal: crude protein 43.9%, crude fat 6.14%; Cottonseed meal: crude protein 55.6%, crude fat 4.49%; Wheat flour: crude protein 17.4%, crude fat 3.95%; Rice bran: crude protein 16.3%, crude fat 21.71%. <sup>2</sup> Provided by Beijing Dabeinong Technology Group Co., Ltd. (Huaian, China). Mineral (g/kg of premix) premix: calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulfate, 2 g; ferrous sulfate, 0.9 g; zinc sulfate, 0.06 g; cupric sulfate, 0.02 g; manganese sulfate, 0.03 g; sodium selenate, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

**Table A2.** Primer sequences used for qRT-PCR.

| Gene Name        | Primer Sequence (5'-3')                              | L   | E (%)  | NCBI Number    |
|------------------|------------------------------------------------------|-----|--------|----------------|
| <i>wtap</i>      | F: AGAGCTCAAGAGCAGCCAAG<br>R: GTTCAGAGGCCGTTGAAGGA   | 200 | 108.60 | XM_048206845.1 |
| <i>fto</i>       | F: GATTCTGCAGCTGGTGGACT<br>R: GTCTGTCTGTGCTGCTGTCT   | 181 | 103.50 | XM_048184627.1 |
| <i>alkbh5</i>    | F: GATCGATGAGGTGGTTGCCA<br>R: TACGTGTAGCCCTCTCCGAA   | 105 | 109.20 | XM_048197746.1 |
| <i>ythdf2</i>    | F: GGACAAGTGGAAGGGACGTT<br>R: TCCAGTGGAACCTCCTGAGT   | 141 | 100.40 | XM_048191909.1 |
| <i>caspase-8</i> | F: GCAGATGGACAAGGGAACG<br>R: TGGCGAAAGGACTGGTAATG    | 137 | 109.80 | XM_048200598.1 |
| <i>bcl-2</i>     | F: CGTCTACCTGGACAACCACA<br>R: GCGTTTCTGTGCAATGAGTG   | 190 | 106.50 | XM_048179299.1 |
| <i>sirt1</i>     | F: TCGGTTTCATTACAGCAGCACA<br>R: ATGATGATCTGCCACAGCGT | 120 | 102.90 | XM_048203988.1 |

Table A2. Cont.

| Gene Name      | Primer Sequence (5'-3')                               | L   | E (%)  | NCBI Number    |
|----------------|-------------------------------------------------------|-----|--------|----------------|
| <i>beclin1</i> | F: ATGAAAGCACGATGGAGGGTT<br>R: CCCTCGCTGCTATCCAACCTG  | 187 | 109.40 | XM_048187618.1 |
| <i>foxo4</i>   | F: GCTTCACAGGGATCCACTCC<br>R: AGCTTGGCTGCTGGACATAG    | 282 | 109.40 | XM_048175885.1 |
| <i>atg9a</i>   | F: TCTACCTGTGCGCTTTTCGTT<br>R: CCGTTCCTCCACGTTTGTA    | 156 | 108.80 | XM_048200701.1 |
| <i>β-actin</i> | F: TCGTCCACCGCAAATGCTTCTA<br>R: CCGTCACCTTCACCGTTCAGT | 152 | 106.60 | AY170122.2     |

Note: F: forward primer; R: reverse primer; L: amplification sequence length; E: amplification efficiency

## Appendix B

### Appendix B.1. Supplementary Explanation on Water Quality Index of the Feeding Trial

One-third of the tank water was replaced bi-daily to maintain optimal water conditions during the feeding trial. Water quality was monitored weekly, with the following parameters maintained: 26–28 °C temperature, dissolved O<sub>2</sub> concentration ≥ 6 mg/L, ammonia nitrogen levels ≤ 0.05 mg/L, pH between 7.0 and 7.5, and a photoperiod simulating the natural light-dark cycle (12 h light:12 h dark).

### Appendix B.2. Supplementary Explanation on Sample Collection Procedures

Plasma was separated by centrifugation (3000 rpm, 10 min, 4 °C) and divided into three aliquots (−20 °C for biochemical analysis; −80 °C for free amino acids (FAA) and metabolite analyses). Liver tissues from three randomly selected fish per tank were excised and divided into five portions. One portion was stored at −20 °C for antioxidant capacity analysis, while the remaining four were stored at −80 °C for total m<sup>6</sup>A level quantification, SAM content measurement, gene expression profiling, and m<sup>6</sup>A methylome analysis. Dorsal muscle samples were collected post skin removal (both flanks) and stored at −80 °C for amino acid hydrolysis. Moreover, three other fish were selected from each tank for immediate liver dissection, and mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) levels were measured.

### Appendix B.3. Supplementary Explanation on Sample Treatment Procedures for Hydrolyzed Amino Acids (HAA) and Free Amino Acids (FAA) Analysis

The amino acid composition and muscle content (including taurine) were quantified after acid hydrolysis. Muscle samples (0.1 g) were treated with 8 mL of 6 M HCl, flushed with N<sub>2</sub> gas, and hydrolyzed at 120 °C for 22–24 h. After hydrolysis, 4.8 mL of 10 M NaOH was added for neutralization, followed by dilution to 25 mL with distilled water. The solution was filtered through double-layer filter paper, and the HAA were analysed using an amino acid analyser (Agilent 1100, USA).

High-performance liquid chromatography (HPLC) was used to measure FAA composition and content in plasma. Plasma samples were diluted with an equal volume of 10 g/100 mL trichloroacetic acid (final concentration: 5%) and filtered using double-layer filter paper. Filtrates were analyzed using a Waters 2695 HPLC system (USA). Mobile phase A consisted of 0.0225% (v/v) triethylamine and 0.8% (m/v) sodium acetate, while mobile phase B comprised a mixture of 2% (m/v) sodium acetate buffer (pH 7.2), acetonitrile, and methanol in a 1:2:2 (v/v) ratio.

### Appendix B.4. Supplementary Explanation on Plasma Metabolomics Analysis Procedures

Plasma metabolites were analyzed using LC-MS. Plasma samples (100 µL) were mixed with 400 µL of 80% methanol aqueous solution, vortexed, and centrifuged for 20 min at

15,000× *g*. A portion of the supernatant was then diluted with mass spectrometry-grade water to adjust the methanol concentration to 53%, followed by a second centrifugation at 15,000× *g* for 20 min. The final supernatant was examined for LC-MS analysis using a Vanquish UHPLC system coupled with a mass spectrometer (Q Exactive™ HF-X, Germany).

#### Appendix B.5. Supplementary Explanation on Hepatic m<sup>6</sup>A Level and SAM Content Analysis Procedures

Total RNA was isolated from hepatic tissue *via* the RNAiso Plus extraction kit (TaKaRa, Dalian, China) as per the manufacturer's protocol. The concentration and purity of the extracted RNA were evaluated using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). An RNA input of 200 ng was used to quantify N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) modifications in hepatic RNA using the EpiQuik™ m<sup>6</sup>A RNA Methylation Quantification Kit (Colorimetric; Beijing Chunye Technology Co., Ltd.). The assay procedure included sequential steps of RNA immobilization (binding to assay wells), m<sup>6</sup>A-specific antibody capture, and signal detection based on colorimetric reading for quantitative analysis.

Liver SAM content was quantified using an ELISA-based approach. Liver tissues (0.1 g) were homogenized in 0.9 mL of sterile physiological saline (4 °C) and processed in an ice bath. The homogenate was spun at 3000 rpm for 10 min to obtain a 10% liver homogenate and quantified using a fish SAM ELISA kit (Shanghai ChaoRui Biotechnology Co., Ltd.). Tissue homogenate, standard solutions, and HRP-labeled detection antibodies were sequentially added to SAM antibody-coated wells. After incubation and washing, TMB substrate was added for chromogenic development. Absorbance was recorded at 450 nm using a microplate reader, and SAM concentrations were calculated accordingly.

#### Appendix B.6. Supplementary Explanation on Hepatic Gene Expression Analysis Procedures

Gene expression levels in liver tissue were determined *via* qRT-PCR analysis. Total content of RNA was extracted using the RNAiso Plus kit (TaKaRa Biotech Co., Ltd., Dalian, China) as per the manufacturer's protocol. The concentration and purity of extracted RNA were evaluated using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from the purified RNA using the PrimeScript™ RT reagent kit (TaKaRa Biotech Co., Ltd., Dalian, China). Target gene transcripts were quantified using a CFX96 Touch real-time PCR detection system (Bio-Rad, USA). Primers were designed using the NCBI Primer Blast tool (Table A2) and synthesised by Shanghai BioEngineering Co., Ltd. *β-actin* was selected as the housekeeping gene, and relative transcription levels were analyzed using the 2<sup>−ΔΔCT</sup> method.

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