Dietary Riboflavin Requirement of Juvenile Grass Carp (Postprint)

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Abstract

Six purified diets with riboflavin levels of 0.54, 2.32, 4.08, 5.78, 9.28, and 19.35 mg/kg were formulated and fed to juvenile grass carp with an initial average weight of (11.21$±$0.16) g for 8 weeks to determine the dietary riboflavin requirement of juvenile grass carp by investigating the effects of riboflavin on growth, D-amino acid oxidase (D-AAO) activity in the hepatopancreas, digestive enzyme activity in the intestine, and body composition. Each diet had three replicates, with 20 fish per replicate. The results showed that the survival rate of juvenile grass carp in the 0.54 and 2.32 mg/kg groups was significantly lower than that in the other groups ($P<0.05$). With increasing dietary riboflavin content, the weight gain rate, specific growth rate, feed efficiency, D-AAO activity in the hepatopancreas, and digestive enzyme activity in the intestine of juvenile grass carp all increased initially and then plateaued, reaching maximum values when the dietary riboflavin content was 5.78 mg/kg. The hepatosomatic index in the 5.78 mg/kg group was significantly higher than that in the 0.54 mg/kg group ($P<0.05$), but dietary riboflavin content had no significant effect on the viserosomatic index, condition factor, or the moisture, crude protein, crude lipid, and crude ash contents of whole fish ($P>0.05$). Broken-line regression analysis indicated that the dietary riboflavin requirement for optimal growth of juvenile grass carp was 5.54 mg/kg, while the dietary riboflavin requirement for optimal D-AAO activity in the hepatopancreas was 5.99 mg/kg.

Full Text

Dietary Riboflavin Requirement of Juvenile Grass Carp (Ctenopharyngodon idellus)

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Abstract

Six purified diets containing riboflavin levels of 0.54, 2.32, 4.08, 5.78, 9.28, and 19.35 mg/kg were formulated and fed to juvenile grass carp with an initial mean weight of (11.21$±$0.16) g for 8 weeks to determine the dietary riboflavin requirement based on growth performance, hepatopancreas D-amino acid oxidase (D-AAO) activity, intestinal digestive enzyme activities, and body composition. Each diet was assigned to three replicates with 20 fish per replicate. The results showed that survival rates in the 0.54 and 2.32 mg/kg groups were significantly lower than those in the other groups ($P<0.05$). Weight gain rate, specific growth rate, feed efficiency, hepatopancreas D-AAO activity, and intestinal digestive enzyme activities all increased initially and then plateaued with increasing dietary riboflavin content, reaching maximum values when dietary riboflavin content was 5.78 mg/kg. The hepatosomatic index in the 5.78 mg/kg group was significantly higher than that in the 0.54 mg/kg group ($P<0.05$), while dietary riboflavin content had no significant effects on viscerasomatic index, condition factor, or whole-body moisture, crude protein, crude lipid, and ash contents ($P>0.05$). Broken-line regression analysis indicated that the dietary riboflavin requirement for optimal growth of juvenile grass carp was 5.54 mg/kg, and the requirement for optimal hepatopancreas D-AAO activity was 5.99 mg/kg.

Key words: grass carp (Ctenopharyngodon idellus); riboflavin; requirement; growth; D-amino acid oxidase

Riboflavin (vitamin B2) is an essential water-soluble vitamin for animals that exists in the body as components of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD serve as prosthetic groups for many oxidoreductases (such as flavoproteins) and are widely involved in various oxidation-reduction reactions in the body, acting as hydrogen carriers. Therefore, riboflavin can promote the metabolism of carbohydrates, fats, and proteins, and plays a role in maintaining normal functions of skin, mucous membranes, and vision. When dietary riboflavin is insufficient, fish develop deficiency symptoms. The symptoms of riboflavin deficiency vary among fish species, with the only common symptoms being poor growth and anorexia; other deficiency symptoms are specific to particular species and include high mortality, uncoordinated swimming, photophobia, cataracts, fin erosion, short body, and darkened skin color [1].

To date, numerous scholars have investigated riboflavin requirements in fish.

The riboflavin requirements of common carp (Cyprinus carpio) [2], rainbow trout (Oncorhynchus mykiss) [2-4], blue tilapia (Oreochromis aureus) [5], hybrid tilapia (Oreochromis mossambicus × Oreochromis niloticus) [6], channel catfish (Ictalurus punctatus) [7], yellowtail (Seriola quinqueradiata) [8], hybrid striped bass (Morone chrysops × Morone saxatilis) [9], large yellow croaker (Pseudosciaena crocea R.) [10], Japanese seabass (Lateolabrax japonicus) [10], gibel carp (Carassius auratus gibelio) [11], and jade perch (Scortum barcoo) [12] have been determined, with requirements ranging from 2.7 to 11.0 mg/kg. D-amino acid oxidase (D-AAO) is a typical flavoprotein that uses FAD as a prosthetic group and oxidizes the amino group of D-amino acids to produce corresponding keto acids and ammonia [13]. This enzyme exhibits high activity in fish hepatopancreas, and its activity is closely related to riboflavin status; therefore, hepatopancreas D-AAO activity is considered a sensitive indicator for evaluating riboflavin requirements in fish [14]. Grass carp (Ctenopharyngodon idellus), as one of the four major traditional Chinese carps, ranks first in freshwater aquaculture production and holds an important position in the aquaculture industry. This study investigated the effects of dietary riboflavin content on growth performance, hepatopancreas D-AAO activity, intestinal digestive enzyme activities, and body composition of juvenile grass carp to explore the dietary riboflavin requirement and provide a theoretical basis for improving the development technology of formulated feeds for grass carp.

1.1 Experimental Diets

A basal diet was formulated using vitamin-free casein and gelatin as protein sources, fish oil and corn oil as lipid sources, and dextrin as carbohydrate source (Table 1). Riboflavin (Sigma, USA) was supplemented to the basal diet at levels of 0, 2, 4, 6, 10, and 20 mg/kg, resulting in six isonitrogenous and isoenergetic purified diets. The actual riboflavin contents in the diets, measured by high-performance liquid chromatography [15], were 0.54, 2.32, 4.08, 5.78, 9.28, and 19.35 mg/kg. During diet preparation, all ingredients were ground to pass through a 60-mesh sieve, mixed thoroughly according to formula proportions, and blended with appropriate water. The mixture was extruded into 1.2 mm diameter strips using a meat grinder, air-dried at room temperature, and finally cut into pellets of 0.5-1.2 mm diameter. The diets were stored at -20°C until use.

Table 1 Composition and nutrient levels of the basal diet (DM basis) %

Item	Content
Ingredient	
Casein (vitamin free)	38.00
Gelatin	12.00
Dextrin	28.00
Fish oil	3.00
Corn oil	3.00

Item	Content
Vitamin premix[1]	1.00
Mineral premix[2]	4.00
Choline chloride	0.50
Microcrystalline cellulose	10.50
Total	**100.00**
Nutrient level	
Dry matter	92.18
Crude protein	38.24
Crude lipid	6.83
Ash	4.92

[1] One kilogram of vitamin premix contained the following: VA 0.80 g, VD_3 0.06 g, VE 4.00 g, VK_3 8.00 g, coated VC 20.00 g, thiamin 2.00 g, pantothenic acid 6.00 g, pyridoxine 2.00 g, folic acid 0.50 g, niacin 15.00 g, VB_{12} 0.02 g, inositol 40.00 g, microcrystalline cellulose 901.62 g.

[2] One kilogram of mineral premix contained the following: $FeSO_4 \cdot 7H_2O$ 15 g, $CuSO_4 \cdot H_2O$ 0.3 g, $ZnSO_4 \cdot 7H_2O$ 10 g, $MnSO_4 \cdot H_2O$ 0.5 g, NaCl 30 g, $MgSO_4$ 40 g, $Ca(H_2PO_4)_2$ 400 g, KI 0.05 g, Na_2SeO_3 0.005 g, $CoCl \cdot 6H_2O$ 0.5 g, zeolite powder 503.645 g.

1.2 Feeding Management

The feeding trial was conducted in an indoor flow-through aquaculture system. Experimental fish were hatchery-reared juveniles that were acclimated to the basal diet (without riboflavin supplementation) for two weeks prior to the trial. Three hundred sixty healthy juvenile grass carp with uniform size and an initial mean weight of (11.21$±$0.16) g were randomly stocked into 18 cylindrical fiberglass tanks, with 20 fish per tank and 200 L water per tank. Each experimental diet was fed to three tanks. The trial lasted for 8 weeks. Fish were fed twice daily at 09:00 and 15:00 at a feeding rate of 4-6% of body weight, which was adjusted appropriately based on feeding response and growth. Throughout the experimental period, a flow-through system was maintained with a water flow rate of approximately 1.0 L/min per tank. Water temperature was 25-30°C, pH 7.3-7.6, dissolved oxygen concentration 6.2-7.4 mg/L, total ammonia nitrogen 0.05-0.10 mg/L, and nitrite nitrogen 0.02-0.07 mg/L.

1.3 Sample Collection and Analysis

At the end of the trial, fish were fasted for 24 h. Total weight and remaining fish number in each tank were recorded. Five fish were randomly sampled from each tank for whole-body proximate composition analysis. The remaining fish were individually measured for body length and weight, then dissected on an ice

tray to obtain viscera, hepatopancreas, and entire intestine for calculation of viscerasomatic index, hepatosomatic index, and condition factor. Hepatopancreas and entire intestine samples were collected, freed of fat and intestinal contents, and stored at -70°C for determination of hepatopancreas D-AAO activity and intestinal digestive enzyme activities.

Proximate composition of experimental diets and whole-body samples was determined according to AOAC (1984) [16] methods: moisture content by oven drying at 105°C, crude protein by Kjeldahl method, crude lipid by Soxhlet extraction using anhydrous ether as solvent, and ash content by muffle furnace incineration at 550°C.

Frozen hepatopancreas and intestine samples were thawed, blotted dry, weighed, and homogenized with 10 volumes of phosphate buffer solution (PBS) using a glass homogenizer in an ice bath. The homogenate was centrifuged at 10,000 r/min for 10 min at 4°C, and the resulting supernatant was collected as crude enzyme extract and stored at 4°C until analysis. D-AAO activity was determined by the keto acid method of Nagata et al. [17] and defined as the amount of enzyme that produced 1 mol pyruvate per minute per gram of tissue protein at pH 8.3 and 37°C, expressed as U/g prot. Protease activity was measured by the Folin method [18] and defined as the amount of enzyme that hydrolyzed casein to produce 1 g tyrosine per minute per milligram of protein at pH 7.5 and 37°C, expressed as U/mg prot. Amylase activity was determined by the Bernfeld method [19] and defined as the amount of enzyme that hydrolyzed soluble starch to produce 1 mol maltose per minute per milligram of protein at pH 6.9 and 25°C, expressed as U/mg prot. Lipase activity was measured using a commercial kit from Nanjing Jiancheng Bioengineering Institute and defined as the amount of enzyme that hydrolyzed 1 mol substrate per minute per gram of protein at 37°C, expressed as U/g prot. Protein concentration in crude enzyme extracts was determined by the Coomassie brilliant blue method [20] using bovine serum albumin as standard.

1.4 Calculation Formulas

Weight gain ratio (WGR, %) = 100 × (final mean weight - initial mean weight) / initial mean weight

Specific growth rate (SGR, %/d) = 100 × (ln final mean weight - ln initial mean weight) / experimental days

Survival rate (SR, %) = 100 × final fish number / initial fish number

Feed efficiency rate (FER) = (final total weight + dead fish total weight - initial total weight) / total feed intake

Viscerasomatic index (VSI, %) = 100 × viscera weight / body weight

Hepatosomatic index (HSI, %) = 100 × hepatopancreas weight / body weight

Condition factor (CF, g/cm^3) = 100 × body weight / body length^3

1.5 Statistical Analysis

Experimental data are presented as mean ± standard deviation (SD). One-way analysis of variance (one-way ANOVA) was performed using SPSS 13.0 statistical software. When significant differences were detected, Duncan' s multiple range test was applied for post-hoc comparisons, with $P<0.05$ considered statistically significant. Broken-line regression model analysis was conducted using weight gain rate and hepatopancreas D-AAO activity as response criteria to determine the dietary riboflavin requirement of juvenile grass carp.

2.1 Effects of Dietary Riboflavin Content on Growth Performance, Feed Efficiency, and Body Profile Indices

Juvenile grass carp fed diets containing 0.54 and 2.32 mg/kg riboflavin exhibited uncoordinated swimming, abnormal convulsions, and subsequent mortality during the mid-to-late culture period, along with poor feed intake and relatively slow growth. As shown in Table 2 , survival rate of juvenile grass carp increased significantly ($P<0.05$) and then plateaued ($P>0.05$) with increasing dietary riboflavin content. Weight gain rate, specific growth rate, and feed efficiency all increased initially and then stabilized with increasing dietary riboflavin content, with no significant changes observed when dietary riboflavin content reached 5.78 mg/kg and above ($P>0.05$). Dietary riboflavin content significantly affected hepatosomatic index ($P<0.05$), with the highest value observed in the 5.78 mg/kg group, which was significantly higher than that in the 0.54 mg/kg group ($P<0.05$). However, dietary riboflavin content had no significant effects on viscerasomatic index, condition factor, or whole-body moisture, crude protein, crude lipid, and ash contents ($P>0.05$). Using weight gain rate as the response criterion, broken-line regression analysis determined that the dietary riboflavin requirement for optimal growth of juvenile grass carp was 5.54 mg/kg (Figure 1 [Figure 1: see original paper]).

Table 2 Effects of dietary riboflavin content on growth performance, feed efficiency rate and body profile indices of juvenile grass carp

Item	Dietary riboflavin content (mg/kg)					
	0.54	2.32	4.08	5.78	9.28	19.35
Initial body weight (g)	11.07$±0.13	11.12±0.15	11.22±0.22	11.31±0.11	11.19±0.16	11.33±0.12		$Finalbodyweight(g)$	34.09±1

Values with different letter superscripts in the same row indicate significant differences ($P<0.05$). The same notation applies to subsequent tables.

2.2 Effects of Dietary Riboflavin Content on Hepatopancreas D-AAO Activity and Intestinal Digestive Enzyme Activities

As shown in Table 3 , hepatopancreas D-AAO activity and intestinal protease, amylase, and lipase activities of juvenile grass carp all increased initially and then plateaued with increasing dietary riboflavin content, reaching maximum values when dietary riboflavin content was 5.78 mg/kg. Using hepatopancreas D-AAO activity as the response criterion, broken-line regression analysis determined that the dietary riboflavin requirement of juvenile grass carp was 5.99 mg/kg (Figure 2 [Figure 2: see original paper]).

Table 3 Effects of dietary riboflavin content on hepatopancreas D-amino acid oxidase and intestinal digestive enzyme activities of juvenile grass carp

Item	Dietary riboflavin content (mg/kg)					
	0.54	2.32	4.08	5.78	9.28	19.35
D-AAO (U/g prot)	2.32$±0.10^a	2.89±0.06^b	3.13±0.08^c	3.77±0.12^d	3.71±0.07^d	3.63±0.10^d ||*Protease(U/mgprot)*|42.62±

2.3 Effects of Dietary Riboflavin Content on Body Composition

As shown in Table 4 , dietary riboflavin content had no significant effects on whole-body moisture, crude protein, crude lipid, or ash contents of juvenile grass carp (P>0.05).

Table 4 Effects of dietary riboflavin content on body composition of juvenile grass carp %

Item	Dietary riboflavin content (mg/kg)					
	0.54	2.32	4.08	5.78	9.28	19.35
Moisture	74.83$±1.01	74.48±1.26	74.13±0.76	73.40±0.61	73.38±0.98	73.38±0.78 ||*Crudeprotein*|14.55±0.71|14

In this study, riboflavin-deficient juvenile grass carp exhibited anorexia and relatively slow growth, consistent with most previously reported findings in fish [1], further confirming that poor growth and anorexia may be typical symptoms of riboflavin deficiency in fish. Riboflavin is widely involved in various oxidation-reduction reactions in the body in the forms of FMN and FAD. When riboflavin is deficient, the metabolism of carbohydrates, fats, and proteins may be affected, potentially influencing feed intake and growth in fish. During the mid-to-late culture period, juvenile grass carp exhibited uncoordinated swimming, abnormal convulsions, and subsequent mortality. Similar symptoms have been reported in rainbow trout [4], while other symptoms such as photophobia, cataracts, fin

erosion, short body, and darkened skin color were not observed in this study, possibly due to differences in fish species, size, and experimental conditions.

Riboflavin deficiency in juvenile grass carp resulted in poor growth, reduced feed efficiency, and increased mortality, similar to results reported in common carp [2], rainbow trout [2-4], and hybrid striped bass [9]. This study determined that the dietary riboflavin requirement for optimal growth of juvenile grass carp was 5.54 mg/kg, which is similar to the requirement for optimal growth of jade perch (5.73 mg/kg) [12], slightly higher than that of gibel carp (3.76 mg/kg) [11], hybrid striped bass (4.10 mg/kg) [9], channel catfish (4.30 mg/kg) [7], and Japanese seabass (4.89 mg/kg) [10], but slightly lower than that of large yellow croaker (6.23 mg/kg) [10]. The riboflavin requirement for fish growth may be related to factors such as species, size, experimental conditions, and evaluation criteria. Additionally, Serrini et al. [7] suggested that appropriate dietary nutrient composition could reduce the riboflavin requirement in fish.

As a flavoprotein, D-AAO activity has been used by many researchers to determine riboflavin requirements in fish [4-5,7,9-12]. In this study, dietary riboflavin content significantly affected hepatopancreas D-AAO activity in juvenile grass carp, and the response fit well with the broken-line model, indicating that hepatopancreas D-AAO activity is a sensitive indicator for evaluating riboflavin requirement in juvenile grass carp. This result is consistent with findings in rainbow trout [4], blue tilapia [5], channel catfish [7], hybrid striped bass [9], Japanese seabass [10], large yellow croaker [10], gibel carp [11], and jade perch [12]. D-AAO has broad substrate specificity and can oxidatively deaminate all D-amino acids except acidic amino acids D-glutamate and D-aspartate, thereby completing amino acid metabolism [13]. When dietary riboflavin is deficient, the oxidative deamination reaction mediated by FAD-dependent D-AAO would be blocked, affecting normal metabolism in fish. In this study, the growth trend of juvenile grass carp was basically consistent with the change in hepatopancreas D-AAO activity, suggesting that dietary riboflavin content may indirectly affect growth by influencing hepatopancreas D-AAO activity.

The intestine plays an important role in nutrient digestion and absorption in agastric fish, and its digestive capacity is closely related to digestive enzyme activities [21]. As an agastric fish, this study specifically investigated the effects of dietary riboflavin content on intestinal protease, amylase, and lipase activities in juvenile grass carp. To date, only Li et al. [22] have reported relevant findings in Jian carp (Cyprinus carpio var. Jian). The results of this study are generally consistent with their findings, showing that intestinal protease, amylase, and lipase activities all increased initially and then plateaued with increasing dietary riboflavin content. Li et al. [22] suggested that this might be the result of riboflavin content affecting the growth and development of hepatopancreas, as most fish lack gastric glands and their digestive enzymes are produced by the hepatopancreas; therefore, digestive enzyme activities are closely related to hepatopancreas growth and development. The finding that hepatosomatic index in juvenile grass carp increased initially and then plateaued with increasing

dietary riboflavin content in this study supports this viewpoint. The digestion of food in aquatic animals mainly depends on the catalytic decomposition by digestive enzymes; thus, digestive enzyme activities directly affect feed conversion efficiency and consequently fish growth [23]. In this study, the change trends of three digestive enzyme activities in the intestine of juvenile grass carp were basically consistent with the growth trend, suggesting that dietary riboflavin content may indirectly affect growth by influencing digestive enzyme activities.

Under the conditions of this study, dietary riboflavin content improved growth performance and feed utilization efficiency of juvenile grass carp. The dietary riboflavin requirement for optimal growth was 5.54 mg/kg, while the requirement based on hepatopancreas D-AAO activity was 5.99 mg/kg.

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Note: Figure translations are in progress. See original paper for figures.