Effect of Dietary Carbohydrate/Protein Ratios and Feeding Frequency on Carbohydrate Metabolism of Common Carp

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Abstract. A 8-week 3×2 two-factorial experiment was conducted to investigate effects of dietary carbohydrate to protein ratio (C5%/P32%, C10%/P30%, C20%/P28%) and feeding frequencies (fourth daily and twice daily) on the hepatic carbohydrate metabolic enzyme activities, regulatory factor contents related to glycometabolism, the insulin receptor (IR), GLUT2, hexokinase (HK), GLUT2 and SGLT1 mRNA expression levels of common carp with initial average weight of 55.37±3.55 g. Three replicates with 150 common carp were randomly allotted to one treatment with combination of dietary carbohydrate level and feeding frequency in a complete randomized design. The results indicated that: (1) Dietary C/P ratio and feeding frequency significantly influenced the amylase activities of midgut ($P<0.05$). Amylase activities of midgut of fish fed fourth daily were significantly lower than those fed twice daily ($P<0.05$). (2) The phosphofructokinase (PFK) and glucose 6 phosphate dehydrogenase (G6PD) activities were significantly affected only by dietary C/P ratio. In addition, the phosphoenolpyruvate carboxykinase (PEPCK) and Glycogen synthase (GS) activities were significantly affected by dietary C/P ratio and feeding frequency, and there was a significant positive correlation between those activities and dietary C/P ratio ($P<0.05$). (3) The IR, GLUT2 and HK mRNA expression levels of hepatopancreas and GLUT2 and SGLT1 mRNA expression levels of intestine were significantly affected by dietary C/P ratio and feeding frequency, which were significantly increased with increasing feeding frequency ($P<0.05$). Furthermore, the highest values of those expression levels were observed in the C10%/P30% group and these were significantly higher than those in other groups ($P<0.05$). Above all, fish fed C10%/P30% diet was suitable with feeding frequency at fourth daily in this study.

1. Introduction

Carbohydrate to protein ratio is an important indicator of evaluating the nutritive value of fish diet. On the one hand, appropriate carbohydrate level in diet is regarded as ideal energy-providing substance, satisfying basic metabolism of fish, conducive to growth, and improving dietary utilization [1]; on the other hand, moderate carbohydrate to protein ratio can enhance protein sparing effect of carbohydrate, and reduce ammonia-nitrogen excretion to water body. Therefore, it is of great economic and ecological significance to explore appropriate carbohydrate to protein ratio in the diet [2-4]. In actual aquaculture production, aquaculture effect is closely related to feeding frequency selected according to feeding rhythms of animals cultured [5]. Rational feeding frequency not only can accelerate the growth rate of fish and influence nutritional ingredients of fish, but also increase dietary utilization [6] and reduce environmental pollution [7]. As a result, it is crucial to determine a rational feeding frequency in aquaculture management. Studies showed that with the change in nutrition composition of diet, feeding frequency suitable for cultured fish varied correspondingly [7], determining fish growth, metabolism and dietary utilization by influencing the intake and absorption of nutrient
substance by fish. Therefore, in aquatic animal culture, in order to reduce culturing cost, it is significant to develop a reasonable feeding strategy and study an appropriate carbohydrate to protein ratio of fish diet and feeding frequency.

Common carp is one of the dominant freshwater aquaculture species in China. Recently, there are more and more studies of nutrition of its diet such as protein and carbohydrate and feeding strategy. Although the protein-sparing of dietary carbohydrate[8] or feed frequency[9] have been well demonstrated in common carp, most studies concerned mainly on one or other of two factors, and the interaction effect of two factors on growth, digestion and metabolism of common carp have received insufficient attention currently. Therefore, on the basis of studying the influence of interaction between carbohydrate to protein ratio of diet and feeding frequency on amylase activities, key enzyme activities of glycometabolism, and mRNA expression of glucose transporter in intestine of common carp, glycometabolism mechanism of common carp under different feeding frequencies and carbohydrate to protein ratios was explored, providing a theoretical basis for exploring an efficient and reliable feeding frequency and dietary formulation of common carp.

2. Ingredients and Methods

2.1. Experimental Diets
Taking fish meal, soybean meal, rapeseed meal, cotton meal and peanut meal as protein source, soybean oil as the lipid source, and wheat starch as carbohydrate source, three groups of equal-energy and equal-fat experimental diet with the carbohydrate/protein level of C5% / P32%, C10% / P30% and C20% / P28% (C - carbohydrate, P - protein) respectively were prepared (Table 1). Diet was prepared in Tianjin Tianxiang Aquaculture Co., Ltd. First, all dry raw materials were smashed with a pulverizer, passed through a 60-mesh screen, and were mixed evenly with a mixing machine according to the principle of step-by-step scaling up. Then soybean oil was added according to dietary formula ratio and mixed completely in the mixed machine according to the principle of step-by-step scaling up. Then soybean oil was added according to dietary formula ratio and mixed completely in the mixed machine. Next, sinking pellet diet with the diameter of 3.00 mm was made from the mixture with a feed stuff cuber (Jiangsu Muyang Group, MUZLM V4). After natural withering, the diet was preserved in a refrigerator at a temperature of - 20 °C.

| Ingredients (%) | Diet | C5P32 | C10P30 | C20P28 |
|----------------|------|-------|--------|--------|
| Fish meal      | 7    | 5     | 3      |
| Soybean meal   | 22   | 22    | 22     |
| Peanut meal    | 15   | 15    | 15     |
| Cotton meal    | 12   | 12    | 12     |
| Rapeseed meal  | 13   | 13    | 13     |
| Microcrystalline Cellulose | 12 | 8.8   | 0.6    |
| DDGS          | 3    | 3     | 3      |
| Soybean oil    | 5    | 5.2   | 5.4    |
| Premix         | 2    | 2     | 2      |
| Wheat starch   | 5    | 10    | 20     |
| CMC-Na         | 2    | 2     | 2      |
| Monocalcium phosphate | 2 | 2     | 2      |

| Nutrient levels | C5P32 | C10P30 | C20P28 |
|-----------------|-------|--------|--------|
| Crude protein (% DM) | 31.94 | 30.46 | 28.38 |
| Crude lipid (% DM)  | 6.66  | 6.69  | 6.67  |
| Energy (MJ/kg DM)   | 16.21 | 16.23 | 16.25 |

Note: 1Fish meal: 62.3% crude protein, 12% crude lipid; soybean meal: 43.7% crude protein; peanut meal: 46.2% crude protein; cotton meal: 43.3% crude protein; rapeseed meal: 36.2% crude protein; 2DDGS: dried distillers grains with solubles, 26% crude protein, 8% crude lipid; 3same as cheng et al.
(2017)[4]; 4 calculated as protein: 24 kJ/g; fat: 38 kJ/g; and starch: 17 kJ/g.

2.2. Experimental Fish and Aquaculture Management

Experimental fish were purchased from Tianjin Huanxin Aquatic Product Improved Variety Station. Temporary culture and aquaculture test were finished in Tianjin Tianxiang Aquaculture Co., Ltd. After disinfection treatment, fish were cultured in a net cage temporarily (3 m × 3 m × 3 m) for 7 days of acclimatization to make them adapt to the aquaculture environment gradually. During acclimatization, commercial diets with 32% protein were fed to fish.

After 7 days of temporary culture, fish were fasted for 24 h. 900 experimental healthy carps with a uniform specification and initial weight of (55.73 ± 3.55) g were put into submersible cages with the size of 1.0 m × 1.0 m × 1.5 m and divided into 6 groups, with three replicates in each group. During the whole experimental period, a fixed daily feeding rate (4% BW·d⁻¹) was adopted. A 3×2 two-factorial experiment was designed. The feeding frequency of each group was twice a day (8:00 am, 17:00 pm) and four times a day (8:00 am, 11:30 am, 14:00 pm, 17:00 pm), which were named C5% /P32%/F2, C5% /P32%/F4, C10% /P30%/F2, C10% /P30%/F4, C20% /P28%/F2 and C20% /P28%/F4 respectively (C - carbohydrate, P - protein, F - feeding frequency). Each treatment had three replicates. During the experiment, weight of fish in each net cage was evaluated once every 14 days and feeding amount was adjusted according to the determined feeding rate. Water temperature was 28°C - 32°C. Dissolved oxygen was about 6.0 mg·L⁻¹ and pH was 7.6 - 8.0.

2.3. Sample Collection

After 8 weeks of culture, the experimental fish were fasted for 24 hours. Then the experimental fish were counted and weighed to calculate survival rate and weight gain rate; from each cage, 20 fish were randomly selected respectively and their body lengths were measured to calculate fatness; tail venous blood was collected into centrifuge tubes and was centrifuged for 20 minutes with a benchtop high speed refrigerated centrifuge at 4 °C with the revolving speed of 4500 r·min⁻¹ to obtain supernatant and prepare serum; experimental fish after blood drawing were dissected rapidly in an ice plate to obtain the hepatopancreas which were weighed to calculate liver and body weight ratio. In addition, the intestines were obtained, and samples were preserved in a refrigerator with a ultra-low temperature of - 80 °C.

2.4. Measurement of Biochemical Indexes

In the experiment, enzyme linked immunosorbent assay (ELISA) kit that was used to measure the activities of carbohydrate metabolic enzymes in the hepatopancreas, and concentrations of relevant hormone in serum (insulin, glucagon, growth hormone) and regulatory factors (insulin receptor, insulin-like growth factor) was produced by Shanghai Enzyme-linked Biotechnology Co., Ltd. Its main principle is to measure the activities of enzymes and concentrations of substances in serum or tissue-related liquid samples with a double antibody sandwich method.

2.5. Gene Expression of Insulin Receptor, Relevant Transporter and Metabolic Enzymes

After the aquaculture experiment, 24 hours of fasting was carried out and then each group was fed with respective diet. 2 hours from then, from each group, three fish were selected randomly. Their livers and intestines were obtained and ground in liquid nitrogen. Total RNA of liver tissues was extracted with Trizol method. According to the instruction of TaKaRa PrimeScript™ 1st Strand cDNA Synthesis Kit (Code: 6110A), total RNA was transcribed into cDNA reversely.

According to conserved sequences of common carp's insulin receptor (IR) (EU009571.1), glucose transporter (GLUT2) (AF247730.1), sodium-glucose cotransporter (SGLT1) (JN867793.1), hexokinase (HK) (AF119837.1) and β-actin (M24113) in Genbank, primers were designed, IR: 5’ CAACCTCTGGTGATGG 3’ (F) and 5’ CCATCTGGATTTTCA 3’ (R), GLUT2: 5’ ACTCTCTGTTGGTTTCA 3’ (F) and 5’ CCATCTCACGCTTCTTCA 3’ (R), SGLT1: 5’ GACTAAAGAAGAAGAGGAG 3’ (F) and 5’ CCAGAGGATTGAGGAGGATA 3’ (R), HK: 5’ GAGAAGAAGAAAGAGGAG 3’ (F) and 5’ CCAGAGGATTGAGGAGGATA 3’ (R), β-actin: 5’ CCGTGACATCAAGGAGA 3’ (F) and 5’ GATACCGCAAGATTCCATAC 3’ (R). All primers
were synthesized by Suzhou GENEWIZ Biotechnology Co. Ltd.

Real time fluorescent quantitative PCR was measured with SYBR Green-dyed under the Bio-Rad iQ5 Real Time PCR System according to the instruction of SYBR® Premix DimerEraser™ (Perfect Real Time) (Code: RR091A) kit. The data were analyzed and compared with $\Delta\Delta Ct$, to obtain relative expression levels of genes in each template. The calculation formula was: Relative expression level $= 2^{-\Delta\Delta Ct} = 2^{-(Ct_{Process}-Ct_{Reference})-(Ct_{Control}-Ct_{Reference})}$. The average of three replicates was calculated and adopted.

2.6. Data Analysis

All data were analysed using SPSS 17.0 statistical software package for windows and are presented as the mean ± SD (n = 3). Data from each treatment were submitted to two-way analysis of variance (ANOVA). When a significant interaction was observed, data were analysed using one-way ANOVA followed by Duncan's multiple range tests to inspect all differences among the dietary treatments. P-values equal to or less than 0.05 indicated statistical significance.

3. Results and Analysis

3.1. Effects of Dietary Carbohydrate to Protein Ratios and Feeding Frequency on the Amylase Activities in Intestine of Common Carp

Amylase activities in foregut and hindgut of fish were not affected by either dietary carbohydrate to protein ratios or feeding frequency (Table 2, $P > 0.05$). Amylase activities in midgut of fish maintained at a feed frequency of 4 meal/d were significantly lower than those of fish at 2 meal/d ($P < 0.05$). Besides, amylase activities in midgut of fish were significantly affected by diet composition with the lowest observed in fish fed with diet C20P28 ($P < 0.05$).

Table 2. Effects of dietary carbohydrate to protein ratios and feeding frequency on the amylase activities in intestine of common carp (μg/ml)

| groups | Foegut  | Midgut  | Hindgut |
|--------|---------|---------|---------|
| C5% /P32%/F4 | 100.82±31.90 | 101.36±50.90 | 68.95±23.64 |
| C10% /P30%/F4 | 124.28±40.70 | 130.16±35.90 | 83.66±63.16 |
| C20% /P28%/F4 | 119.72±57.60 | 60.54±21.91 | 75.97±48.57 |
| C5% /P32%/F2 | 128.83±48.87 | 149.17±16.57 | 121.72±16.59 |
| C10% /P30%/F2 | 157.81±64.91 | 175.15±26.79 | 124.79±26.15 |
| C20% /P28%/F2 | 148.85±39.94 | 90.63±49.44 | 107.11±49.70 |

Main effects

| C/P | Foegut | Midgut | Hindgut |
|-----|--------|--------|---------|
| C5% /P32 | 115.88 | 139.66$^a$ | 105.34 |
| C10% /P30 | 146.26 | 138.25$^a$ | 104.26 |
| C20% /P28 | 134.29 | 63.08$^a$ | 91.54 |

Frequency

| Frequency | Foegut | Midgut | Hindgut |
|-----------|--------|--------|---------|
| 4 | 114.94 | 89.02$^p$ | 86.19 |
| 2 | 145.16 | 138.32$^p$ | 127.87 |

Two-way ANOVA

| Frequency | C/P | Interaction |
|-----------|-----|-------------|
| 0.84 | 0.02 | 0.06 |
| 0.87 | 0.01 | 0.90 |
| 0.34 | 0.46 | 0.87 |

3.2. Effects of Dietary Carbohydrate to Protein Ratios and Feeding Frequency on the Activities of Carbohydrate Metabolic Enzymes in the Hepatopancreas of Common Carp

As shown in Table 3, dietary C/P ratio had significant influence on the activity of PFK and G6PD of the hepatopancreas of common carps ($P < 0.05$), and had no significant influence on PK activity ($P > 0.05$); feeding frequency had no significant influence on the activity of G6PD and PK of the hepatopancreas of common carps ($P > 0.05$). Interaction between dietary C/P ratio and feeding
frequency had no significant influence on key enzyme activities of glucose decomposition approach ($P > 0.05$). Under each dietary C/P ratio, the activities of PFK, G6PD and PK of hepatopancreas increased when the feeding frequency increased from twice a day to four times a day, but the difference was not significant ($P > 0.05$). In the two feeding groups, the activities of PFK, G6PD and PK of hepatopancreas decreased with the increase in dietary C/P ratio. The activities of PFK and G6PD of the C5% / P32% group were significantly higher than that of the C20% / P28% group ($P < 0.05$). In addition, there was significant difference in the activities of G6PD between the C10% / P30% / F2 group and the C20% / P28% / F2 group ($P < 0.05$). PK activity did not decrease significantly with the increase of dietary C/P ratio ($P > 0.05$).

Dietary C/P ratio and feeding frequency had significant influence on PEPCK and GS activities in hepatopancreas of common carps ($P < 0.05$), but did not have significant influence on the interaction between PEPCK and GS activities therein ($P > 0.05$). Under each dietary C/P ratio, the activities of PEPCK and GS of hepatopancreas increased significantly when the feeding frequency increased from twice a day to four times a day ($P < 0.05$). In the C10% / P30% group, PEPCK and GS activities of the F4 group were significantly higher than those of the F2 group ($P < 0.05$); in the C20% / P28% group, PEPCK activities of the F4 group were significantly higher than those of the F2 group ($P < 0.05$). In the two feeding groups, PEPCK and GS activities in hepatopancreas decreased significantly with the increase of dietary C/P ratio ($P < 0.05$). There was significant difference in PEPCK activities between the C5% / P32% group and the C20% / P28% group ($P < 0.05$); the difference in GS activities among three groups with different C/P ratios was significant ($P < 0.05$).

### Table 3. Effects of dietary carbohydrate to protein ratios and feeding frequency on key enzyme activities of carbohydrate metabolic enzymes in the hepatopancreas of common carp (U·g$^{-1}$)

| groups          | PFK     | PK       | G6PD     | PEPCK    | GS       |
|-----------------|---------|----------|----------|----------|----------|
| C5% / P32% / F4 | 370.17±25.92 | 365.85±19.81 | 10.81±0.82 | 43.31±1.30 | 66.79±0.30 |
| C10% / P30% / F4| 304.07±17.78 | 329.67±21.90 | 8.36±0.23 | 34.26±1.09 | 59.58±0.86 |
| C20% / P28% / F4| 213.30±22.80 | 308.74±29.62 | 7.66±0.28 | 26.70±7.88 | 23.47±3.57 |
| C5% / P32% / F2 | 309.56±17.93 | 317.61±15.65 | 10.45±0.37 | 38.78±4.64 | 64.79±4.64 |
| C10% / P30% / F2| 245.86±21.89 | 296.80±22.37 | 8.84±0.20 | 22.44±2.81 | 46.83±3.24 |
| C20% / P28% / F2| 208.37±21.15 | 290.66±21.79 | 7.17±0.28 | 17.83±1.26 | 19.00±2.04 |

Main effects
- C/P
  - C5% / P32
    - $339.86^x$
    - $10.63^x$
    - $331.32^x$
    - $41.05^x$
    - $65.79^x$
  - C10% / P30
    - $274.97^y$
    - $8.60^y$
    - $323.64^y$
    - $28.35^y$
    - $53.20^y$
  - C20% / P28
    - $210.84^z$
    - $7.42^z$
    - $299.70^z$
    - $22.26^z$
    - $21.24^z$
- Frequency
  - 4
    - $295.84^p$
    - $8.94^p$
    - $334.75^p$
    - $34.76^p$
    - $49.95^p$
  - 2
    - $254.59^q$
    - $8.82^q$
    - $301.69^q$
    - $26.35^q$
    - $43.54^q$

Two-way ANOVA
- Frequency
  - 0.04
  - 0.12
  - 0.75
  - 0.01
  - 0.00
- C/P
  - 0.00
  - 0.42
  - 0.00
  - 0.00
  - 0.04
- Interaction
  - 0.37
  - 0.46
  - 0.52
  - 0.50
  - 0.07

3.3. Effects of Dietary Carbohydrate to Protein Ratios and Feeding Frequency on Hormones and Regulatory Factors Related To Serum Glycometabolism of Common Carp

As shown in Table 4, dietary C/P ratio and feeding frequency had significant influence on INS, GC and GH concentrations in serum ($P < 0.05$), and interaction between dietary C/P ratio and feeding frequency had significant influence on GH concentration ($P < 0.05$). Under each dietary C/P ratio, with the increase of feeding frequency, INS concentration in serum increased significantly ($P < 0.05$), but GC concentration and growth hormone did not increase significantly ($P > 0.05$). In the C20% / P28% group, INS concentration of the F4 group was significantly higher than that of the F2 group ($P < 0.05$); in the C10% / P30% group, GH concentration of the F4 group was significantly lower than that of the F2 group ($P < 0.05$). In the two feeding groups, serum INS contents increased significantly with the increase of dietary C/P ratio ($P < 0.05$), while GC and GH contents decreased significantly ($P$
< 0.05).

Dietary C/P ratio and feeding frequency had significant influence on serum GLU, ISR, and IGF-1 concentrations ($P < 0.05$), but interaction between dietary C/P ratio and feeding frequency did not have significant influence on above indexes ($P > 0.05$). Under each dietary C/P ratio, GLU concentration decreased significantly ($P < 0.05$) when the feeding frequency increased from twice a day to four times a day, while IST and IGF-1 concentrations increased significantly ($P < 0.05$). In the C5% /P32% group, GLU and IGF-1 concentrations of the F4 group were significantly lower than those of the F2 group ($P < 0.05$); in the C10% /P30% group, ISR and IGF-1 concentrations of the F4 group were significantly higher than those of the F2 group ($P < 0.05$); in the C20% /P28% group, GLU concentration of the F4 group decreased significantly ($P < 0.05$). In the two feeding groups, GLU concentrations increased significantly with the increase of dietary C/P ratio, and that of the C10% /P30% group and C20% /P28% group was significantly higher than that of the C5% /P32% group ($P < 0.05$); ISR concentration of the C10% /P30% group was the highest, and was significantly higher than that of the other two groups ($P < 0.05$); IGF-1 concentration of the C20% /P28% group was the lowest, and was significantly lower than that of the C5% /P32% group ($P < 0.05$).

**Table 4.** Effects of dietary carbohydrate to protein ratios and feeding frequency on hormone contents related to serum glycometabolism of common carp

| groups   | INS/ (mmol·L$^{-1}$) | GC/ (ng·L$^{-1}$) | GH/ (μg·L$^{-1}$) | GLU/ (mmol·L$^{-1}$) | ISR/ (nmol·L$^{-1}$) | IGF-1/ (μg·L$^{-1}$) |
|----------|-----------------------|-------------------|-------------------|-----------------------|----------------------|----------------------|
| C5% /P32% /F4 | 24.70±0.35             | 611.20±51.27      | 10.86±0.72        | 6.25±0.29             | 86.58±2.51           | 27.28±1.30          |
| C10% /P30% /F4 | 27.80±0.60             | 702.55±9.44       | 14.73±0.60        | 8.92±0.97             | 116.95±13.21         | 23.91±1.18          |
| C20% /P28% /F4 | 31.90±1.30             | 728.74±9.23       | 17.98±0.61        | 8.79±0.84             | 81.87±2.59           | 17.39±0.31          |
| C5% /P32% /F2   | 23.10±0.71             | 634.49±12.83      | 13.33±1.23        | 8.08±0.11             | 75.49±2.59           | 23.19±0.89          |
| C10% /P30% /F2   | 25.90±0.40             | 757.12±12.28      | 17.81±0.09        | 9.17±0.63             | 91.98±3.56           | 19.17±1.39          |
| C20% /P28% /F2   | 28.40±0.40             | 777.37±7.43       | 26.03±1.32        | 10.66±0.36             | 69.35±2.98           | 17.32±1.53          |

3.4. Effects of Dietary Carbohydrate to Protein Ratios and Feeding Frequency on Glycometabolism Related Gene Expression Levels of Common Carp

As shown in Table 5, dietary C/P ratio and feeding frequency had significant influence on IR, GLUT2 and HK mRNA expression levels in hepatopancreas of common carp ($P < 0.05$). In addition, interaction between dietary C/P ratio and feeding frequency had significant influence on IR mRNA expression level in hepatopancreas ($P < 0.05$). IR, GLUT2 and HK mRNA expression levels in hepatopancreas increased significantly when the feeding frequency increased from twice a day to four times a day ($P < 0.05$). With the increase of dietary C/P ratio, IR, GLUT2 and HK mRNA expression levels in hepatopancreas of the C10% /P30% group were the highest and significantly higher than those of the other two groups ($P < 0.05$).

Dietary C/P ratio and feeding frequency had significant influence on the GLUT2 and SGLT mRNA expression levels in intestine ($P < 0.05$), but they did not had significant interactive effect on the GLUT2 and SGLT1 mRNA expression levels ($P > 0.05$). With the increase of feeding frequency, the GLUT2 and SGLT1 mRNA expression levels in intestine increased significantly ($P < 0.05$). In the C10% /P30% group, the GLUT2 and SGLT1 mRNA expression levels in the intestine of the F4 group were significantly higher than those of the F2 group ($P < 0.05$). With the increase of dietary C/P ratio,
the GLUT2 and SGLT1 mRNA expression levels in intestine increased first and then decreased, and those of the C10% /P30% group were the highest and significantly higher than those of the other two groups ($P < 0.05$).

**Table 5.** Effects of dietary carbohydrate to protein ratios and feeding frequency on glycometabolism related gene expression levels of common carp

| Groups          | Hepatopancreas |   | Intestine |   |   |
|-----------------|----------------|---|-----------|---|---|
|                 | IR  | GLUT2 | HK | GLUT2 | SGLT1 |
| C5% /P32%/F4    | 1.00±0.00$^b$ | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 | 1.00±0.00 |
| C10% /P30%/F4   | 1.80±0.34$^a$ | 1.81±0.26 | 1.39±0.19 | 2.37±0.59 | 4.16±1.58 |
| C20% /P28%/F4   | 0.54±0.10$x$ | 0.97±0.08 | 0.84±0.04 | 0.68±0.08 | 1.29±0.13 |
| C5% /P32%/F2    | 0.11±0.00$^c$ | 0.53±0.14 | 0.05±0.01 | 0.13±0.02 | 0.45±0.05 |
| C10% /P30%/F2   | 0.42±0.05$^c$ | 1.05±0.15 | 0.20±0.02 | 0.68±0.04 | 2.06±1.18 |
| C20% /P28%/F2   | 0.20±0.03$^c$ | 0.47±0.09 | 0.08±0.02 | 0.21±0.08 | 0.33±0.06 |

Main effects

| C/P             | Frequency | 4   | 2   | 4   |
|-----------------|-----------|-----|-----|-----|
| C5% /P32        | 1.11$^p$  | 1.26$^p$ | 1.08$^p$ | 1.34$^p$ | 2.15$^p$ |
| C10% /P30%      | 0.34$^q$  | 0.68$^q$ | 0.11$^q$ | 0.34$^q$ | 0.94$^q$ |
| C20% /P28%      | 1.11$^p$  | 1.26$^p$ | 1.08$^p$ | 1.34$^p$ | 2.15$^p$ |

Two-way ANOVA

| Frequency | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| C/P       | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Interaction | 0.03 | 0.57 | 0.09 | 0.12 | 0.30 |

4. Discussion

As for fish, 2-hour absorption percentage is key to dietary carbohydrate absorption and utilization[10]. In the experiment, dietary C/P ratio and feeding frequency were improved to increase 2-hour carbohydrate absorption percentage of fish. Activity of digestive enzyme is the primary factor reflecting absorption and utilization of carbohydrate in fish.

The increase of dietary C/P ratio can increase the contacting amount and time between dietary carbohydrate and amylase in intestine. But study in *Silurus meridionalis* Chen[11] and study of *Pseudosciaena crocea* R. [12] found that amylase activity did not increase with carbohydrate level, instead, it has certain conservation. However, in the study, with the increase of C/P ratio, amylase activities in intestine first increased and then decreased. Moreover, amylase in midgut was significantly affected by dietary C/P ratio, and amylase activity of the C10% /P30% group was the highest and significantly higher than that of the C20% /P28% group. This indicated that a too high concentration of carbohydrate in diet inhibits amylase activity to a certain extent, which was similar to the study in *Silurus meridionalis* Chen [11] and study of *Pseudosciaena crocea* R[12].

Feeding frequency increases 2-hour dietary carbohydrate absorption percentage of fish by reasonably controlling feeding speed of fish. However, increase in feeding frequency leads to certain dietary restriction effect, and changes in digestive enzyme affect the utilization of dietary nutrient substances by fish. The study indicated that under each dietary C/P ratio, activities of amylase in foregut, midgut and hindgut of the F4 group decreased. Cui[13] pointed out that increase in feeding frequency led to increase in amylase activity in the liver and activity of digestive enzyme in the intestine of Actipenser gueldenstaedtii. Difference in feeding habit of the experimental fish may be the main reason for the above difference.

In the experiment, after 24 hours of fasting, blood glucose levels of common carp showed that: C20% /P28% group > C10% /P30% group > C5% /P32% group, which reflected the digestion and absorption rate of dietary starch by fish to some extent[14]. The key to full use of starch by fish is whether glycometabolism regulation and relevant hormones regulation match changes in blood glucose. The experiment revealed that during regulating key enzymes of glucose synthesis approach, activities of key enzyme of gluconeogenesis - PEPCK and key enzyme of glycogen synthesis - GS
decreased significantly with the increase in dietary C/P ratio. This revealed that in the premise of maintaining dietary energy constant, appropriate increase in C/P ratio can inhibit gluconeogenesis, reduce the activity of key enzyme of gluconeogenesis, which conformed to findings in studies of common carp[15-16] and Rachycentron canadum[17]; during regulating key enzymes of glucose decomposition approach, the activities of key enzymes PFK and PK during glycolysis and the activity of key enzyme G6PD of the pentose phosphate pathway decreased significantly with the increase in dietary C/P ratio. However, Cheng[4] stated that increase in dietary corn starch level promoted the increase in PK activity in hepatopancreas of common carp. Enes[18] reported that the increase in dietary carbohydrate level induced the enhancement of G6PD activity of fish. It is inferred that difference in fish's adaptability to different carbohydrates results in the above differences. However, it is clear that besides glucose decomposition and synthesis approaches, adaptability to hormone regulation also affects the ability of glycometabolism of fish. The experiment found that concentrations of insulin and IGF-1 with the function of reducing blood glucose increased significantly with C/P ratio, which conformed to the results of study by Baños[19]. However, significant increase in concentration of glucagon that can inhibit glucose utilization shows that regulating mechanism of glycometabolism in fish is still not perfect.

Hung and Storebakken[20] reported that under continuous feeding conditions, G6PD activities in the livers of Oncorhynchus mykiss of the glucose and maltose groups were higher than that of the F4 group, which indicated that increase in feeding frequency can enhance fish's ability to make use of low-molecular carbohydrate to some extent. The experiment indicated that compared with the F2 group, PFK, PK and G6PD activities and insulin and IGF-1 concentrations in hepatopancreas of the F4 group were significantly higher, while glucagon and growth hormone concentrations were significantly lower. This reveals that proper increase of feeding frequency promotes fish's ability to digest and absorb polysaccharides such as starch. Therefore, after 24 hours of fasting, blood glucose level of the F4 group was lower, which proves that increasing feeding frequency, is conducive to the saving effect of dietary carbohydrate on protein.

Galgarber pointed out that SGLT1 and GLUT2 mRNA expressions in animals were influenced by nutrition supply[21]. The study indicated that amylase activities in foregut, midgut and hindgut of the C10% /P30% group were all the highest. Meanwhile, mRNA expression levels of SGLT1 and GLUT2 in the intestine of the C10% /P30% group were the highest. Hepatopancreas is the key tissue to regulate blood glucose concentration in fish. Glycolysis, gluconeogenesis and pentose phosphate cycle in glycometabolism and insulin secretion are all carried out in hepatopancreas. Therefore, glucose transporter, insulin receptor and HK mRNA expressions in hepatopancreas also determine glycometabolism ability of hepatopancreas. Moreover, the experiment also indicated that when dietary C/P ratio was C5% /P32% - C10% /P30%, GLUT2, IR and HK mRNA expression levels were positively correlated to blood glucose concentration and insulin secretion and increased significantly. Study on Oncorhynchus mykiss[22] also obtained similar findings; however, when C/P ratio was over C10% /P30%, changes in GLUT2, IR and HK mRNA expression levels were opposite to changes in blood glucose concentration and insulin secretion. Changes in above mRNA expression levels were same with the changes in amylase activity in intestine, which reveals that a too high C/P ratio restricts mRNA expression of glucose transporter. Therefore, this may be one reason why fish has the constitution of high glucose intolerance.

5. Conclusion
Above experimental results shows that increase in feeding frequency inhibits amylase activity in the intestine to some extent. However, mRNA expression levels of SGLT1 and GLUT2 in intestine, GLUT2, IR and HK in hepatopancreas, and key enzymes activities of glycolysis increased significantly with feeding frequency. This indicates that increase in feeding frequency inhibits the utilization of nutrient substance in fish, but fish makes full use of limited nutrient substance by regulating factors related to glycometabolism. In this study, diets containing 10% carbohydrate and 30% protein are suitable for common carp and its feeding frequency is fourth daily. It is necessary to further study on the regulatory mechanism by the influence of dietary carbohydrate to protein ratio and feeding frequency.
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7. References

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