Effects of Dietary Lysine Levels on Growth Performance and Glycolipid Metabolism via the AKT/FoxO1 Pathway in Juvenile Largemouth Bass, *Micropterus salmoides*

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A 56-day feeding experiment was conducted to determine the dietary lysine requirement of juvenile largemouth bass (*Micropterus salmoides*) and investigate the effects of dietary lysine on growth, whole-body composition, and hepatic gene expression related to glycolipid metabolism via the AKT/FoxO1 pathway. The juveniles (17.34 ± 0.02 g) were fed six graded lysine levels (2.11% (control), 2.56%, 2.92%, 3.33%, 3.68%, and 4.09%, dry diet). The results showed that the 3.33% dietary lysine level significantly increased the final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) and improved the feed conversion ratio (FCR) compared with the control group. The whole-body composition was not significantly affected by dietary lysine levels, while lowest hepatic lipid contents were found in the 2.92% and 3.33% dietary lysine groups. Regarding glycolipid metabolism, compared with the control group, 3.33% dietary lysine improved the protein kinase B (AKT) and inhibited the forkhead box O1 (FoxO1), thus upregulated the pyruvate kinase (PK) mRNA levels to enhance glycolysis. Furthermore, sterol-regulatory element binding protein-1c (SREBP1c) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were downregulated by 3.33% dietary lysine, which caused the downregulation of lipid synthesis-related genes acetyl-CoA carboxylase-1 (ACC) and stearyl-CoA desaturase (SCD) mRNA. In addition, 3.33% dietary lysine promoted the expression of the lipolysis-related genes peroxisome proliferator-activated receptor-α (PPAR-α) and carnitine palmitoyl transferase-1 (CPT1). According to the quadratic regression analysis based on the FCR and SGR values, the optimal dietary lysine levels were estimated to be 3.03% and 3.07% of the diet (6.39% and 6.48% of dietary protein), respectively.

1. Introduction

Due to the lack of the corresponding transaminase, many fish cannot synthesize lysine by themselves and must obtain it from food. Hence, lysine (Lys) is usually the first limiting essential amino acid that is insufficient in commonly used raw materials for fish feed, especially in plant protein sources [1]. In fish, lysine deficiency could decrease growth performance and reduce the feed conversion ratio [2]. This result has been verified in other reports by Forster and Ogata [3] and Ruchimat et al. [4] on juvenile red sea bream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*), respectively. Moreover, previous studies on other fish species observed that feeding excessive lysine reduced the growth rate [5, 6]. Berge et al. [7] explained that antagonistic reactions occur between amino acids when a single amino acid is in excess;
thus, the antagonistic effect of lysine and arginine is particularly apparent. Therefore, it is necessary to continue to evaluate the optimal addition amount of lysine in different fishes to obtain a more comprehensive aquatic feed. In previous studies, Gridale-Hellada et al. [8] reported that the lysine requirement of Atlantic cod (Gadus morhua) is 2.67% of the diet (5.34% of the dietary protein). Cao et al. [9] reported that the lysine requirement of juvenile yellow catfish (Pelteobagrus fulvidraco) was 3.31% of the diet or 8.32% of the dietary protein. However, for the same fish, different culture specifications and ages have different optimal requirements for lysine. Dai [10] found that the lysine requirements of tongue sole (Cynoglossus semilaevis) are 2.75%-2.85% of the diet (initial weight 3.66 g), 2.43%-2.49% of the diet (initial weight 113.0 g), and 2.46%-2.58% of the diet (initial weight 347.3 g). Thus, research on lysine requirements should also focus on the size and age of fish species to create a more accurate nutrition database.

As a growth amino acid, lysine has a variety of biological functions. It is necessary for maintaining the growth performance, development, and immunity of the body [11]. In addition, lysine also has many other biological functions. For example, lysine can be converted to carnitine in animals, and carnitine participates in energy metabolism. Early studies found that feeding low lysine and carnitine-free diets can cause fatty liver and growth retardation in rats [12]. However, another study showed that adding an appropriate amount of lysine at a 0.2% level to feed can promote the growth of rats, increase the content of carnitine in muscle tissues, and reduce the level of triglycerides in tissues [13]. Lysine can also be broken down into glucose and participate in glucose metabolism [14]. Similar findings were observed in grass carp (Ctenopharyngodon idellus), and an appropriate amount of dietary lysine (2.44% of diet) promoted glycolysis and lipolysis [15]. These processes are also regulated by the AKT pathway and FoxO1 pathway [16, 17]. AKT is one of the most important and multifunctional kinases. It plays a role in maintaining the steady state of lipid metabolism and has a direct regulatory effect on multiple factors associated with glucose metabolism. FoxO1 also plays a role in mediating transcription in glucose and lipid metabolism diseases; it is involved in gluconeogenesis, glycogenolysis, and lipogenesis. To date, many studies have examined the effects of nutrients on glucose and lipid metabolism through the regulation of the AKT pathway in several fish species [18, 19]. Nevertheless, literature regarding the regulation of AKT/FoxO1 by dietary lysine treatments and its potential consequences on glycolipid metabolism in fish species is extremely scarce.

Largemouth bass, Micropterus salmoides, is a carnivorous warm-water fish species that was introduced to China in the early 1980s. Because of its fast growth and high economic value, it has been one of the most economically important fish species in China, accounting for the vast majority of fish (99%) of the Asian market [20]. In 2020, the annual output of largemouth bass in China reached 619,000 tons, an increase of 29% compared with 2019 [21]. With an increase in production each year, the nutritional requirements of largemouth bass have attracted more attention. To date, few studies have reported the optimal lysine requirement of largemouth bass. Dai [10] suggested that the lysine requirement was 4.9% of dietary protein for largemouth bass fry (1.29 g). Moreover, it has been revealed that supplementation with lysine in feed could improve growth performance and regulate glycolipid metabolism in juvenile largemouth bass [23, 24]. As a carnivorous fish, largemouth bass can also have liver diseases caused by glycolipid metabolism disorders [25]. In addition, the dietary lysine level requirement for largemouth bass at the juvenile stage is still unknown, and few reports about the effects of dietary lysine levels on glycolipid metabolism have been published.

Overall, determining the optimal lysine requirement helps fish growth and is more conducive to the regulation of glycolipid metabolism, reducing the incidence of disease. Consequently, the objective of this study was to determine the optimal lysine requirement of juvenile largemouth bass and estimate the effects of dietary lysine levels on glycolipid metabolism through the AKT/FoxO1 signaling pathway.

2. Materials and Methods

2.1. Experimental Diets. Six practical diets were prepared containing six lysine levels (2.11%, 2.56%, 2.92%, 3.33%, 3.68%, and 4.09%, dry diet). The main protein sources were fish meal, rapeseed meal, and soybean meal. Fish oil was the main lipid source, and wheat flour and cassava starch were the main carbohydrate sources (Table 1). A combination of crystalline amino acids, excluding lysine, was supplemented to simulate the whole-body amino acid pattern of largemouth bass (Table 2). All ingredients were ground into powder through 60 mash-sieve and then thoroughly mixed with fish oil and water to produce stiff dough. The dough was forced through a pelletizer (F-26 (II), South China University of Technology, China) and dried in a ventilated oven [26]. After drying at 45°C for 24 h, the diets were stored at −20°C until use.

2.2. Experimental Design and Conditions. Experimental fish were obtained from the breeding farm of the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences and temporarily kept in floating cages (2 m × 2 m × 1 m) for two weeks, and the fish were fed with commercial feed (obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 47%, crude lipid 12%) to acclimate to the experimental conditions. Before the rearing trial, 540 juvenile largemouth bass (initial weight 17.34 ± 0.02 g) were divided into 18 floating cages (1 m × 1 m × 1 m). Three replicates were performed for each lysine supplementation group, and each cage contained 30 fish. During the experimental period, the juveniles were fed two times (7:00 and 17:00) daily at a feeding rate of 5% of their weight for 8 weeks. The fish were counted and weighed every two weeks to adjust the feeding amount. During this experiment, the water temperature was kept at 28 ± 2°C, the pH values were kept at 7–7.5, the dissolved oxygen levels were higher than 6.0 mg/L, and the photoperiod was the same as natural light.
2.3. Sample Collection. After the 8-week feeding, all juvenile largemouth bass were fasted for 24 h. Then, all juvenile largemouth bass were quantified and weighed, and plasma and liver tissues were quickly collected from three fish per cage. Blood was collected from three of the sampled fish using caudal venipuncture and then immediately centrifuged at 3,000 rpm for 10 min at 4°C. Finally, the upper plasma was transferred into new tubes. In addition, liver tissues were dissected and removed. All plasma and liver tissues were stored at −80°C until analysis. Another two fish were collected from each cage and stored in a −20°C freezer for whole body composition analysis.

2.4. Chemical Analysis. The experimental diets, ingredients, whole-body composition, and liver lipid content were determined by established methods based on the AOAC [27]. The plasma biochemical parameters include triglycerides (TG), total cholesterol (TC), and glucose (GLU).

Table 1: Formulation and proximate composition (% dry matter) of experimental diets.

| Ingredients                        | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 |
|-----------------------------------|--------|--------|--------|--------|--------|--------|
| Fish meal¹                         | 30.00  | 30.00  | 30.00  | 30.00  | 30.00  | 30.00  |
| Rapeseed meal¹                     | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   |
| Soybean meal¹                      | 10.00  | 10.00  | 10.00  | 10.00  | 10.00  | 10.00  |
| Wheat flour¹                       | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   |
| Cassava starch                     | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   |
| Corn gluten meal¹                  | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   | 8.00   |
| Fish oil                           | 5.00   | 5.00   | 5.00   | 5.00   | 5.00   | 5.00   |
| Shrimp paste                       | 2.00   | 2.00   | 2.00   | 2.00   | 2.00   | 2.00   |
| Amino acid premix                  | 8.58   | 8.58   | 8.58   | 8.58   | 8.58   | 8.58   |
| Choline chloride                   | 0.10   | 0.10   | 0.10   | 0.10   | 0.10   | 0.10   |
| Vitamin premix³                    | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   |
| Mineral premix³                    | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   |
| Calcium dihydrogen phosphate      | 2.50   | 2.50   | 2.50   | 2.50   | 2.50   | 2.50   |
| Microcrystalline cellulose         | 0.81   | 0.81   | 0.81   | 0.81   | 0.81   | 0.81   |
| Rice bran                          | 5.00   | 5.00   | 5.00   | 5.00   | 5.00   | 5.00   |
| Ethoxy quinoline                   | 0.01   | 0.01   | 0.01   | 0.01   | 0.01   | 0.01   |
| Glycine⁴                          | 2.00   | 1.60   | 1.20   | 0.80   | 0.40   | 0.00   |
| L-Lysine⁵                          | 0.00   | 0.40   | 0.80   | 1.20   | 1.60   | 2.00   |
| Bentonite                          | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   | 0.00   |
| Total                             | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

Analyzed proximate composition

| Lysine (%)                        | 2.11   | 2.56   | 2.92   | 3.33   | 3.68   | 4.09   |
| Dry matter (%)                    | 93.94  | 92.99  | 93.08  | 93.83  | 94.15  | 94.05  |
| Crude protein (%)                 | 47.48  | 47.32  | 47.40  | 47.49  | 47.42  | 47.51  |
| Crude lipid (%)                   | 10.73  | 10.56  | 10.03  | 10.77  | 11.30  | 11.19  |
| Crude ash (%)                     | 9.45   | 9.80   | 9.42   | 9.55   | 9.62   | 9.70   |
| Crude fiber (%)                   | 3.11   | 3.14   | 3.05   | 3.22   | 3.18   | 3.09   |
| NFE (%)                           | 22.71  | 22.17  | 23.18  | 22.80  | 22.63  | 22.56  |
| Gross energy (MJ/kg)              | 19.71  | 19.76  | 19.64  | 19.68  | 19.80  | 19.76  |

¹Fish meal, obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 65.6%, crude lipid 9.5%; rapeseed meal obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 39.2%, crude lipid 6.1%; soybean meal obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 50.8%, crude lipid 4.3%; corn gluten meal obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 60.6%; wheat flour obtained from Wuxi Tongwei Feedstuffs Co., Ltd., Wuxi, China, crude protein 13.1%, crude lipid 4.0%. ²Vitamin premix (IU or mg/kg of diet): vitamin A, 800,000 IU; vitamin D3, 150,000-250,000 IU; vitamin E, 4,500 IU; vitamin K3, 600 mg; thiamin, 800 mg; riboflavin, 800 mg; calcium pantothenate, 2,000 mg; pyridoxine HCl, 2,500 mg; cyanocobalamin, 8 mg; biotin, 16 mg; folic acid, 400 mg; niacin, 2,800 mg; inositol, 10,000 mg; vitamin C, 10,000 mg. ³Mineral mix (g/kg of diet): magnesium sulphate, 1.0-1.5%; ferrous sulphate, 15-30 g; zinc sulphate, 8-13.5 g; cupric sulphate, 0.35-0.8 g; manganese sulphate, 2-6 g; rice chaff and zeolite were used as a carrier. ⁴Glycine, obtained from Feeer Co., Ltd. (Shanghai, China). ⁵L-lysine, obtained from Feeer Co., Ltd. (Shanghai, China). ⁶NFE (nitrogen free extract, %) = dry matter (%) – (crude protein % + crude lipid % + crude ash %) + crude fiber %).
Table 2: Amino acid composition of ingredients (% dry matter).

| Amino acid  | 30% FM$^1$ | 8% RM$^1$ | 10% SM$^1$ | 8% WF$^1$ | 8% CGM$^1$ | 5% RB$^1$ | CAPP$^2$ | Total | 47% WBP$^3$ |
|-------------|------------|-----------|------------|-----------|-----------|-----------|---------|-------|-------------|
| EAA$^4$     |            |           |            |           |           |           |         |       |             |
| Arginine    | 1.20       | 0.20      | 0.37       | 0.08      | 0.15      | 0.06      | 0.75    | 2.06  | 2.81        |
| Histidine   | 0.45       | 0.09      | 0.13       | 0.04      | 0.10      | 0.02      | 0.33    | 0.83  | 1.17        |
| Isoleucine  | 0.83       | 0.13      | 0.24       | 0.07      | 0.20      | 0.03      | 0.55    | 1.49  | 2.04        |
| Leucine     | 1.45       | 0.23      | 0.39       | 0.14      | 0.80      | 0.05      | 0.43    | 3.07  | 3.50        |
| Lysine      | 1.51       | 0.18      | 0.31       | 0.05      | 0.07      | 0.03      | Variable| 2.16  | 3.50        |
| Methionine  | 0.55       | 0.07      | 0.07       | 0.03      | 0.13      | 0.01      | 0.25    | 0.86  | 1.11        |
| Phenylalanine | 0.79     | 0.13      | 0.27       | 0.10      | 0.30      | 0.03      | 0.33    | 1.62  | 1.95        |
| Threonine   | 0.82       | 0.14      | 0.20       | 0.06      | 0.16      | 0.03      | 0.59    | 1.41  | 2.00        |
| Valine      | 0.96       | 0.17      | 0.25       | 0.09      | 0.22      | 0.04      | 0.53    | 1.72  | 2.25        |
| Tryptophan  | 0.21       | 0.05      | 0.07       | 0.02      | 0.02      | 0.01      | 0.12    | 0.38  | 0.50        |
| NEAA$^5$    |            |           |            |           |           |           |         |       |             |
| Aspartic acid | 2.11     | 0.56      | 0.75       | 0.11      | 0.41      | 0.15      | 0.33    | 4.09  | 4.42        |
| Serine      | 0.77       | 0.14      | 0.26       | 0.10      | 0.25      | 0.03      | 0.47    | 1.55  | 2.02        |
| Glycine     | 1.99       | 0.36      | 0.41       | 0.03      | 0.23      | 0.04      | 0.85    | 3.06  | 3.90        |
| Alanine     | 1.33       | 0.14      | 0.22       | 0.06      | 0.43      | 0.04      | 1.08    | 2.23  | 3.30        |
| Cystine     | 0.18       | 0.08      | 0.07       | 0.05      | 0.08      | 0.02      | 0.00    | 0.48  | 0.27        |
| Glutamic acid | 3.25     | 0.67      | 1.08       | 0.73      | 1.22      | 0.13      | 1.42    | 7.08  | 8.50        |
| Proline     | 0.91       | 0.21      | 0.26       | 0.23      | 0.44      | 0.03      | 0.56    | 2.08  | 2.64        |

$^1$FM: fish meal; RM: rapeseed meal; SM: soybean meal; WF: wheat flour; CGM: corn gluten meal; RB: rice bran; $^2$CAPP: crystalline amino acid premix; $^3$WBP: whole-body protein; $^4$EAA: essential amino acid; $^5$NEAA: nonessential amino acid.

Table 3: The chemical analysis used in the experiment.

| Items               | Methods                         | Assay kits/testing equipment                                                                 |
|---------------------|---------------------------------|---------------------------------------------------------------------------------------------|
| Composition of diets/ingredients | Drying method                   | Electric blast drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) |
| Moisture            |                                 | Auto Kjeldahl apparatus: Hanon K1100 (Jinan Hanon Instruments Co., Ltd., Jinan, China)          |
| Protein             | Kjeldahl                        | Auto fat analy: Hanon SOX606 (Jinan Hanon Instruments Co., Ltd., Jinan, China)               |
| Lipid               | Soxhlet                         | Muffle: XL-2A (Hangzhou Zhuoqi Instrument Co., Ltd., Hangzhou, China)                        |
| Ash                 | Combustion                      | Oxygen bomb calorimeter: IKA C6000 (IKA Works Guangzhou, Guangzhou, China)                  |
| Gross energy        | Combustion                      | Amino acid analyzer: SYKAM S-433D (Sykam GmbH, Munich, Germany)                               |
| Amino acids (except tryptophan) | Acid hydrolysis               |                                               |                                               |
| Tryptophan          | Alkali hydrolysis               |                                               |                                               |
| Liver               |                                 | Auto fat analy: Hanon SOX606 (Jinan Hanon Instruments Co., Ltd., Jinan, China)               |
| Lipid               | Soxhlet                         |                                               |                                               |
| Plasma parameters   |                                 |                                               |                                               |
| TG$^1$              | International Federation of Clinical Chemistry recommended | Assay kits purchased from Mindray Medical International Ltd. (Shenzhen, China); Mindray BS-400 automatic biochemical analyzer (Mindray Medical International Ltd., Shenzhen, China) |
| TC$^1$              |                                 |                                               |                                               |
| GLU$^1$             |                                 |                                               |                                               |

$^1$TG: total triglyceride; TC: total cholesterol; GLU: glucose.
and qRT–PCR was performed on a CFX96 real-time PCR detection system thermocycler (Bio-Rad). The primer pairs used in this experiment are presented in Table 4. GAPDH was selected as a reference, and there was no difference in its expression among the different treatments. Finally, Pfaffi’s mathematical model [28] was selected to calculate the relative expression of the target genes in this experiment.

2.5. Statistical Analysis. The data were subjected to normality and homogeneity tests where necessary, and then, the experimental data (means ± SEM) were analyzed using the SPSS 24.0 statistical software for one-way analysis of variance (ANOVA). When the difference was significant (P < 0.05), Tukey’s multiple comparisons were performed. After comparing the estimation coefficient (R²) among the linear regression model (SGR, 0.015; FCR, 0.026), broken-linear regression model (SGR, 0.137; FCR, 0.185), and quadratic regression model (SGR, 0.836; FCR, 0.935), the quadratic regression model was selected to determine the optimum dietary lysine requirement of juvenile largemouth bass.

### 3. Results

#### 3.1. Growth Performance. As shown in Table 5, the juvenile largemouth bass fed 3.33% dietary lysine gained the maximum FBW, WGR, and SGR (P < 0.05), and the FCR reached the lowest value (P < 0.05). In addition, no obvious changes were observed in FI and SR among all the dietary groups (P > 0.05). According to the quadratic regression analysis based on the FCR and SGR, the optimal dietary lysine levels were estimated to be 3.03% (Figure 1) and 3.07% (Figure 2) of the diet (6.39% and 6.48% of dietary protein), respectively.

#### 3.2. Whole-Body Composition and Hepatic Lipid Content. The body composition data of juvenile largemouth bass fed the experimental diets are shown in Table 6. The moisture, lipid, protein, and ash contents were not significantly affected by lysine treatments (P > 0.05).

Figure 3 shows the hepatic lipid content of juvenile largemouth bass fed experimental diets. The liver lipid content decreased with increasing dietary lysine up to 3.33% and then gradually increased thereafter. The control group had the highest hepatic lipid content (2.11%) among all the groups (P < 0.05).

#### 3.3. Plasma Total Triglycerides, Total Cholesterol, and Glucose Levels. The plasma TG, TC, and GLU levels of juvenile largemouth bass fed the experimental diets are shown in Figure 4. Plasma TG levels were significantly decreased in fish fed 2.56% dietary lysine (P < 0.05, Figure 4(a)). Compared with the control group, plasma TC levels were decreased in the groups fed a diet supplemented with lysine, but there were no significant changes among all dietary treatment groups (P > 0.05, Figure 4(b)). Figure 4(c) shows that the plasma GLU levels were not obviously affected by the lysine treatments (P > 0.05).

#### 3.4. Relative mRNA Expression of AKT and FoxO1. The hepatic mRNA expression levels of AKT and FoxO1 are displayed in Figure 5. Figure 5 shows that AKT mRNA levels increased with increasing dietary lysine levels, and the maximum value was observed in the 4.09% dietary group (P < 0.05). In addition, the FoxO1 mRNA levels showed a decreasing trend with increasing levels of dietary lysine, and the control group (2.11% dietary lysine) demonstrated the highest expression levels of FoxO1 (P < 0.05).

#### 3.5. Relative mRNA Expression of Glucose Metabolism-Related Genes. The expression levels of hepatic glucose metabolism-related genes are presented in Figure 6. The PK mRNA levels increased with increasing dietary lysine
up to 3.68% (P < 0.05) and then gradually decreased thereafter. The expression levels of GK showed a similar tendency to those of PK; however, there were no significant differences among all dietary treatments (P > 0.05). In addition, at the highest level of dietary lysine (4.09%), the PEPCK mRNA level was upregulated in comparison with that in the control group (P < 0.05).

3.6. Relative mRNA Expression of Lipid Metabolism-Related Genes. The mRNA expression levels of hepatic lipid metabolism-related genes are shown in Figures 7 and 8. As shown in Figure 7, the lipolysis-related genes PPAR-α and CPT1 were both regulated by dietary lysine levels. The mRNA expression levels of PPAR-α increased with increasing dietary lysine, and the highest level was observed in the 4.09% dietary lysine group (P < 0.05). The mRNA expression levels of CPT1 reached their maximum value when the fish were fed 2.92% and 3.33% dietary lysine (P > 0.05). Figures 8(a) and 8(b) present the mRNA expression levels of hepatic lipid synthesis-related genes. The highest mRNA expression level of SREBP1c was observed in the control group (2.11% dietary lysine). When the fish were

Table 5: Growth performance of juvenile largemouth bass fed with the experimental diets.

| Lysine levels (%) | IBW (g)\(^1\) | FBW (g)\(^2\) | WGR \(^3\) | SGR ( % day\(^{-1}\))\(^4\) | FCR\(^5\) | FI (g fish\(^{-1}\) d\(^{-1}\))\(^6\) | SR (%)\(^7\) |
|------------------|----------------|----------------|-----------|-----------------|----------|-----------------|----------|
| 2.11             | 17.26 ± 0.03   | 50.77 ± 0.41\(^{ab}\) | 194.2 ± 2.70\(^a\) | 1.93 ± 0.02\(^a\) | 1.24 ± 0.01\(^{ab}\) | 0.64 ± 0.02 | 100.0 ± 0.00 |
| 2.56             | 17.36 ± 0.06   | 52.42 ± 0.72\(^{abc}\) | 202.0 ± 4.52\(^{ab}\) | 1.97 ± 0.03\(^{ab}\) | 1.19 ± 0.02\(^{ab}\) | 0.64 ± 0.01 | 98.9 ± 1.11 |
| 2.92             | 17.37 ± 0.07   | 53.38 ± 0.15\(^{bc}\) | 207.4 ± 1.28\(^{ab}\) | 2.01 ± 0.01\(^{ab}\) | 1.17 ± 0.00\(^{ab}\) | 0.64 ± 0.00 | 98.9 ± 1.11 |
| 3.33             | 17.30 ± 0.10   | 54.63 ± 1.00\(^b\)  | 215.8 ± 7.29\(^{b}\) | 2.05 ± 0.04\(^{b}\) | 1.15 ± 0.04\(^b\)  | 0.64 ± 0.01 | 96.7 ± 1.92 |
| 3.68             | 17.36 ± 0.03   | 52.13 ± 0.25\(^{abc}\) | 200.4 ± 0.91\(^{ab}\) | 1.96 ± 0.01\(^{ab}\) | 1.21 ± 0.01\(^{ab}\) | 0.65 ± 0.01 | 100.0 ± 0.00 |
| 4.09             | 17.40 ± 0.04   | 50.51 ± 0.51\(^a\)  | 190.3 ± 2.29\(^a\)  | 1.90 ± 0.01\(^a\)  | 1.26 ± 0.02\(^{ab}\) | 0.66 ± 0.01 | 100.0 ± 0.00 |

Regressions

\[
Y_{FBW} = -3.365X^2 + 20.842X + 21.535
\]

\[
Y_{WGR} = -19.217X^2 + 118.2X + 28.525
\]

\[
Y_{SGR} = -0.1135X^2 + 0.698X + 0.9489
\]

\[
Y_{FCR} = 0.089X^2 - 0.539X + 1.9348
\]

\[
Y_{FI} = 0.0126X^2 - 0.0688X + 0.7302
\]

All data are mean value of three replicates ± SEM. Means in the same column with different superscripts are significantly different (P < 0.05). \(^1\)IBW: initial body weight. \(^2\)FBW: final body weight. \(^3\)Weight gain rate (WGR) (%) = 100 × (final body weight (g) – initial body weight (g))/initial body weight (g). \(^4\)Specific growth rate (SGR) (% day\(^{-1}\)) = 100 × [(In (final body weight (g)) – In (initial body weight (g)))/days]. \(^5\)Feed conversion ratio (FCR) = dry feed fed (g) / (final body weight (g) – initial body weight (g)). \(^6\)Feed intake (FI) (g fish\(^{-1}\) d\(^{-1}\)) = dry feed fed (g)/(final body weight (g) + initial body weight (g)/2 × days). \(^7\)Survival rate (SR) (%) = 100 × (survival fish number/total fish number).

Figure 1: Quadratic regression analysis of feed conversion ratio (FCR) against graded different levels of dietary lysine.
Figure 3: The lipid contents in the liver of juvenile largemouth bass fed with experimental diets.

Table 6: Whole body composition of juvenile largemouth bass fed with the experimental diets.

| Lysine levels (%) | Moisture (%) | Protein (%) | Lipid (%) | Ash (%) |
|-------------------|--------------|-------------|-----------|---------|
| 2.11              | 70.45 ± 0.17 | 17.49 ± 0.49 | 7.93 ± 0.23 | 4.89 ± 0.12 |
| 2.56              | 70.03 ± 0.28 | 17.67 ± 0.37 | 7.85 ± 0.24 | 4.86 ± 0.31 |
| 2.92              | 70.38 ± 0.07 | 17.70 ± 0.29 | 7.87 ± 0.04 | 4.18 ± 0.38 |
| 3.33              | 69.91 ± 0.25 | 17.28 ± 0.11 | 7.87 ± 0.32 | 4.67 ± 0.20 |
| 3.68              | 70.61 ± 0.31 | 17.22 ± 0.38 | 7.02 ± 0.34 | 4.46 ± 0.25 |
| 4.09              | 70.14 ± 0.12 | 17.96 ± 0.20 | 7.49 ± 0.49 | 4.63 ± 0.26 |

Regressions

\[ Y_{\text{Moisture}} = 0.1314X^2 - 0.8445X + 71.55 \]
\[ R^2 = 0.051 \quad P = 0.846 \]

\[ Y_{\text{Protein}} = 0.2095X^2 - 1.2459X + 19.332 \]
\[ R^2 = 0.144 \quad P = 0.608 \]

\[ Y_{\text{Lipid}} = -0.076X^2 + 0.1324X + 8.0303 \]
\[ R^2 = 0.487 \quad P = 0.237 \]

\[ Y_{\text{Ash}} = 0.3144X^2 - 2.0961X + 7.9543 \]
\[ R^2 = 0.428 \quad P = 0.376 \]

All data are mean value of three replicates ± SEM. Means in the same column with different superscripts are significantly different \((P < 0.05)\).
Figure 4: Plasma total triglycerides (TG) (a), total cholesterol (TC) (b), and glucose (GLU) (c) contents of juvenile largemouth bass fed with the experimental diets.
fed 3.33% dietary lysine, the mRNA expression levels of SREBP1c were significantly decreased ($P < 0.05$). Compared with the control group, the mRNA expression levels of PPAR-γ were markedly decreased in the 3.33% dietary lysine group ($P < 0.05$). The ACC and SCD mRNA expression levels also showed a similar phenomenon in that the relative

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**Figure 5:** Relative mRNA expression of AKT/FoxO1 pathway in the liver of juvenile largemouth bass.

**Figure 6:** Relative mRNA expression of glucose metabolism in the liver of juvenile largemouth bass.
expression levels began to decrease as the amount of dietary lysine increased. The mRNA expression levels of ACC were lowest in the 3.33% dietary lysine group ($P < 0.05$), and the mRNA expression levels of SCD gradually decreased with increasing levels of dietary lysine in comparison with that in the control group ($P < 0.05$). Moreover, the FAS mRNA levels were not affected by the dietary lysine treatments ($P > 0.05$).

4. Discussion

4.1. Growth Performance of Largemouth Bass. In this experiment, the best growth performance was observed in the 3.33% dietary lysine group, as the FCR was the lowest in this group. In addition, the growth of largemouth bass was reduced in the low- and high-level lysine groups. Our experimental results indicate that appropriate lysine supplementation could promote the growth of juvenile largemouth bass and enhance feed utilization. Similar findings were also observed in juvenile totoaba (*Totoaba macdonaldi*) [29] and juvenile silver pompano (*Trachinotus blochii*) [30]. In addition, this study also demonstrated that high lysine levels reduced the growth performance of largemouth bass. Excessive lysine may cause arginine-lysine antagonism, or the increased lysine degradation consumes more energy in fish [31, 32]. According to the quadratic regression analysis of the FCR and SGR for dietary lysine, the optimal dietary lysine levels were estimated to be 3.03% (6.39% of dietary protein) and 3.07% (6.48% of dietary protein) of diet. However, these results are different from those reported in the previous study by Dairiki et al. [22] in largemouth bass fry (1.29 g, 4.9% of dietary protein). In their study, the largemouth bass fry were cultured in indoor tanks in a closed circulation system, and the main protein source was tilapia fillet. The different growth stages of aquatic animals could cause a wide variation in nutrient requirements. Moreover, dietary formulation, culture conditions, and dietary amino acid balance could also be potential causes for these observed differences [33].

4.2. Body Composition of Largemouth Bass. A previous study reported that the proximate body composition of cultured fish was influenced by numerous factors, including environmental conditions and diets [34]. Some authors reported that lysine supplementation could increase body protein contents and decrease body lipid contents in fish [35, 36]. Besides, it has also been found that lysine supplementation increases the protein and lipid contents [37]. However, in this study, the whole-body moisture, lipid, protein, and ash contents were not affected by the dietary lysine treatments. This result was the same as other studies reported by Prabu et al. [38] in GIFT tilapia (*Oreochromis niloticus*) and Li et al. [39] in juvenile grass carp (*Ctenopharyngodon idella*), where the whole-body composition was not notably affected by dietary lysine supplementation. The differences may be related to diet formulations, species, and farming conditions, and the specific mechanism needs further study.

4.3. AKT/FoxO1 Signaling Pathway. In recent years, research on glycolipid metabolism and the regulation of largemouth bass has become a hot spot in aquaculture. In some carnivorous fish, the liver has a low ability to regulate glycolysis.
and gluconeogenesis, and this could result in serious metabolic disorders, including glycogenic liver disease and hepatic steatosis, in long-term culture [40]. In our experiment, the regulatory effect of dietary lysine on hepatic glyco-

lipid metabolism in largemouth bass through the AKT/FoxO1 pathway was explored. The AKT signaling pathway is a key pathway by which insulin controls hepatic lipid metabolism [41], and AKT is also a kinase that is well-

**Figure 8:** Relative mRNA expression of lipid synthesis in the liver of juvenile largemouth bass.
known to be involved in improving glucose metabolism [42]. In addition, as a key molecule downstream of the AKT signaling pathway, FoxO1 also plays a transcriptional mediating role in glucose and lipid metabolism diseases, including gluconeogenesis, glycogenolysis, and lipogenesis [17]. Our experimental results showed that AKT mRNA expression increased with increasing lysine levels. The highest level was observed in the high-lysine group, while FoxO1 mRNA showed the opposite trend. Wang et al. [43] reported that compared to a low-lysine diet, lysine supplementation increased the mRNA expression levels of AKT, which was consistent with our findings. Furthermore, FoxO1 is regulated by the upstream signaling molecule AKT. After AKT phosphorylates FoxO1, FoxO1 is translocated from the nucleus. This translocation inhibits its transcriptional activity and decreases the expression of FoxO1 [44]. These results indicate that lysine supplementation could activate AKT, and the activation of AKT suppresses the mRNA expression of FoxO1, which then influences hepatic glucose and lipid metabolism in juvenile largemouth bass.

4.4. Glucose Metabolism. Glycolysis and gluconeogenesis are the decomposition and synthesis of sugar in their physiological functions [45]. Glycolysis is the only method of glucose metabolism in all organisms. Gluconeogenesis refers to the conversion of nonsugar substances into glucose, mainly in the liver [46]. In our experiment, the mRNA expression level of the glycolysis-related gene PK increased with increasing dietary lysine up to 3.68% and decreased thereafter. These results suggest that appropriate lysine supplementation could enhance the glycolytic ability of juvenile largemouth bass. A previous study on largemouth bass also showed that lysine supplementation could promote the expression of PK [24]. In addition, the mRNA levels of the gluconeogenesis-related gene PEPCK were upregulated with high dietary lysine levels (4.09% dietary lysine level); its expression was higher in this group than in all other groups. Moreover, a high plasma GLU content was observed in the high lysine supplementation group, and this finding is similar to the findings of previous studies on the essential amino acids arginine [47] and tryptophan [48] in blunt snout bream (Megalobrama amblycephala). Our previous study indicated that gluconeogenesis could be stimulated by high lysine levels in grass carp [15]. Furthermore, the FoxO1 transcription factor is thought to positively regulate the expression of PEPCK in the liver [49] and can also regulate the expression of key glycolysis enzymes [50]. Our results indicated that FoxO1 inhibition promoted glycolysis, while gluconeogenesis did not change significantly in the 3.33% or 3.68% dietary lysine group compared with the control group (2.11%). In contrast, the high lysine group (4.09%) promotes the expression of PEPCK mRNA. Normally, the inhibition of FoxO1 weakens the PEPCK activity; the differences may be caused by the lysine addition amount. A previous study in grass carp [15] found that high lysine levels could promote gluconeogenesis. Try to analyze the reasons, lysine is a cationic or basic amino acid with a long side chain capable of providing energy through the gluconeogenesis pathway [51], which might directly cause the high expression of the PEPCK mRNA in the high lysine treatment group. However, information regarding the regulation of FoxO1 on glucose homeostasis in fish is still scarce; thus, the regulatory mechanism of the AKT/FoxO1 pathway on hepatic glucose metabolism needs further verification.

4.5. Lipid Metabolism. Regarding lipid metabolism, FoxO1 can inhibit lipid synthesis by regulating the expression of SREBP1c [52]. The transcription factor SREBP1c participates in the synthesis of fatty acids, triglycerides, and cholesterol [53]. In this experiment, 3.33% dietary lysine inhibited FoxO1 expression and significantly suppressed the mRNA expression of SREBP1c. In addition, as the downstream transcription factors of SREBP1c, the expression levels of ACC and SCD mRNA were also downregulated significantly when juvenile largemouth bass were fed 3.33% dietary lysine. In mammals, appropriate lysine supplementation in feed can promote growth, increase the content of carnitine, and reduce triglyceride levels in tissues [13]. Previous studies in fish also found that optimal dietary lysine could reduce lipid accumulation in some fish species, including tambaqui (Colossoma macropomum) [54] and Nile tilapia [55]. These findings agree with our findings in juvenile largemouth bass. In addition, PPAR-γ can cooperate with SREBP1c to regulate lipid synthesis [56] and positively regulate downstream lipid synthesis-related transcription factors (FAS, ACC, and SCD) that are also regulated by FoxO1 [57]. In the present study, appropriate lysine supplementation (3.33%, 3.68%, and 4.09%) inhibited the mRNA expression of PPAR-γ and SREBP1c, which resulted in a decrease in hepatic lipid deposition.

Moreover, PPAR-α plays an important role in lipolysis and is expressed in many tissues [58]. In this study, as lysine supplementation increased, PPAR-α expression gradually increased. Additionally, the expression of the downstream transcription factor CPT1 was significantly increased when dietary lysine levels were increased to 2.92% and 3.33%. Our results indicate that appropriate dietary lysine could activate lipolysis-related genes to promote lipolysis, which might explain the low plasma TG and TC contents that were observed when the appropriate amount of lysine was supplemented in this study. Our results show that lysine supplementation could reduce the plasma TG content, but plasma TC contents were not influenced. As Zhou et al. [36] reported, lysine promotes the synthesis of carnitine, which can reduce the TG and TC contents and convert fatty acids into energy for the body. Thus, as a regulatory factor in the synthesis of carnitine, lysine indirectly regulates the plasma TG and TC contents. Furthermore, based on the hepatic lipid contents among all lysine treatments compared with the control group (Figure 3), the appropriate dietary lysine supplementation could reduce lipid accumulation in the liver of juvenile largemouth bass.

5. Conclusions

Overall, the optimal dietary lysine level for juvenile largemouth bass was determined to be 3.03% and 3.07% of the diet, according to quadratic regression analysis based on
the FCR and SGR, respectively. Appropriate dietary lysine levels (3.33%) promoted glycolysis without affecting gluconeogenesis, inhibited lipid synthesis, and promoted lipolysis in the liver by regulating the AKT/FoxO1 pathway (Figure 9), resulting in decreased hepatic lipid content. Conversely, high lysine levels do not effectively regulate glycolipid levels and may lead to amino acid imbalances, causing arginine-lysine antagonism or increased lysine degradation, forcing the fish to consume more energy, and thus leading to reduced growth performance.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Authors’ Contributions**

Mingchun Ren designed the study. Dongyu Huang carried out the experiments and wrote the manuscript. Hualiang Liang reviewed the manuscript. Jian Zhu, Songlin Li, Xiaoru Chen, and Yongli Wang provided technical assistance. Xianping Ge provided technical guidance. All authors contributed to the article and approved the submitted version.

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