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Dietary calcium requirements of bighead carp (*Aristichthys nobilis*)

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Abstract

To investigate the dietary calcium requirement of bighead carp (*Aristichthys nobilis*), six purified diets were formulated to contain different concentrations of calcium (0.09% (control), 0.43%, 0.76%, 1.12%, 1.44%, and 1.79% of dry diets). Each diet was hand-fed to triplicate 30 fish with average initial body weight (3.31 ± 0.09 g) for 60 days. The results showed that weight gain (WG) and specific growth rate (SGR) significantly increased when the dietary calcium level was from 0.09% to 0.76% ($P < 0.05$). The phosphorus and calcium contents of the whole fish body were highest in the 0.76% and 1.12% groups, respectively ($P < 0.05$). The serum phosphorus content in the 1.79% group was significantly lower than in other groups ($P < 0.05$). As dietary calcium content was from 0.09% to 0.76%, the activities of lipase and proteinase in the intestine significantly increased ($P < 0.05$). In contrast, the glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities were significantly decreased ($P < 0.05$). Based on quadratic curve model analysis with WG and WGR as the appraising criteria, the appropriate dietary requirement of calcium for the bighead carp larvae (3.31 ± 0.09 g) was 1.01% - 1.02%.

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Introduction

Calcium (Ca) is required by aquatic organisms for growth, bone mineralization, and other physiological functions such as blood clotting, muscle functioning, and the transmission of nerve impulses (NCR 2011). Ca plays a key role in the ionic regulation of freshwater fish because it influences biological membrane permeability and inhibits diffusive efflux and excessive loss of ions to the surrounding water (Wood & McDonald, 1988). It is also essential for activating several enzymes and stimulating muscle contraction (Hossain & Yoshimatsu, 2014). Approximately 99% of the body calcium of teleost is incorporated into bone and scales, which may act as internal calcium and phosphorus reservoirs (Flik & Fenwick, 1986). Ca is present dissolved in water. Unlike terrestrial animals, marine and freshwater fish can obtain all or part of Ca from water to meet their requirement for growth and immunity (Flik et al., 1995). However, dietary supplementation of Ca is still required by fish because of the low concentration of Ca in the water and Ca demand in intensive farming (Ichikawa & Oguri, 1961; Steffens, 1997). Signs of impaired growth and poor skeleton mineralization may occur in aquatic organisms when fed a diet with calcium deficiency (Vielma & Lall, 1998; Ye et al., 2006). The overdose of dietary calcium harms average growth and the absorption of other mineral nutrients, such as phosphorus, magnesium, and zinc (Hardy & Shearer, 2011; Iii & Phillips, 1989; Vielma & Lall, 1998). Meanwhile, excessive supplementation of calcium in the diet increases farming costs and releases mineral elements into the aquatic environment (Cheng et al., 2006). Consequently, studying the calcium requirements of cultured fish is extremely important.

Bighead carp (*Aristichthys nobilis*) is one of China Field's most important aquaculture species (Tong & Sun, 2015). Furthermore, it is also distributed in Asia, Europe, and America (Kolar & Chapman, 2005). The total production of bighead carp has rapidly increased and reached approximately 3.13 million tons in 2020 in China (Fisheries Bureau of Agriculture Ministry of China 2021). In recent years, the nutrient requirements of bighead carp have been reported, such as protein (Santiago & Reyes, 1991), vitamins (Santiago & Gonzal, 2001), and phosphorus (Ji et al., 2017). However, there needs to be more information on dietary Ca requirements for bighead carp.

In this study, six purified diets containing different concentrations of dietary Ca were prepared and hand-fed to bighead carp for 60 days to assess the effects of dietary Ca levels on growth performance, body and muscle composition, vertebra calcium and phosphorus contents, the activities of digestive enzymes, and blood biochemical parameters. This experiment will obtain the Ca requirement of bighead carp and provide the scientific basis for its feed.

Materials and Methods

Experimental diet

As shown in **Table 1**, the experimental diets (43.99% crude protein, 6.87% crude lipid) were formulated to contain different Ca levels (0.09% (control), 0.43%, 0.76%, 1.12%, 1.44% and 1.79% of dry diets) by using the same source (calcium chloride). Dietary protein was supplied by casein and soybean meal, and corn and soybean oil (2:1) were mixed as fat sources. Six experimental feeds were recorded as T1, T2, T3, T4, T5, and T6, respectively. Dry ingredients were ground into powder, weighed accurately, and mixed thoroughly, and then corn oil and soybean oil were added to the mixture. After blending, calcium chloride dissolved in water and was added and blended thoroughly. The mash was extruded through extruding machine with a 0.63 mm diameter template, air-dried at low temperature, broken into pellets, and stored at 4 °C.

Table 1 Composition and nutrient levels of basal diets (dry basis, %)

Ingredients（%）	Experiment feed group					
	T1	T2	T3	T4	T5	T6
Casein	50.00	50.00	50.00	50.00	50.00	50.00
Soybean meal	10.50	10.50	10.50	10.50	10.50	10.50
Dextrin	20.00	20.00	20.00	20.00	20.00	20.00
Corn oil	4.00	4.00	4.00	4.00	4.00	4.00
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix [1]	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin mix [2]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Monosodium phosphate	4.64	4.64	4.64	4.64	4.64	4.64
Calcium chloride	0	1.11	2.22	3.33	4.44	5.55
Cellulose	5.86	4.75	3.64	2.53	1.42	0.31
Total	100.0	100.0	100.0	100.0	100.0	100.0
Proximate composition						
Crude lipid	6.87	6.85	6.86	6.87	6.87	6.87
Crude protein	43.99	43.99	43.96	43.95	43.97	43.96
Crude ash	7.60	7.99	7.78	7.67	8.01	8.20
Moisture	8.93	8.92	9.01	8.93	8.92	8.95
Calcium	0.09	0.43	0.76	1.12	1.44	1.79

[1] Mineral premix (g/kg of mixture): $MgSO_4$, 20.00 g; $FeSO_4 \cdot 7H_2O$, 20.00 g; $ZnSO_4 \cdot 7H_2O$, 2.00 g; $MnSO_4 \cdot H_2O$, 2.00 g; $CuSO_4 \cdot 5H_2O$, 4.00 g; $KAl(SO_4)_2 \cdot 12H_2O$, 0.60 g; KCl, 64.00 g; $CoCl_2 \cdot 6H_2O$, 0.40 g; KI, 0.60 g; microcrystalline cellulose, 886.40 g

[2] Vitamin premix (g/kg of mixture): vitamin A, 700,000 IU; vitamin D_3, 350,000 IU; vitamin K, 3.50 g; vitamin E, 16.00 g; vitamin B_1, 3.50 g; vitamin B_2, 7.00 g; vitamin B_6, 7.00 g; vitamin B_{12}, 7.00 mg; biotin, 0.03 g; folic acid, 1.60 g; nicotinic acid, 35.00 g; Ca-D- pantothenic acid salt, 16.00 g; inositol, 35.00 g; starch, 840.12 g

Experimental Procedure

The feeding trial was performed at the experimental base of Wuhan Polytechnic University (Wuhan, China). Juvenile bighead carp were obtained from a local commercial farm. These fingerlings were dipped in povidone iodine as a prevention against diseases. Fish were maintained in a concrete pool (2 m× 1.5 m× 1.5 m) with a constant flow of filtered water (25-28 °C) and fed the T1 diet for 35 days to acclimatization.

The formal trial was conducted in a circulating system consisting of 18 cylindrical plastic tanks (diameter, 80 cm; height, 60 cm). At the beginning of feeding trial, the fish were fasted for 24 h. Then, a total of 540 fish of similar size (initial body weight, 3.31 ± 0.09 g) were randomly selected, weighed, and divided into 18 tanks (n = 30), and were fed six different diets, resulting in three tanks for each diet. To reduce pellet waste, fish were slowly hand-fed until they appeared to be satiated by observing their feeding behavior, and care was taken to ensure that no uneaten food remained after feeding for 2 hours. The number of diets supplied was recorded daily. The fish were fed thrice daily at 08:30, 12:00, and 16:00 (natural photoperiod). The feeding trial lasted 60 days. During the experiment, aeration was provided

to each tank to maintain a dissolved oxygen level of 7-8 mg/L. The water temperature was maintained at about 25-28 °C and recorded daily. The pH was maintained at 7.0-7.5, and the ammonia-N content was monitored once a week and below 0.2 mg/L.

Sample collection

Fish were deprived for 24 h before sampling. Five fish from each replicate tank were randomly collected and anesthetized with 100 mg L^{-1} of MS-222, and the blood samples were collected from the caudal vein using a 1-mL syringe. The collected blood samples from each replicate tank were pooled in Eppendorf tubes, clotted, and then centrifuged at 3500 × g for 10 min at 4 °C. Serum was separated and stored at -80 °C until analysis. After serum samples were obtained, the sampled fish were dissected immediately, and the intestine and muscle were collected and frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. Then these fishes were sacrificed to obtain the vertebrae samples. Subsequently, fish were cooked in a microwave oven for 5 min to remove the vertebrae for the pectoral and caudal fins collection. Vertebrae samples were lightly scrubbed, rinsed with deionized water, oven-dried for 2 h at 105 °C, and then solvent extracted and dried at 60 °C. Vertebrae samples were then ground for mineral analysis. Another five fish from each replicate tank were randomly collected and stored at -20 °C for whole-body composition analysis. The initial weight and final weight of each tank fish were recorded to calculate weight gain (WG), specific growth rate (SGR), condition factor (CF), and hepatopancreas somatic indices (HSI).

Analytical methods

The contents of moisture, crude protein, crude lipid, and ash of the experimental diets and final body samples were determined using standard methods (AOAC 2005). Moisture contents were dried in an oven at 105 °C until constant weight; crude protein (N×6.25) contents were measured by the Kjeldahl method after acid digestion; crude lipid contents were measured by ether extraction using Soxhlet; ash contents were measured by combustion at 550 °C for 5h. Calcium content was measured using EDTA complexometry. Phosphorus content was determined using the molybdovanadate method (AOAC 2005). The activity of serum alkaline phosphatase and concentration of total cholesterol (T-CHO), total protein (TP), albumin (ALB), globulin (GLB), triglycerides (TG), and glucose (Glu), and calcium content were measured using automatic biochemical analyzer (HITEC7100, Japan). The activities of lipase, amylase, and protease, and the total protein concentration in the intestine, were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China) according to the manufacturer's instructions.

Statistics Analysis

All data are presented as means ± S.E.M. and subjected to one-way analysis of variance (SPSS for Windows, ver22.0, U.S.A.) to determine if significant differences occurred in treatments. If a significant difference was identified, differences among means were compared by Duncan's multiple range test (Duncan, 1955) at $P < 0.05$. Excel 2016 and Prism7 software for charts.

Results

Growth Performance

The growth performances of bighead carp are shown in **Table 2**. Fish-fed diets without Ca supplement had significantly lower weight gain (WG) and specific growth rate (SGR) than fish-fed diets with Ca supplementation ($P < 0.05$). The WG and SGR of the T3 group were the highest of all groups, significantly higher than those in the T1, T5, and T6 groups ($P < 0.05$) and not significantly different than those in the T2 and T4 groups ($P > 0.05$). WG and SGR improved as dietary calcium concentrations increased from T1 to T3, but there were no further

improvements thereafter. As shown in **Figures 1 and 2**, when considering the WG and SGR between dietary Ca levels in regression analysis, the appropriate dietary requirement of Ca for the bighead carp larvae was 1.01% - 1.02%. The level of dietary calcium had a significant effect on the HSI of bighead carp ($P < 0.05$). HSI increased with the increasing dietary Ca level up to the T3 group and thereafter showed a decreasing trend. There was no significant difference in condition factor (CF) and viscerosomatic index (VSI) among the treatments ($P > 0.05$).

Table 2 Effect of different calcium levels on growth and visceral indicators of bighead carp

Diet	Initial weight (g)	Final weight (g)	WG (%)	SGR (%/day)	CF (%)	HSI（%）	VSI（%）
T1	3.34±0.06	8.87±0.53^{a}	165.67±15.96^{a}	1.63±0.10^{a}	1.91±0.05	1.94±0.08^{a}	11.14±0.11
T2	3.31±0.04	10.39±0.55^{bc}	213.80±16.71^{bc}	1.90±0.09^{bc}	1.91±0.10	1.98±0.06^{a}	11.35±0.15
T3	3.30±0.05	11.14±0.38^{c}	237.58±11.37^{c}	2.02±0.06^{c}	1.93±0.05	2.13±0.10^{b}	11.36±0.13
T4	3.29±0.08	10.33±0.26^{bc}	213.98±7.90^{bc}	1.91±0.04^{bc}	1.92±0.08	2.04±0.07^{ab}	11.28±0.12
T5	3.32±0.06	9.94±0.27^{b}	199.30±7.98^{b}	1.83±0.04^{b}	1.91±0.02	1.94±0.10^{a}	11.12±0.10
T6	3.29±0.08	9.62±0.53^{ab}	192.30±16.05^{b}	1.79±0.09^{b}	1.92±0.06	1.95±0.08^{a}	11.17±0.14

All data were expressed as mean ± SD (n=3), (P < 0.05) indicates statistically significant differences between the control and exposure. Significant differences were analysis of variance using SPSS package program.

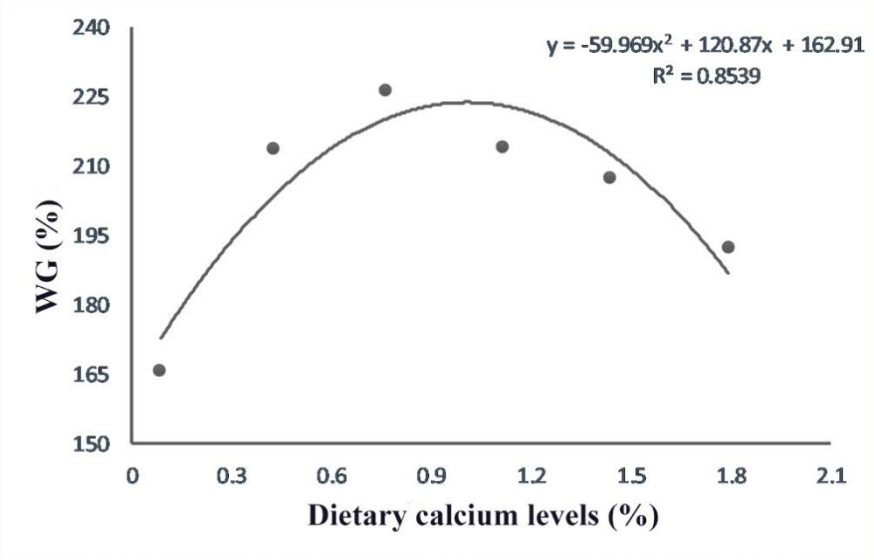

Figure 1 Quadratic curve analysis of the relationship between dietary calcium content and weight gain of bighead carp

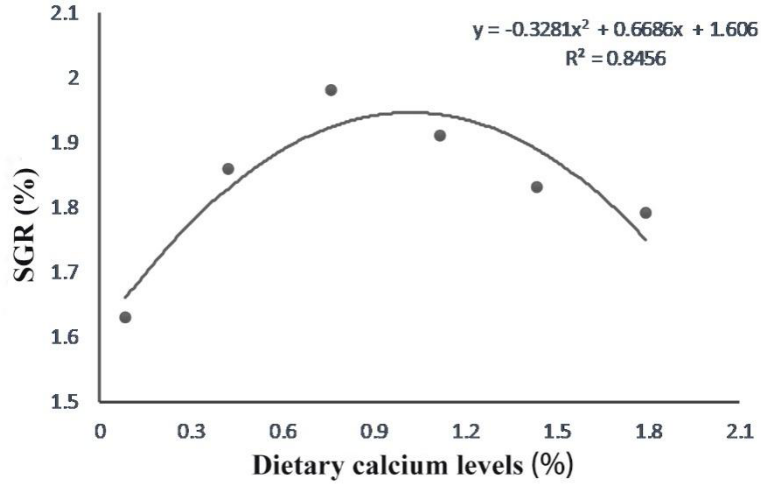

Figure 2 Quadratic curve analysis of the relationship between dietary calcium content and specific growth rate of bighead carp

Whole fish body and muscle composition

The whole fish body composition of bighead carp fed diets containing different concentrations of calcium is shown in **Table 3**. With increasing dietary calcium levels, whole fish crude lipid content showed a downward trend. It was the lowest in the T6 group, which was significantly lower than those in the T1, T2, and T3 groups ($P < 0.05$), and had no significant difference from those in the T4 and T5 groups ($P > 0.05$). Whole fish crude ash content increased initially and then stabilized, reaching significant increases in the T4 group (1.12%). With the increased dietary calcium level from T1 to T5, the phosphorus content increased and decreased. The phosphorus content in the T3 group (0.76%) was the highest, significantly higher than that in the T1 group (0.09%) ($P < 0.05$), but not significantly different from other groups ($P > 0.05$). The T4 group (1.12%) had the highest calcium content, which was significantly higher than that in the T1, T2, and T6 groups ($P < 0.05$), and there was no significant difference compared with T3 and T5 groups ($P > 0.05$). Moreover, moisture and crude protein contents were not significantly affected by diets containing different concentrations of calcium ($P > 0.05$).

Table 3 Effect of different calcium levels on whole body and composition (dry basis, %)

Indexes	Experiment feed group					
	T1	T2	T3	T4	T5	T6
Crude protein (%)	59.31±0.48	59.25±0.22	59.70±0.34	59.01±0.64	58.61±0.28	58.42±0.26
Crude lipid (%)	20.21±0.48^{c}	19.65±0.65^{bc}	19.17±0.36^{b}	18.64±0.45^{a}	18.60±0.52^{a}	18.28±0.35^{a}
Crude ash (%)	11.50±0.27^{a}	11.84±0.29^{b}	12.48±0.24^{c}	12.74±0.35^{c}	12.33±0.24^{bc}	12.31±0.21^{bc}
Moisture (%)	69.53±0.43	70.09±0.36	69.65±0.42	69.53±0.29	69.64±0.44	69.58±0.26
Phosphorus (%)	1.67±0.06^{a}	1.72±0.05^{ab}	1.80±0.06^{b}	1.73±0.06^{ab}	1.69±0.07^{ab}	1.72±0.08^{ab}
Calcium (%)	3.92±0.09^{a}	4.03±0.11^{ab}	4.21±0.05^{cd}	4.34±0.07^{d}	4.26±0.05^{cd}	4.13±0.07^{bc}

Data were presented as mean (n = 3) ± SEM, and values of different treatments within the same row having different superscripts are statistically different at P < 0.05.

The muscle composition of bighead carp was significantly affected by dietary treatments (**Table 4**; $P < 0.05$). With increasing dietary calcium levels, muscle crude lipid content showed a downward trend. It was the lowest in the T6 group, which was significantly lower than those in the T1, T2, and T3 groups ($P < 0.05$) and had no significant difference from those in the T4 and T5 groups ($P > 0.05$). Phosphorus and calcium contents in T4 group (1.12%) were significantly higher than those in the T1 group and T1, T2, T5, and T6 groups, respectively ($P < 0.05$). Moisture, crude protein, and crude ash levels did not differ significantly among the treatments ($P > 0.05$).

Table 4 Effect of different calcium levels on muscle composition (dry basis, %)

Indexes	Experiment feed group					
	T1	T2	T3	T4	T5	T6
Crude protein (%)	52.73±0.63	53.29±0.74	53.38±0.70	53.82±0.72	53.32±0.56	52.90±0.70
Crude lipid (%)	15.57±0.65^{b}	15.66±0.56^{b}	15.30±0.48^{b}	14.77±0.55^{ab}	14.85±0.51^{ab}	13.90±0.48^{a}
Crude ash (%)	7.57±0.20	7.74±0.12	7.97±0.22^{c}	7.84±0.21	7.57±0.25	7.61±0.28
Moisture (%)	76.57±0.61	77.09±0.40	76.61±0.56	76.92±0.19	76.88±0.54	76.69±0.42
Phosphorus (%)	0.93±0.02^{a}	0.95±0.01^{ab}	0.99±0.04^{b}	1.00±0.06^{b}	0.94±0.00^{ab}	0.96±0.02^{ab}
Calcium (%)	0.47±0.02^{a}	0.50±0.02^{ab}	0.58±0.05^{c}	0.60±0.02^{c}	0.50±0.01^{ab}	0.52±0.02^{b}

Data were presented as mean (n = 3) ± SEM, and values of different treatments within the same row having different superscripts are statistically different at P < 0.05.

Mineral contents

Ash and mineral contents of vertebrae for the various treatments are presented in **Table 5**. With the increased dietary calcium level, phosphorus (P) and calcium contents increased initially and then decreased. Phosphorus content in the T3 group (0.76%) was the highest, significantly higher than that in the T1, T2, T5, and T6 groups ($P < 0.05$), and there was no significant difference with that in T4 group ($P > 0.05$). The calcium content in the T4 group (1.12%) was the highest, which was significantly higher than that in the T1, T2, T5, and T6

groups ($P < 0.05$), and there was no significant difference with that in the T3 group ($P > 0.05$). Dietary Ca levels did not significantly affect Vertebrae ash content ($P > 0.05$).

Table 5 Effect of different calcium levels on calcium and phosphorus in vertebrae (dry basis, %)

Group	Crude ash (%)	Phosphorus (%)	Calcium (%)
T1	38.47±1.06	6.61±0.25^{a}	12.90±0.42^{a}
T2	38.95±1.07	6.84±0.19^{ab}	14.36±0.50^{bc}
T3	40.76±1.02	7.48±0.18^{c}	15.22±0.40^{cd}
T4	40.53±1.99	7.16±0.37^{bc}	15.41±0.67^{d}
T5	39.16±1.36	6.81±0.17^{ab}	14.33±0.59^{bc}
T6	38.83±1.09	6.75±0.22^{ab}	14.14±0.33^{b}

Data were presented as mean (n = 3) ± SEM, and values of different treatments within the same row having different superscripts are statistically different at P < 0.05.

Biochemical composition of serum

As shown in **Table 6**, dietary calcium levels had significant effects on the contents of serum glucose (GLU) and phosphorus and the activity of alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) ($P < 0.05$). However, serum calcium content was similar in all treatments ($P > 0.05$). As dietary calcium levels increased, serum ALP activity decreased initially and then increased, reaching a significant decrease in the T3 group ($P < 0.05$). Phosphorus content decreased, reaching the lowest level in the T6 group, which was significantly lower than the other treatment groups ($P < 0.05$). The serum GOT and GPT activities were significantly decreased initially and then increased. The activity was the highest in the T1 group and significantly higher than in the T2-T5 groups ($P < 0.05$). The T6 group (1.79%) had the highest serum glucose content, which was significantly higher than that in the T1-T4 groups ($P < 0.05$), and there was no significant difference compared with the T5 group ($P > 0.05$).

Table 6 Effect of different calcium levels on plasma inorganic ions and ALP, GOT and GPT activity

Group	Calcium (mmol/L)	Phosphorus (mmol/L)	ALP (U/L)	GOT (U/L)	GPT (U/L)	Glucose (mmol/L)
T1	1.93±0.04	2.91±0.10^{c}	31.49±1.97^{c}	49.96±4.31^{c}	9.52±0.86^{c}	5.72±0.3^{a}
T2	1.95±0.04	2.80±0.07^{bc}	29.88±1.64^{c}	34.51±2.91^{b}	5.18±0.65^{ab}	5.25±0.2^{a}
T3	1.96±0.05	2.67±0.08^{b}	24.27±1.27^{a}	31.08±2.78^{a}	4.35±0.66^{a}	5.56±0.3^{a}
T4	1.97±0.07	2.69±0.08^{b}	26.77±1.09^{ab}	29.17±2.17^{a}	3.78±0.64^{a}	5.35±0.5^{a}
T5	2.00±0.16	2.65±0.10^{b}	26.93±1.08^{ab}	39.28±2.64^{b}	6.19±0.93^{b}	7.75±0.4^{b}
T6	1.97±0.06	2.25±0.05^{a}	27.30±1.44^{b}	46.26±3.11^{c}	10.57±0.85^{c}	8.28±0.4^{b}

Data were presented as mean (n = 3) ± SEM, and values of different treatments within the same row having different superscripts are statistically different at P < 0.05.

Activities of digestive enzymes in the intestine

As shown in **Table 7**, lipase activity in the intestine was increased initially and then decreased with increasing dietary calcium levels. Lipase activity in the T3 group was significantly higher than in the T1, T2, T5, and T6 groups ($P < 0.05$). Protease activity in the intestine in the T3 group was significantly higher than those in the T1, T2, T5, and T6 groups ($P < 0.05$), and no significant difference compared with the T4 group ($P > 0.05$). Diet calcium levels did not significantly influence amylase activities in the intestine ($P > 0.05$).

Table 7 Effect of different calcium levels on digestive enzyme activity

Group	Amylase（U/mgProt）	Protease（U/gProt）	Lipase（U/gProt）
T1	0.77±0.24	$0.89±0.31^{a}$	$41.36±077^{a}$
T2	0.86±0.33	$0.92±0.33^{ab}$	$42.58±0.91^{ab}$
T3	0.80±0.21	$1.15±0.28^{c}$	$44.78±0.55^{c}$
T4	0.75±0.23	$0.97±0.17^{bc}$	$43.68±0.72^{bc}$
T5	0.78±0.12	$1.01±0.30^{b}$	$42.55±1.23^{ab}$
T6	0.79±0.18	$0.99±0.24^{b}$	$41.74±1.26^{a}$

Data were presented as mean (n = 3) ± SEM, and values of different treatments within the same row having different superscripts are statistically different at P < 0.05.

Discussion

Growth performance

The present experiment showed that Ca supplementation significantly influenced WG and SGR of bighead carp. With increasing dietary calcium levels, WG and SGR increased initially and then decreased. These results indicated that both calcium deficiency and calcium excess inhibited the growth performance of bighead carp. Similar results have been reported in *Tilapia aurea* (Robinson & Rawles, 1984) and *Cichlasoma urophthalmus* (Chavez-Sanchez & Martinez-Palacios, 2000). In this experiment, the appropriate dietary calcium requirement for bighead carp larvae (3.31 ± 0.09 g) was 1.01% – 1.02%. The optimal calcium requirement in the present study was similar to that of grass carp (4.52 g; 1.04%) (Liang & Tian, 2011). However, it was higher than those of channel catfish (0.45%) (Robinson & Rawles, 1986), Japanese flounder (0.1% - 0.25%) (Hossain & Furuichi, 1999), and blue tilapia (0.7%) (Edwin et al., 1987), but lower than catfish (1.5%) (Andrews, 1973). Such differences may be due to differences in fish species, growth stages, and dietary formulations. Liang et al. (2018) studied the bighead carp weighing 105.52 ± 0.33 g. They considered that the appropriate calcium requirement was 1.26%, which was higher than the result of this experiment. The reason may be related to the fish size and the calcium content in the water.

In this experiment, dietary calcium had no significant effect on the condition factor of bighead carp, and the same conclusion was reached in the study of the GIFT tilapia (Yao et al., 2012). This indicated that dietary calcium level was not easy to affect the obesity of fish. In this experiment, HSI values increased with the increasing dietary Ca level up to 0.76%, and thereafter showed a decreasing trend. As far as we know, it has not previously been reported that the mechanism of the effect of dietary calcium level on hepatopancreas somatic indices. However, the study confirmed that there existed a specific negative correlation

between dietary phosphorus level and hepatopancreas somatic indices (Roy & Lall, 2003). It has been reported that inhibited P absorption with increasing dietary Ca levels (Hua & Bureau, 2006; Nakamura & Yoshikazu, 1982; Pornngam & Satoh, 1993). Therefore, dietary calcium might improve the hepatopancreatic somatic indices of bighead carp by affecting phosphorus absorption.

Whole fish body and muscle composition

In the present study, crude lipid content in whole fish and muscle was the lowest in the T6 group (1.79%), which validated that dietary calcium inhibited fatty acids absorption by chelating fatty acids to form soap salts (Tancharoenrat & Ravindran, 2014). Whole fish crude ash content increased initially and then stabilized. However, previous studies have found no significant differences in Atlantic cod's whole fish ash content with increased dietary calcium (Kousoulaki & Fjelldal, 2010), which may be related to differences in species, age, test environmental conditions, and dietary composition. Dietary Ca supplementation had no significant effects on protein and moisture contents in whole fish and muscle. Similar results were reported in juvenile jade perch (Song & Mao, 2009) and fingerling scorpion fish (Hossain & Furuichi, 2000). Because at the developmental stage of fish, Ca is needed at an increased level for building the hard tissues and other physiological functions. It has also been reported that the protein content of perch fed with a high calcium diet (3.1%) was significantly reduced (Song & Zhang, 2017). The maximum calcium level in this experiment was 1.79%, which was less than 3.1%, which may have affected the test results. The present experiment showed that dietary calcium did not significantly affect muscle ash content. Similar results have been reported in juvenile grouper (Ye et al., 2006). This finding suggested that dietary Ca was not entirely deposited in the body or that the Ca deposited did not change ash content.

Mineral contents

With the increased dietary calcium level, phosphorus and calcium in whole fish and muscle contents increased initially and then decreased. Similar results were reported in the tilapia (Robinson & Labomascus, 1987). It has previously been reported that inhibited P absorption with increasing dietary Ca levels (Nakamura & Yoshikazu, 1982; Robinson & Labomascus, 1987). As an interpretation of this inhibitory effect, the formation of calcium phosphate by Ca and phosphate ions may be considered, thus affecting the absorption of calcium and phosphorus by fish (Andrews, 1973).

Ca is directly involved in developing and maintaining the skeletal system and participates in several physiological processes in fishes. Bones and scales account for much of the body's calcium concentration in fish. The present study demonstrated that with the increased dietary calcium level, phosphorus and calcium contents increased initially and then decreased. However, Berntssen et al. (2015) fed Atlantic salmon with a high-calcium diet and found no changes in calcium and phosphorus content in the bone. The difference might be because the juvenile bighead carp in this experiment was in the rapid growth phase, and the calcium and phosphorus contents in the spine were more sensitive to the changes of feed calcium level.

Biochemical composition of serum

Blood parameters are considered convincible indicators for fish's physiological conditions and health status in response to dietary supplements (Congleton & Wagner, 2006; Kader & Koshio, 2010). Blood Ca and phosphorus levels are often used to measure the Ca and P nutritional status of animals and reflect the absorption of Ca and P in the bone (Zhang et al., 2006). In our study, serum Ca concentrations of bighead carp fed different Ca diets were similar (0.09%-1.79%), suggesting Ca homeostasis in fish. In teleost fish, Ca uptake from the environment usually balances Ca's diffusional and urinary losses such that serum Ca levels are regulated within narrow limits. Similar results were reported in the Atlantic salmon (Berntssen & Waagb, 2015) and grass bighead carp (Liang et al., 2012). Nakamura et al.

(1982) reported that Ca interacts with other essential dietary minerals, particularly P. In our study, serum P levels showed decreasing trend with increasing dietary Ca levels. Nakamura et al. (1982) observed a negative and linear relationship between the amount of P absorbed and the dietary Ca content in carp. When there is an excess of dietary Ca supplementation, the P is not absorbed by the intestine, which could explain the reason for the result.

ALP, existing in many tissues such as bone, liver, and intestine, is an important indicator of bone metabolism and plays an essential role in the mineralization of aquatic animals. Its activity is related to the activity of the bone regeneration (Urea & Hruby, 1996). When the metabolism of osteoblasts was exuberant, the partial entry of ALP from bone into blood led to the increase of serum (Griffiths, 1989). In the present study, serum ALP activities in the T1 (0.09%) and T2 (0.43%) groups were significantly higher than in other groups, suggesting that these low dietary Ca levels did not meet the bone salt depositional requirements of bighead carp, which maintained osteoblasts. Song et al. (2017) and Liang et al. (2018) also indicated that lower Ca-containing diets also increased plasma ALP activity.

High activities of blood GOT, and GPT generally indicates a weakening or damage of normal liver function in fish species (Kim & Lee, 2009). In the current study, when the feed calcium levels were 0.09% and 1.79%, the serum GOT, and GPT activities were significantly higher than those in other groups, which indicated that both low and high dietary calcium levels could damage the normal liver function of bighead carp. It has been studied that nutritional stress can increase plasma glucose levels in fish (Hossain & Furuichi, 2000). In this study, the plasma glucose content was the highest when the feed calcium level was 1.79%, indicating that excessive feed calcium level would cause nutritional pressure on bighead carp, leading to slow growth.

Activities of digestive enzymes in the intestine

The activities of digestive enzymes are affected by different feeding habits, dietary nutrient composition and the contents of nutrients (NRC, 2011). In this experiment, when the dietary calcium level was 0.77%, the protease and lipase activities in the intestine of bighead carp were the highest, while WG and SGR were also the highest, indicating that the intestinal digestive enzyme activities could reflect the calcium absorption capacity of bighead carp. Similar results have been reported in juvenile discus fish (Liu et al., 2018). However, Hu et al. (2006) fed *Portunus trituberculatus* with a high-calcium diet and found that the protease activity increased. The difference might be because of the different types of experimental subjects and the ion concentration. Many metal ions were activators or inhibitors of digestive enzymes, and the effect of ions was not the same at different concentrations.

In conclusion, the Quadratic curve model analysis based on weight gain and specific growth rate showed that bighead carp's optimal dietary calcium requirement ranged from 1.01% to 1.02% under this trial. It was beneficial for growth and bone development, secreting digestive enzymes, avoiding damage to liver function, and improving the calcium absorption capacity of bighead carp.

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