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Effects of neutral phytase on growth performance and phosphorus utilization in crucian carp (*Carassius auratus*)*

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Abstract: A feeding trial was conducted for nine weeks to investigate the effects of partially replacing $Ca(H_2PO_4)_2$ with neutral phytase on the growth performance, phosphorus utilization, nutrient digestibility, serum biochemical parameters, bone and carcass mineral composition, and digestive-enzyme-specific activity in crucian carp (*Carassius auratus*). The diets prepared with 0.8%, 0%, and 1.8% $Ca(H_2PO_4)_2$ (1%=1 g/100 g) supplements were regarded as the P_1E_0, negative control (NC), and positive control (PC) groups, respectively; the other three experimental diets were prepared with the addition of 200, 300, and 500 U/kg of neutral phytase, respectively, based on the P_1E_0 group. Three hundred and eighty-four fish ((1.50±0.01) g) were randomly distributed in the six treatments with four replicates each. The fish were initially fed with 2%–3% diets of their body weight per day, with feeding twice daily (08:00 and 16:00), under a 12-h light/12-h dark cycle at the temperature of (27.56±0.89) °C. The results showed that supplemental phytase at different levels in the diet improved the final body weight, average daily gain, feed conversion ratio, phosphorus utilization, and protein efficiency ratio of crucian carp (P<0.05). Phytase supplementation increased the mineral content in serum (P), bone (P, Ca), and carcass (P, Ca, Zn, Na, and Mg) (P<0.05); the trypsin and chymotrypsin activity soared when fed with the phytase-supplemented diets (P<0.05). We may conclude that supplemental dietary neutral phytase improved the growth performance, phosphorus utilization as well as nutrient utilization in crucian carp, and it can be considered an important nutritional replacement for $Ca(H_2PO_4)_2$.

Key words: Neutral phytase; Crucian carp (*Carassius auratus*); Growth performance; Phosphorus utilization; Apparent digestibility coefficient; Body composition

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1 Introduction

Fish meal is one of the main sources of protein in animal feeds. However, plant proteins are used as substitute protein sources in feeds owing to the limited and unpredictable supply of fish meal. The primary problem with using plant proteins is the existence of anti-nutritional factors, such as phytate, which is the main form of storage for phosphorus (P) in plants (Baruah *et al*., 2004; Cao *et al*., 2007; Zou *et al*., 2008). Under physiological conditions, due to differences in pH and metal cations, phytate exists in the forms of free acid, phytic acid, and phytin (Lori *et al*., 2001). Animals can have difficulty in utilizing the P in phytate (phytate-P) because of the negligible native phytase activity in vivo (Wang *et al*., 2009; Gonzalez-Vega *et al*., 2015). One of the solutions to this problem is the addition of $Ca(H_2PO_4)_2$ into feeds, which significantly increases the amount of available P within the diet. Nonetheless, most of the dietary P ends up being excreted into the water supply, which represents a major environmental concern in terms of water safety for man and animals (Liebert and Portz, 2005; Cao *et al*., 2007). Furthermore, the bioavailability of essential minerals such as calcium (Ca), zinc

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(Zn), iron (Fe), and magnesium (Mg) may also be disadvantageously affected by the formation of insoluble chelate complexes with phytate (Spinelli *et al*., 1983; Cao *et al*., 2007; Liu *et al*., 2012). Finally, $Ca(H_2PO_4)_2$ is a nonrenewable mineral, and its price has risen sharply in the past few years (Xu *et al*., 2014). Phytase, a group of enzymes known as myo-inositol hexaphosphate phosphohydrolase, is capable of hydrolyzing phytate into myoinositol and inositol phosphates, thus representing a promising approach for increasing the amounts of bioavailable P and, at the same time, reducing the quantities of P that are excreted (Lori *et al*., 2001; Huang *et al*., 2009).

The effects of phytase on the growth, nutrient utilization, mineral bioavailability, as well as phosphorus bioavailability, have been demonstrated in livestock and fish studies with most of them based on a diet of corn-soybean meal diets (Qian *et al*., 1997; Cheng and Hardy, 2003; Cao *et al*., 2008; Gonzalez-Vega *et al*., 2015). Before the 1990s, however, phytase supplementation was mainly considered as a way of improving the utilization of phytate-P in poultry and pig diets, whereas it was used less in the fish diet due to the lack of research information and the constraints on manufacturing (Baruah *et al*., 2004; 2005; Cao *et al*., 2007). After the mid-1990s, a growing number of studies have focused on the effects of adding phytase for utilization of nutrients. Thus, studies of growth performance based on phytase-added diet were conducted in some aquacultural species (Vielma *et al*., 1998; Cheng and Hardy, 2002; Cao *et al*., 2007). Currently, research focuses mainly on the effects of phytase within the domain of dose-response studies and the most efficient ways for supplementation, the alimentary systems in different fish development stage, as well as the source and variety of phytase for fish (Debnath *et al*., 2005; Shao *et al*., 2008; Wang *et al*., 2009; Liu *et al*., 2012; 2013; 2014).

Crucian carp (*Carassius auratus*) is an important and popular aquaculture species in Europe (Tarkan *et al*., 2016) and Asia, especially in China. However, there are not sufficient studies that reference phytase's application in the diet of crucian carp. In particular, neutral phytase with an optimum pH of 7.0 can tolerate an elevated temperature during manufacturing and adapt to the intestinal pH of crucian carp, which is a stomachless fish (Huang *et al*., 2009). Therefore, the aim of this study is to evaluate the effect of neutral phytase on the growth performance, nutrient digestibility of feed, phosphorus utilization, body and blood biochemical indexes, and intestinal digestive enzyme activity of crucian carp with regard to a lack of available P. This study also aims to examine the scope for reducing aquaculture pollution by partially replacing $Ca(H_2PO_4)_2$ with neutral phytase, thereby minimizing the discharge of P into the environment.

2 Materials and methods

2.1 Preparation of diets

The ingredients and their levels of inclusion in the experimental diets are shown in Table 1. According to previous studies, the addition of about 1.8% $Ca(H_2PO_4)_2$ (1%=1 g/100 g, the same below) to the basal diet was proved to be sufficient and suitable for the optimal growth of crucian carp (Zhang *et al*., 2001). Therefore, in the present experiment, we added 0% (negative control (NC)), 0.8% (P_1E_0), and 1.8% (positive control (PC)) $Ca(H_2PO_4)_2$ in the first three groups, respectively. The neutral phytase was obtained from the Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China and the enzyme activity was 3000 U/g. This enzyme-relevant gene, which was originated from *Pedobacter nyackensis* MJ11 CGMCC 2503, was expressed in *Pichia pastoris*, and then the recombinant protein was purified. Neutral phytase was evenly mixed in the premix before being pelletized, and we added 200 (P_1E_1), 300 (P_1E_2), and 500 U/kg (P_1E_3) in the last three groups, respectively, based on the P_1E_0 group. The nutrient requirements followed the standard of Zhejiang Xinxin Feed Co., Ltd., Jiaxing, China and were formulated to contain 32% crude protein, 5% crude lipid, and 1% total P at least, 6% crude fiber and 10% ash at most. Soybean meal and fish meal were used as the main protein sources. Phospholipid was used as the lipid source in the basal diet. In addition, the crude protein, crude lipid, crude fiber, ash, and gross energy of the six experimental diets were kept at similar levels. The diets were made using large fish-compound-feeding production equipment and the outlet temperature was (80±5) °C. The pellets were of 1.8 mm in diameter, and were stored in a cool and dry place.

2.2 Experimental design

The experiment with the crucian carp was conducted at the Xiaoxiang Aquaculture Farm in Hangzhou, Zhejiang Province, China. The fish were acclimatized for two weeks in a net cage at the Zhejiang Xinxin Feed Co., Ltd. and fed with the PC feed. A total of 384 fish ((1.50±0.01) g) were selected after acclimatization. Then the fish were randomly assigned to each fiberglass tank (50 cm×50 cm×50 cm). In total, there were four tanks for each treatment, with 16 fish per tank. All fish were initially fed at 2%–3% diets of their body weight per day, and then the level was adjusted weekly according to the actual feeding performance, with feeding twice a day (08:00 and 16:00), under a 12-h light/12-h dark cycle, for nine weeks. The water was refreshed weekly by 50% replacement of water volume, and intermittent aeration was supplied by an air compressor every day at 7:00, 11:00, 15:00, and 23:00 (30 min at each time point). The water quality was maintained by siphoning the leftover food and wastes when collecting feces, and also monitored daily by qualitative ammonia detection with test paper. In the test period, the water temperature was (27.56±0.89) °C and pH was 7.5±0.2. The dissolved oxygen was (7.45±0.09) mg/L.

2.3 Analysis of dietary chemical composition

Three samples of each of the six experimental diets were subjected to the determination of moisture content, after oven drying at 105 °C for 24 h. The approximate composition of the diets, including crude protein, crude lipid, crude fiber, crude ash, and nitrogen-free extract, was measured according to AOAC (2005) standard method. Gross energy values were measured using an oxygen bomb calorimeter (1281, Parr, USA). The values are reported as a percentage on a dry matter basis.

2.4 Sampling procedures

At the end of the experiments, the fish were counted and weighed after a 24-h starvation. Then five fish were randomly selected from each cage and killed on an ice tray. The feed conversion ratio (FCR) was measured from the dry weight of the diet consumed. In order to measure the apparent digestibility coefficient (ADC), feces were collected at night after the crucian carp excreted, and the acid insoluble ash (AIA) was used as an indicator. Blood samples were collected from the tail vein and allowed to coagulate at room temperature for 30 min. Serum was separated by centrifugation at 3000*g* for 10 min and transferred into 1.0 ml microcentrifuge tubes. The visceral organs (including liver, foregut, and hindgut) and carcass (without visceral organs) were quickly collected from each individual fish on an ice-cold surface. Then, the samples were divided into aliquots and snap-frozen in liquid nitrogen. Frozen tissues and serum samples were stored at −80 °C prior to analysis.

2.5 Analysis of serum parameters and mineral composition of carcass and bone

The serum indexes included alkaline phosphatase (AKP) activity, serum calcium and phosphorus contents. AKP activity was assessed by the time-dependent formation of *p*-nitrophenolate (which absorbs light at 405 nm in an alkaline solution) from *p*-nitrophenyl phosphate (PNPP) according to the method of Ma *et al*. (2014). Serum calcium and phosphorus contents were determined using an automatic biochemical analyzer (Abbott-Aeroset, Abbott Park, IL, USA) at the Campus Hospital of Zhejiang University, Zijingang Campus, Hangzhou, China.

The carcass and bone were washed with distilled water and dried at 105 °C, then ground in a mortar. The Zn, Fe, Mg, Na, Ca, and P contents were determined according to the AOAC (2005) standard method.

2.6 Digestive enzyme assays

The frozen foregut was dissected by scissors and then the tissue was homogenized using a micro-homogenizer (S10; Scientz, China) in the presence of 20% (0.2 g/ml) saline. The homogenate was centrifuged at 5000*g* at 4 °C for 30 min. The lipid layer on the surface was removed, and the supernatant was kept at −20 °C for digestive enzyme activity determination after two days.

The activity of α-amylase (EC 3.2.1.1) was determined based on the method of Ma *et al*. (2014) with an amylase assay kit C016 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The α-amylase activity was calculated as unit (U) per milligram (mg) of protein, where 1 U was defined as 10 mg amylum decomposed per 30 min at 37 °C. The activities of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were also determined using the assay kits A080-2 and A080-3 (Nanjing Jiancheng Bioengineering Institute,

Nanjing, China). Trypsin activity was calculated as U per mg of protein, where 1 U was defined as the trypsin in 1 mg of protein giving an absorbance change of 0.003 at 37 °C, pH 8.0; chymotrypsin activity was calculated as U per mg of protein, where 1 U was defined as 1 mg of protein that decomposes into 1 μg of amino acid per minute at 37 °C.

Protein concentration (mg/ml) was determined using the method of Lowry *et al*. (1951). The protein concentrations in the crude enzyme extracts were used for standardization when quantifying the digestive-enzyme-specific activity (U/mg protein).

2.7 Data analysis and calculation of zootechnical parameters

The data were analyzed using SPSS Version 20.0 (SPSS Inc., Chicago, USA). The comparison between groups was made by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) multiple-range test. A level of $P<0.05$ was considered to be statistically significant unless indicated otherwise. Replication was considered at the experimental unit level. Results are expressed as the mean value with a standard deviation (mean±SD (n=3)). The calculation formulae for the growth and feed utilization parameters were as follows: survival rate (SR, %)=$(n_t/n_0)\times100\%$, where n_t is the number at the end of the test (time t) and n_0 is the number at the beginning of the test; average daily gain (ADG, mg/d)=$1000(W_t-W_0)/t$, where W_t means the weight (g) at the end of the experiment (time t) and W_0 means the weight (g) at the beginning; condition factor (CF, g/cm^3)=$100(W_1/L_t^3)$, where W_1 is live body weight (g) and L_t is total body length (cm); viscerosomatic index (VSI, %)=(wet weight of visceral organ/wet body weight)×100%; hepatosomatic index (HSI, %)=(wet weight of liver/wet body weight)×100%; intestosomatic index (ISI, %)=(length of intestine/length of body)×100%; feed conversion ratio (FCR, g feed/g gain)=dry feed consumed (g)/wet weight gain (g); total P intake (mg/g)=dry matter intake (g/tank)× total P in feed (mg/g dry matter)/total weight in tank (g/tank), where the dry matter intake and total weight of fish were measured at the end of the experiment; apparent digested P (mg/g)=intake of P (mg/g)× digestibility of P (%); retained P (%)=(total P accumulation (mg/g)/intake of P (mg/g))×100%; apparent digestibility coefficient (ADC, %)=$[1-(A^*/A\times B/B^*)]\times100\%$, where A means one component content in the feed (g/100 g), A^* means the same component content in feces (g/100 g), B means the AIA content in feed (g/100 g), and B^* means the AIA content in feces (g/100 g).

3 Results

3.1 Chemical compositions of the diets

The calculated chemical composition in terms of crude protein, crude lipid, crude fiber, nitrogen-free extract, and gross energy is shown in Table 1. These constituents had similar levels in the six diets, so the addition of $Ca(H_2PO_4)_2$ and neutral phytase had no effect on the overall nutritional composition of the diet.

3.2 Growth and feed utilization of crucian carp

The growth and feed utilization parameters of crucian carp are given in Table 2. The results show that supplemental phytase at the three levels in the diet all improved the final body weight, ADG, and FCR of crucian carp. The final body weight was significantly more increased in the P_1E_1, P_1E_2, and P_1E_3 than in the NC and P_1E_0 ($P<0.05$). The ADGs of P_1E_1, P_1E_2, and P_1E_3 were significantly increased compared with NC ($P<0.05$); when compared to P_1E_0 and PC, the values were also slightly higher although the difference was nonsignificant ($P>0.05$). The FCRs of the PC, P_1E_1, P_1E_2, and P_1E_3 were significantly decreased ($P<0.05$) compared to the NC and P_1E_0. The SRs of the PC and the phytase-supplemented groups were significantly elevated compared to the P_1E_0 ($P<0.05$). The protein efficiency ratios (PERs) of the phytase-supplemented groups were significantly increased compared to the NC and P_1E_0 ($P<0.05$). There was no significant difference in CF, VSI, or ISI ($P>0.05$), but the HSI for P_1E_1 was significantly lower than that for the non phytase-supplemented groups ($P<0.05$). Furthermore, all the growth parameters listed in Table 2 except HSI showed better conditions but were not more significant for the fish fed with neutral phytase than for the PC ($P>0.05$), and the parameters for the P_1E_1, P_1E_2, or P_1E_3 group were also not significant ($P>0.05$).

3.3 Phosphorus utilization

As shown in Table 3, the total P intake showed an increasing trend with the addition of neutral phytase, while P_1E_3 was significantly higher than all

the other treatment groups (P<0.05). The apparent digestible P intakes of PC, P_1E_1, and P_1E_2 were increased compared to NC and P_1E_0 (P<0.05); P_1E_3 also showed a significant increase compared to all other treatment groups (P<0.05). Fecal phosphorus excretion decreased with the addition of $Ca(H_2PO_4)_2$, where the PC was significantly lower than the NC and P_1E_0 (P<0.05), and in the fish fed with neutral phytase, it was lower than in the non-phytase groups (P<0.05). When it came to total P accumulation rate, the result suggested that there was no significant difference between NC, P_1E_0, and PC. However, it was significantly increased due to the addition of neutral phytase (P<0.05).

Table 1 Formulations by weight of ingredients and the chemical compositions of the diets used for crucian carp

Group	Ingredient (%)									
	Soybean meal	Cottonseed meal	Rapeseed meal	Wheat middling	Fish meal	Wheat	Phospholipid	Grease	Bentonite	$Ca(H_2PO_4)_2$[a]
NC	22	8	21	12	14	16.0	2.5	1.5	2	0
P_1E_0	22	8	21	12	14	15.2	2.5	1.5	2	0.8
PC	22	8	21	12	14	14.2	2.5	1.5	2	1.8
P_1E_1	22	8	21	12	14	15.2	2.5	1.5	2	0.8
P_1E_2	22	8	21	12	14	15.2	2.5	1.5	2	0.8
P_1E_3	22	8	21	12	14	15.2	2.5	1.5	2	0.8

Group	Ingredient (%)		Chemical composition (% on dry matter)[e]							
	Neutral phytase (U/kg)	Premix[b]	CP	EE	CF	Ash	NFE	NPP[c]	T-P[d]	GE (kJ/g)
NC	0	1	32.97	5.85	5.33	5.24	50.61	0.56	0.95	18.42
P_1E_0	0	1	32.87	5.84	5.31	5.23	50.75	0.74	1.14	18.57
PC	0	1	32.81	5.82	5.29	5.25	50.83	0.98	1.39	18.74
P_1E_1	200	1	32.91	5.84	5.31	5.23	50.71	0.74	1.10	18.83
P_1E_2	300	1	32.90	5.83	5.32	5.24	50.71	0.74	1.18	18.79
P_1E_3	500	1	32.90	5.84	5.32	5.22	50.72	0.74	1.16	18.43

The diets are given as dry matter of pretreatment amounts. Ingredients and formulations: Zhejiang Xinxin Feed Co., Ltd., Jiaxing, China. [a] $Ca(H_2PO_4)_2$: the content of available P in $Ca(H_2PO_4)_2$ in NC, P_1E_0, PC, P_1E_1, P_1E_2, and P_1E_3 was 0%, 0.19%, 0.43%, 0.19%, 0.19%, and 0.19%, respectively. [b] Premix: 1 kg of premix contained 15 mg vitamin B_1 (VB_1), 15 mg VB_2, 100 mg niacin, 20 mg VB_6, 4 mg VB_{12} (1%), 50 mg D-pantothenic acid, 1 mg biotin, 200 mg inositol, 5 mg folic acid, 2500 mg choline chloride, 240 mg VC, 20 mg VA, 8 mg VD_3, 300 mg VE, 10 mg VK_3, 400 mg butylated hydroxytoluene (BHT), 162 mg α-cellulose, 3000 mg $MgSO_4$, 2000 mg $NaHCO_3$, 600 mg $FeSO_4$, 350 mg $ZnSO_4$, 180 mg $MnSO_4$, 10 mg KI, 10 mg $Na_2Se_2O_3$, 50 mg $CoCl_2$, 10 mg $CuSO_4$, 1000 mg L-carnitine, and 2000 mg yeast culture. [c] NPP, non-phytate phosphorus, was calculated by the Feed Composition and Nutritional Value of China, 2002 (Wang and Wang, 2003). [d] T-P, total phosphorus, was measured using the AOAC (2005) standard method. [e] Chemical composition: CP, crude protein; EE, ether extract; CF, crude fiber; NFE, nitrogen free extract; GE, gross energy

Table 2 Growth performance parameters of crucian carp when fed with various diets for nine weeks

Group	Initial body weight (g)	Final body weight (g)	Average daily gain (mg/d)	Feed conversion ratio (g feed/g gain)	Survival rate (%)
NC	1.79±0.01	5.27±0.42^{b}	55.26±6.58^{b}	4.01±0.48^{a}	87.50±9.49^{bc}
P_1E_0	1.78±0.01	5.49±0.33^{b}	58.80±5.26^{ab}	4.01±0.33^{a}	81.94±11.45^{c}
PC	1.78±0.01	5.75±0.41^{ab}	63.05±6.38^{ab}	3.30±0.50^{b}	94.44±7.85^{ab}
P_1E_1	1.79±0.01	6.06±0.18^{a}	67.84±2.97^{a}	2.81±0.20^{b}	100.00±0.00^{a}
P_1E_2	1.79±0.01	6.00±0.52^{a}	66.90±8.34^{a}	2.88±0.31^{b}	97.22±3.21^{ab}
P_1E_3	1.78±0.01	6.00±0.05^{a}	66.97±0.78^{a}	3.37±0.48^{b}	95.83±2.78^{ab}

Group	Protein efficiency ratio	Condition factor (g/cm^3)	Viscerosomatic index (%)	Intestosomatic index (%)	Hepatosomatic index (%)
NC	0.87±0.10^{b}	0.62±0.07	155.70±6.54	2.84±0.28	75.53±19.57^{abc}
P_1E_0	0.92±0.08^{b}	0.59±0.10	157.72±13.72	2.81±0.23	80.83±10.49^{ab}
PC	0.99±0.10^{ab}	0.61±0.04	159.01±14.77	2.92±0.44	79.28±11.22^{ab}
P_1E_1	1.06±0.05^{a}	0.58±0.08	154.27±12.13	2.82±0.33	66.80±11.72^{c}
P_1E_2	1.05±0.13^{a}	0.54±0.10	152.25±9.83	2.80±0.34	72.40±9.19^{abc}
P_1E_3	1.05±0.01^{a}	0.60±0.05	157.74±14.35	2.98±0.43	73.37±11.77^{abc}

Data are expressed as mean±SD (n=3). The values with different superscript lowercases in the same column are significantly different

Table 3 Phosphorus utilization of crucian carp when fed with various diets for nine weeks

Group	Total P intake (mg/g)	Apparent digestible P intake (mg/g)	Fecal P excretion (mg/g)	Total P accumulation rate (%)
NC	19.87±1.34^{b}	8.99±0.15^{c}	13.96±0.28^{a}	23.74±2.48^{d}
P_1E_0	20.61±1.38^{b}	9.03±0.27^{c}	13.33±0.46^{a}	23.94±2.28^{d}
PC	21.18±1.38^{b}	10.20±0.64^{b}	11.34±0.91^{b}	27.12±3.60^{cd}
P_1E_1	21.16±0.88^{b}	9.91±0.03^{b}	9.96±0.32^{c}	32.94±2.10^{ab}
P_1E_2	21.83±0.67^{b}	10.67±0.81^{b}	9.24±0.37^{d}	34.20±2.26^{a}
P_1E_3	23.78±1.73^{a}	11.73±0.72^{a}	9.09±0.13^{d}	28.83±4.25^{bc}

Data are expressed as mean±SD (n=3). The values with different superscript lowercases in the same column are significantly different

3.4 Apparent digestibility coefficients for nutrients

The ADCs for nutrients in crucian carp after the feeding trial are given in Table 4. Compared to the NC, the ADCs of crude protein, lipid, and T-P for the P_1E_0 showed no significant difference, but the ADCs of crude protein and T-P were dramatically increased in the PC compared to the NC (P<0.05). Compared to the P_1E_0, the ADC of crude protein in the PC, P_1E_2, and P_1E_3 was significantly increased (P<0.05); the ADC of crude lipid in the P_1E_3 was significantly increased (P<0.05); the ADC of total P was significantly increased in the PC and all fish fed with neutral phytase (P<0.05). When compared to PC, the ADC of protein in the P_1E_1 was lower (P<0.05), but the ADC of lipid in the P_1E_3 was significantly increased (P<0.05), and the ADC of T-P in P_1E_2 and P_1E_3 was even higher (P<0.05).

Table 4 Apparent digestibility coefficients of nutrients in crucian carp when fed with various diets for nine weeks

Group	Protein (%)	Lipid (%)	Total P (%)
NC	71.56±0.37^{c}	83.94±0.92^{b}	41.82±1.26^{d}
P_1E_0	72.56±0.54^{bc}	82.34±1.92^{b}	43.39±1.90^{d}
PC	76.11±0.27^{a}	84.25±1.64^{b}	47.15±0.34^{c}
P_1E_1	73.67±0.52^{b}	82.58±1.63^{b}	48.71±0.95^{c}
P_1E_2	76.80±1.26^{a}	84.04±2.46^{b}	54.56±0.99^{b}
P_1E_3	75.87±2.12^{a}	87.94±0.95^{a}	55.99±0.11^{a}

Data are expressed as mean±SD (n=3). The values with different superscript lowercases in the same column are significantly different

3.5 Serum, bone, and carcass compositions

Serum biochemical profiles, as well as bone and carcass compositions of the fish are shown in Table 5. There were no significant differences in the serum Ca or AKP (P>0.05) except that the serum Ca in P_1E_2 was higher than in P_1E_1 (P<0.05). Nevertheless, the serum inorganic P concentration was increased by the addition of $Ca(H_2PO_4)_2$ (P<0.05) and was higher in the fish fed with the phytase-supplemented diet than in the NC and P_1E_0 (P<0.05). Both bone Ca and P showed a significant increase as $Ca(H_2PO_4)_2$ was added (P<0.05), and in fish fed with neutral phytase, was increased compared with NC and P_1E_0 (P<0.05). Supplementation of $Ca(H_2PO_4)_2$ had no effect on the Ca, Zn, Na, Mg, or Fe content in the carcasses of fish (P>0.05). Carcass-Ca in P_1E_1 was close to that in P_1E_0 and PC (P>0.05), while the Ca in P_1E_2 and P_1E_3 was significantly increased (P<0.05) when compared to the non-phytase groups. The P content in carcass was increased by the addition of $Ca(H_2PO_4)_2$ (P<0.05), and the content in P_1E_2 and P_1E_3 was higher than in the fish fed without neutral phytase and P_1E_1 (P<0.05). The carcass-Zn in the three neutral phytase groups was higher than in the non-phytase groups (P<0.05), while the Na content in PC and the neutral phytase groups showed a significant increase over the NC and P_1E_0. No significant increase was observed in the whole body Fe or Mg concentration among all the groups (P>0.05) except that the carcass-Mg in P_1E_3 was higher than that in the other treatment groups.

3.6 Specific activity of digestive enzymes

The specific activity of digestive enzymes is shown in Table 6. The specific activity of α-amylase was similar in the six dietary treatment groups (P>0.05). The activity of chymotrypsin was higher in the P_1E_0 and PC than in the NC (P<0.05), and in the fish fed with neutral phytase it was also slightly higher than that in the non-phytase groups (P<0.05). Both the phytase groups showed greater trypsin activity than the NC and P_1E_0, and this activity in P_1E_3 was dramatically increased over the PC (P<0.05). Altogether, there was no significant difference in digestive enzymes between P_1E_1, P_1E_2, and P_1E_3 (P>0.05).

Table 5 Serum, bone, and carcass compositions of crucian carp when fed with various diets for nine weeks

Group	Serum			Bone	
	Calcium (mmol/L)	Phosphorus (mmol/L)	AKP (U/100 ml)	Phosphorus (%)	Calcium (%)
NC	2.69±0.06^{ab}	3.18±0.15^{d}	7.72±2.35	5.80±0.13^{b}	10.89±0.22^{c}
P_1E_0	2.82±0.09^{ab}	4.13±0.05^{c}	5.79±1.42	5.94±0.20^{b}	10.97±0.30^{c}
PC	2.70±0.73^{ab}	4.52±0.18^{b}	5.35±1.60	6.36±0.11^{a}	11.88±0.12^{b}
P_1E_1	2.25±0.30^{b}	4.49±0.09^{b}	6.71±2.24	6.47±0.13^{a}	12.16±0.14^{ab}
P_1E_2	3.25±0.21^{a}	4.46±0.08^{b}	6.25±0.35	6.43±0.15^{a}	12.06±0.13^{ab}
P_1E_3	2.80±0.55^{ab}	5.04±0.09^{a}	7.09±3.71	6.40±0.13^{a}	12.37±0.21^{a}

Group	Carcass					
	Calcium (%)	Phosphorus (%)	Zinc (mg/kg)	Natrium (g/kg)	Magnesium (g/kg)	Iron (mg/kg)
NC	4.32±0.26^{b}	2.06±0.02^{c}	34.19±1.01^{b}	0.75±0.02^{b}	0.33±0.01^{b}	80.61±2.84
P_1E_0	4.31±0.48^{b}	2.10±0.01^{c}	34.24±0.32^{b}	0.75±0.02^{b}	0.33±0.01^{b}	80.50±4.23
PC	4.49±0.39^{b}	2.21±0.06^{b}	34.49±1.80^{b}	0.77±0.03^{ab}	0.34±0.01^{ab}	81.58±3.97
P_1E_1	4.72±0.37^{b}	2.21±0.01^{b}	37.35±1.51^{a}	0.81±0.00^{a}	0.34±0.00^{ab}	81.72±1.58
P_1E_2	5.42±0.10^{a}	2.29±0.03^{a}	37.86±1.06^{a}	0.80±0.02^{a}	0.34±0.01^{ab}	85.59±1.14
P_1E_3	5.57±0.09^{a}	2.30±0.02^{a}	37.13±1.33^{a}	0.79±0.02^{a}	0.35±0.01^{a}	83.85±0.94

AKP, alkaline phosphatase. Data are expressed as mean±SD (n=3). The values with different superscript lowercases in the same column are significantly different

Table 6 Specific activity of digestive enzymes in crucian carp when fed with various diets for nine weeks

Group	Amylase (U/mg prot)	Chymotrypsin (U/mg prot)	Trypsin (U/mg prot)
NC	33.26±2.48	33.41±2.28^{c}	65.04±7.12^{c}
P_1E_0	32.13±2.53	42.33±1.60^{b}	66.27±8.84^{c}
PC	32.10±0.85	46.66±3.08^{b}	75.41±4.84^{bc}
P_1E_1	32.63±2.20	55.74±3.43^{a}	78.80±9.14^{ab}
P_1E_2	32.66±1.93	53.35±4.34^{a}	82.04±4.93^{ab}
P_1E_3	31.15±2.52	58.81±2.71^{a}	87.30±5.09^{a}

Prot: protein. Data are expressed as mean±SD (n=3). The values with different superscript lowercases in the same column are significantly different

4 Discussion

Our study showed that crucian carp fed with six different diets exhibited differences in their growth performance and feed utilization characteristics. This indicates significant effects for the substitution of neutral phytase for $Ca(H_2PO_4)_2$ on the overall quality of the diet. Our study also demonstrated that phytase supplementation in the diets improved final body weight, ADG, FCR, SR, and PER of crucian carp. There were prior reports on *Pangasius pangasius* (Hamilton) fingerlings (Debnath *et al*., 2005), Nile tilapia *Oreochromis niloticus* (Liebert and Portz, 2005; Cao *et al*., 2008), and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Wang *et al*., 2009), with improved performance in growth, FCR, and PER. The increase could partially be attributed to the promoted digestibility of P and other minerals (Ca, Mg, Fe, etc.) in the diet (Cao *et al*., 2008; Wang *et al*., 2009; Liu *et al*., 2014), the increased ADC of protein (Cheng and Hardy, 2003; Liebert and Portz, 2005; Wang *et al*., 2009), and even the highly increased activity of digestive enzymes after the adjunction of neutral phytase. In the present study, the SR was improved by phytase treatment, in agreement with the results from Robinson *et al*. (2002), who reported that lack of availability of P would decrease fish survival, and vice versa. However, no significant effect was observed in CF (in common with Yoo *et al*. (2005)), VSI, or ISI in the experiment, in contrast to the findings of Liu *et al*. (2014), who reported that phytase treatment increased the CF and decreased the VSI. The HSI showed a decreasing trend when phytase was added, while Yoo *et al*. (2005) reported no significant difference in HSI. In short, the differences in CF, VSI, ISI, and HSI that were observed in the above studies may be attributed to several factors such as the fish variety, age, development stage, diet composition, rearing condition, and duration of experiments (Shao *et al*., 2008).

Phosphorus is one of the most important sources of pollution in fresh water environments (Baruah *et al*., 2004). Therefore, it is very important to reduce dietary P levels and fecal P discharges in order to reduce environmental pollution. The present study clearly showed that P content in the feces of phytase-added groups was significantly decreased compared with the non-phytase groups, as expected, and the effects of phytase-supplemented diets on P utilization were dramatically improved. The total P intake and apparent digestible P intake were increased along with the supplement of $Ca(H_2PO_4)_2$, indicating that they were proportional to the P content in diets. Xu *et al*. (2014) reported that neutral phytase could increase the utilization of P in black sea bream, *Acanthopagrus schlegelii*, which may be attributed to the increased decomposition of phytate. In our study, the fecal P excretion dropped considerably as neutral phytase was added, which means that the utilization of P was increased, in agreement with the early findings of Jackson *et al*. (1996). This study illustrates that the indigestible P can be converted to available P by neutral phytase, which is the same result as Liu *et al*. (2012) and Xu *et al*. (2014). Thus, as Luo *et al*. (2007) reported, our study also found that the total P accumulation went up when neutral phytase was used as a supplement. Higher neutral phytase activity in the diet had no significant effect on P utilization, which may illustrate that 200 U/kg of neutral phytase can hydrolyze a large quantity of phytate.

Phytate can bind protease, thus reducing the bioavailability of various dietary components as well as fish performance. Phytate can also form a complex with protein to form phytate-protein or phytate-mineral-protein complexes that are resistant to proteolytic digestion (Baruah *et al*., 2004; Cao *et al*., 2007). Additionally, phytate may bind amino acids in vivo, which can decrease amino acid availability. Supplementation with phytase in general could improve the availability of protein (Liebert and Portz, 2005), thus ultimately leading to additional improvements in growth performance. There have been many reports on phytase that were related to the positive effects on the ADCs of nutriments (Vielma *et al*., 1998; Wang *et al*., 2009; Liu *et al*., 2012). In this experiment, the ADCs of protein and P were increased in the P_1E_2 and P_1E_3, which was similar to the results achieved by Cheng and Hardy (2003), Yoo *et al*. (2005), and Wang *et al*. (2009), and the elevated level of the ADC for crude protein could explain the increase in PER with the addition of neutral phytase in the diet. The elevated level of the ADC for P in the study also indicated that the indigestible phytate P was successfully converted to available P by phytase (Liu *et al*., 2012), which means that it is possible to improve P retention and reduce P discharge into the water supply when animals are fed with a phytase-supplemented diet. However, phytase addition enhanced the apparent digestibility of lipids when 500 U/kg neutral phytase was added, which is in slight disagreement with Wang *et al*. (2009), who reported that a phytase-supplemented diet imparted a slight but non-significant reduction on the ADC of the lipid content on rainbow trout. It explained that adding phytase may inhibit the activity of lipase and decrease the efficiency of lipase hydrolysis lipid, but this cannot be clarified. All these differences may be attributed to the dose of phytase, and more research should focus on the ADC of crude lipid although it is less of an influence than phytase on fish. Overall, phytase addition improves the nutritional quality of diets for crucian carp.

The ability of phytate to chelate minerals is well known (Lowry *et al*., 1951). In this study, the elevated levels of Ca and P showed the positive impact of neutral phytase on the release of chelate minerals, that is to say supplementation with neutral phytase increased the mineral content in crucian carp. The total Ca, P, Zn, Na, and Mg contents in the carcasses of the phytase-added groups were increased, also indicating that phytase could improve mineral utilization and retention. Lall (2002) considered that a deficiency of several minerals may lead to growth reduction, and the concentration of T-P in blood is a basic index for health and nutritional status in fish (Kumar *et al*., 2010). In this present study, serum P concentrations were higher in the PC, P_1E_1, P_1E_2, and P_1E_3 than in the P_1E_0, which confirms the observation of Cao *et al*. (2008) who reported the same increase in the P content of serum. Liu *et al*. (2013) showed that the T-P content was reduced by decreasing P levels in the diet, while phytase supplement could release minerals from the bound compound (Vielma *et al*., 1998), and therefore increase the concentration of minerals in the whole-body. This means that phytase could hydrolyze phytate and elevate the concentration of minerals

such as Ca, Mg, and Zn in the whole-body (Vielma *et al*., 1998), and the higher P value in the phytase-supplemented diet was the main cause of raised mineral content in the whole body. Bone mineralization is considered the most sensitive criterion for assessing the influence of phytase on mineral utilization (Liu *et al*., 2014). The present study confirmed this point. In sum, the results of mineral content in serum, bone, and carcass all indicate that neutral phytase can raise the bioavailability of minerals. Therefore, phytase has been widely used to improve dietary mineral retention in fish and other animals (Baruah *et al*., 2004; Cao *et al*., 2007; Liu *et al*., 2014).

A phytase-supplemented diet can promote the growth performance of fish. However, a number of experiments have concentrated on the utilizations of Ca, P, and other nutrients, regardless of the significance of digestive enzymes. Lori *et al*. (2001) suggested that the inhibitory effects on various enzymes are due to the binding strength of the complexes formed from phytate. Yoo *et al*. (2005) reported that phytate can restrain the digestive enzymes such as amylase, trypsin, and chymotrypsin, and the addition of phytase could hydrolyze phytate and then eliminate the inhibitory effect on digestive enzymes. In this present study, the chymotrypsin and trypsin levels were sharply increased when phytase was used as a supplement, while amylase showed no change. This result suggests that the utilization of protein was enhanced in fish fed with a diet supplemented with phytase but not carbohydrate. In the study by Niu *et al*. (2010), phytate could be combined with protein to form phytate-protein or phytate-mineral-protein complexes and at the same time release the combination of phytate and digestive enzymes. Although researchers have been interested in phytase adjunction for quite some time, very little literature related to fish diets has been available until now. Liu *et al*. (2013) reported that the increase in digestive enzyme activity might be partly attributed to the release of minerals bound to the phytate by phytase addition. The studies by Hu and Pan (2006) on *Portunus trituberculatus* and Jiang *et al*. (2016) on Dalian mantis shrimp reported that the Ca^{2+}, Mg^{2+}, and even the Zn^{2+} had a significant activation on protease. This may be another important contributor to the higher activity of the chymotrypsin and trypsin in the phytase-supplemented diet.

5 Conclusions

The results of the present experiment indicate that neutral phytase can efficiently convert phytate-P to available P and replace the inorganic P supplementation in the diet for crucian carp without harming, and may even enhance their growth performance, according to the ADCs of both crude protein and P, mineral contents, as well as the activities of trypsin and chymotrypsin. This improved performance in phytase-supplemented diet groups can be attributed to phytase's ability to improve the bioavailabilities of P, protein, and metal ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} in the diet. In sum, the addition of phytase in the diet can be beneficial to crucian carp production systems by maintaining performance without the necessity of increased available P supplementation, and it can minimize P discharge into the environment and ultimately reduce aquaculture pollution.

Compliance with ethics guidelines

Xin-zheng NIE, Sha CHEN, Xiao-xu ZHANG, Bin-yang DAI, and Li-chun QIAN declare that they have no conflict of interest.

All experiments were approved by the Animal Care Committee of Zhejiang University, Hangzhou, China and were conducted in accordance with the Guidelines for the Care and Use of Agricultural Animals for Research and Teaching at Zhejiang University.

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中文概要

题　目：中性植酸酶对鲫鱼生长及磷利用的影响

目　的：比较研究在无机磷（磷酸二氢钙）添加不足时，添加不同剂量中性植酸酶对鲫鱼生长和磷利用的影响，为水产养殖更好地应用植酸酶提供依据并减少磷的排放，保护环境。

创新点：试验采用的中性植酸酶能够耐受饲料加工温度，并从营养物质消化及磷利用等方面研究中性植酸酶对鲫鱼的影响，探究低磷日粮在水产养殖的应用。

方　法：384 条鲫鱼（(1.50±0.01) g）随机分为 6 组，分别饲喂对照组（未添加中性植酸酶）跟试验组（添加不同剂量中性植酸酶）日粮 63 天，比较 3 组间各项指标差异。

结　论：在以植物蛋白为主的日粮中，添加中性植酸酶能够促进植酸磷水解，提高鲫鱼对饲粮中磷、粗蛋白等营养物质的利用，促进钙、磷等矿物质在鲫鱼体中的沉积，降低粪磷排出量，促进鲫鱼生长。说明可以在鲫鱼日粮中添加中性植酸酶，减少无机磷的添加，降低粪磷排放，从而减轻环境污染。

关键词：中性植酸酶；鲫鱼；生长性能；磷利用；表观消化率；鱼体成分