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Optimum Dietary Ca/P Ratio and Phytase for Growth and Bone Mineralization in Juvenile *Clarias gariepinus* Fed Soya Bean-Based Diet

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Abstract

The research investigated the interactive effect of five phytase graded levels of 0 FTU/g (P0), 250 FTU/g (P1), 500 FTU/g (P2), 750 FTU/g (P3), 1000 FTU/g (P4) and four graded levels of roasted soya bean substitution at 25%, 50%, 75% and 100% in place of fish meal, respectively. All basal diets contained 0.20% calcium without added inorganic phosphorus, wheat and amino acids supplements so that the phosphorus, calcium, and Ca/P ratios of the five (5) were: S0 (1.31%, 1.85%, and 1.41), S1 (1.15%, 1.64%, and 1.43), S2 (1.01%, 1.28%, and 1.27), S3 (0.75%, 0.99%, and 1.30), and S4 (0.43%, 0.68%, and 1.59). 1092 fish of average weight 11.55 ± 0.2 g were randomly allocated to all experimental diet and fed 3% body for 84 days. There was no significant difference in final weight, weight gain and FCR S3P1 and S0P0 (P>0.05). Fish fed low phosphorus diet (100% soya bean) showed reduced total weight and weight gain compared to high (0%, 25%, 50%) and medium phosphorus diet (75%) with phytase addition (P<0.05). Fish fed phytase at 50-200 FTU/g and inorganic control had lower weight gain compared to S3P1 (P<0.05). Ca/P ratio correlated negatively with final weight (r=-0.439, P<0.01), weight gain (r=-0.431, P<0.01), SGR (r=-0.138, P>0.05), and PER (r=-0.210, P>0.05). Ca/P of S3P1 (1.41 ± 0.02) was not different from S0P0 (1.41 ± 0.01). Bone phosphorus, calcium and zinc were higher in 250 FTU/g compared to 0 FTU/g, 500 FTU/g, 750 FTU/g and 1000 FTU/g. In conclusion, Phytase improved growth performance (r=0.05) of juvenile Clarias gariepinus with optimum at 250 FTU/g; however, Ca/P affected performance (r=-0.431) and should range between 1.41-1.46 for optimum phytase activity.

Keywords: Phytase; Growth performance; Bone mineralization; Ca/P ratio; Optimum

Introduction

The main source of plant protein in terrestrial and aquatic feed includes soybean meal, corn (gluten), groundnut meal, sunflower meal, canola/rapeseed meal, peas and lupins [1]. Soybean meal represents the highest proportion of plant protein in fish diets owing to high yield, relatively high crude protein content and easy and round the year availability. Soybeans are the most plentiful of oilseed crops, with a worldwide production of 253.1 million metric tonnes in 2012 [2]. However, the major constraints that limit use of plant proteins in animal feed are the presence of anti-nutritional factors such as saponins [3], lectins (Santiago et al.), and phytate being one of importance [1]. They are also limiting in essential amino acids affecting growth and nutrient utilization in fish [4]. Apart from minerals, phytate also forms complexes with proteins and amino acids [5] at acidic pH leading to the formation of phytate-protein aggregates and precipitates, which results in a decreased accessibility for proteases, and consequently in inefficient protein and amino acid digestion [6]. Cao et al. reported that the growth and feed conversion efficiency in commonly cultured fish species, such as carps, tilapias, trout and salmons were negatively affected by the phytate in the diets [7]. Phytase in fish diet can breakdown a portion of the phytate, increasing availability of dietary phytate-phosphorus in diets for fish [1,8,9]. However, very low phytase activities have been reported for legume seeds, hence dietary supplementation is necessary in oil seed meals, particularly soy bean meal to improve protein and mineral digestibility and availability, which is very low compared to fish meal [10]. Although dietary phytase supplementation results in substantial reductions in total phosphorus excretion, a slight increase of soluble phosphorus in the excreta is possible [11]. A precise prescription of appropriate phytase inclusion in diets is needed to release the actually required phosphorus and other nutrients for the animals to avoid or minimize this side effect [12], hence

there is need to investigate the intrinsic (native) phytase in fish diets and the optimum phytase supplementation needed to promote growth and nutrient utilization in fish. In dose–response studies, phytase addition of 250-1500 FTU/g is usually considered feasible in many fish species. The optimum dose changes along with many factors such as fish species, different phytase sources, diet formulation (amount of substrate for phytase) and selected response parameters [7]. Optimum phytase has been determined for Channel catfish [13,14], Nile tilapia [15], Korean rockfish [16], *Pangasius pangasius* [17], and Rainbow trout [18]. Van weerd et al. [19] and Nwanna et al. [20] reported 750 FTU/g (Natuphos 5000) and 8000 FTU/g (Ronozyme 5000) phytase, respectively, as optimum for growth of *Clarias gariepinus*, but did not consider consistently increasing levels of soya bean used in their studies. In the former study, increasing levels of soya bean depressed growth performance, which could be due to the inclusion of wheat in the study. Wheat has a high phytase (1193 FTU/g), according to Eeckhout and de Paepe (1994) [47], which can compromise responses to phytase and reduce its efficacy.

In diet, it is necessary to take into account the Ca/P ratio, because it has important consequences for bone development, which can be

adversely affected when this ratio increases, resulting in aberrations in bone mineral homeostasis and bone metabolism [1]. Dietary levels of Ca:P ratios are crucial to phytase efficacy and activity [21]. Cao et al. [7] reported that data on this aspect in fish are limited. High dietary Ca or a high ratio of Ca: P interferes with phosphorus absorption and reduces the effectiveness of phytase activity [22,23]. High concentration of calcium in fish feed will chelate with phytate to become insoluble complex, or compete with phytase to change the phytase activity center site as an inhibitor, or increase the pH value to inhibit the activity of phytase. Increasing Ca:P ratio depressed *E. coli* derived phytase action in the pig diet and thus significantly depressed weight gain and feed efficiency. The aim of this research, therefore, is to define optimum dietary Ca/P ratio and effective phytase efficacy for optimum growth performance in soya bean diet of juvenile *Clarias gariepinus*.

Materials and Method

Experimental design

The research investigated the interactive effect of five phytase supplemented at graded levels of 0 FTU/g (P0), 250 FTU/g (P1), 500 FTU/g (P2), 750 FTU/g (P3), 1000 FTU/g (P4) and substitution of four graded levels of fish meal with 25%, 50%, 75%, and 100% roasted, full-fat soya bean meal with hulls as S1, S2, S3 and, S4 and fed to duplicate group of juvenile *Clarias gariepinus* in a 4 × 5 factorial design so that there are 21 treatment combinations with treatment code S0P0-S4P4 (S for soya bean, P for phytase). A diet with 100% fish meal (S0), which served as control, was included in the experiment, but with no phytase addition (P0).

Experimental diet

Five (5) iso-nitrogenous (40%) and iso-caloric (~ 18.15kj/g GE) diets serving as controls without dietary phytase were formulated using soya bean as the main plant protein source to replace fish meal at 0%, 25% soya bean meal, 50% soya bean meal, 75% soya bean meal, and 100% soya bean meal, which were labelled as S0, S1, S2, S3 and S4 as basal diets served as controls with no phytase treatment (P0). Another four diets as commercial control was also included in this experimental group to compare growth performance with phytase supplementation in high (S1 and S2), medium (S3), and low phosphorus diet (S4) at 250 FTU/g (P1), 500 FTU/g (P2), 750 FTU/g (P3), and 1000 FTU/g (P4). A fish meal control (S0) was not supplemented with phytase (P0). All basal diets contained 0.20% calcium without added inorganic phosphorus, wheat and amino acids supplemented so that the phosphorus, calcium, and Ca/P ratios of the five (5) were: S0 (1.31%, 1.85%, and 1.41), S1 (1.15%, 1.64%, and 1.43), S2 (1.01%, 1.28%, and 1.27), S3 (0.75%, 0.99%, and 1.30), and S4 (0.43%, 0.68%, and 1.59). Added calcium was lower than requirement of 0.45% for catfish [24] and 0.50 for African catfish (ADCP, 1983). Inorganic phosphorus was not added because phosphorus in feed, particularly at high levels can repress the activity of phytase. In addition, several studies have reported better growth performance with phytase compared to inorganic phosphorus source [12,14]. Moreover, Dicalcium phosphate has also been shown to lower phytase efficacy in poultry [25].

About 10kg of soya bean was purchased from a local grain market with good quality soya bean products. Soybean was subjected to heat treatment by roasting [20] to remove anti-nutritional factors affecting nutrient bioavailability in fish; roasted soybean were grinded, sundried, and packed into plastic bags, and stored at ambient temperature prior to inclusion with other feeding stuff for the fish. Compounded feeds were formulated using Pearson's method and mixed with cold,

distilled water to a moisture content of 14%. Feed were cold pelleted and forced through a 2 mm hand mesh and sundried before phytase supplementation. Liquid Natuphos° phytase (Natuphos 5000L, BASF, the Chemical Co., Germany) were prepared by serial dilution of the phytase concentrations using a dilution factor of 1:10 and bulk density of 1.2 g/cm³ (1.15-1.25 g/cm³) according to BASF to give 0.4917 ml, 0.9833 ml, 1.475 ml, and 1.967 ml/kg diets for 250 FTU/g, 500 FTU/g, 750 FTU/g, and 1000 FTU/g phytase, respectively, which were supplemented in the diets according to the design. Phytase was supplied by BASF, the Chemical Co., Ludwigshafen, Germany, while phytase activity of all experimental groups analysed by BASF SE, Lampertheim, Germany. Both phytase and non phytase-supplemented groups were analysed for total phosphorus, phytate phosphorus [26], calcium, and phytase activity. The control diet, S0 (100% fish meal), did not receive any phytase treatment. The gross and chemical composition of the basal diet is shown in Table 1 below.

Experimental fish

To meet the requirement of the 4 × 5 factorial design in the first experimental group pf roasted , full fat soya bean diets, about 1160 *Clarias gariepinus* fish of average weight (4.5+0.2 g), were procured from a reliable fish farm with no trace of metal contamination and pollution. Fish were acclimated under laboratory conditions for 3 weeks prior to experimental feeding during which time they were fed commercial diet in 10 fish holding tanks supplied with clean water until they attained the juvenile status of average weight 11.55+0.2 g and length 11.79+1.03 cm before experimental feeding with phytase-treated diets. Water temperature, pH and oxygen maintained at optimum range between 25°C-32°C, 7.40-7.45, 4.80-5.0 mg/l. after which all fish were left unfed for 2-3 days prior to start of experiment. After acclimation, 1092 fish were randomly allocated to all 21 treatment combination groups of diets, including control diet, and fed to duplicate groups of fish stocked at 26 fish per tank ~300.30g. Each of the feed rations were shared so that experimental fish were fed twice daily (morning at 08:00 hrs) and evening (evening at 16:00 hrs) to obtain optimum growth with uneaten feed and faeces removed at each feeding period. Experimental fish were weighed biweekly throughout the experiments using an electronic compact balance S. Mettler scale with accuracy of 0.01 g (Model: K-BH). Water was changed once every two (2) days using static water renewal method and water quality monitored and measured for all treatment tanks at the end of the experiment. Fish (n=2) were sampled for bone mineralization after ashing to constant weight [27].

Determination of mineral composition

Duplicate sample of feeds were analyzed for phosphorus, calcium, sodium, copper, and manganese and determined using flame atomic absorption spectrophotometer model Buck 205, Buck Scientific, USA, while phosphorus (P) was estimated spectrophotometrically using molybdovanadate method [28].

Determination of phytate

Phytate in feeds was measured by alkaline picrate method of Oberleas [23]. Sample was extracted with 0.2 N HCl to give (3-30 μg ml⁻¹ phytate solution). 0.5 ml of extract was pipetted into a test tube fitted with a ground glass stopper. 1 ml of ferric solution was added to the tube, which was covered with the stopper and fixed with a clip. The tube was heated in a boiling water bath for 30 minutes. Care was taken to ensure that for the first 5 minutes, the tube remained well stoppered. After cooling in ice water for 15 minutes, the tube was allowed to adjust to room temperature. Once the tube reached room temperature, the

Table 1: Gross and chemical composition of roasted, full-fat soyabean-basal diets for juvenile *Clarias gariepinus*.

Ingredient	S_0 (0%)	S_1 (25%)	S_2 (50%)	S_3 (75%)	S_4 (100%)	S_3 D
Fish meal (66.46%)	54.29	45.38	34.16	19.62	-	19.83
Soyabean meal (full fat, 42.93%)	-	15.13	34.16	58.85	92.15	59.48
Maize (10.24%)	41.71	35.50	27.68	17.54	3.87	16.69
Vit. Min Mix#	0.50	0.50	0.50	0.50	0.50	0.50
Fish oil	1.00	1.00	1.00	1.00	1.00	1.00
$CaCO_3$	0.50	0.50	0.50	0.50	0.50	0.50
Salt	0.50	0.50	0.50	0.50	0.50	0.50
Chromic oxide	0.50	0.50	0.50	0.50	0.50	0.50
Cellulose*	0.20	0.20	0.20	0.20	0.20	0.20
Starch	0.80	0.80	0.80	0.80	0.80	0.80
Chemical composition (%)						
Phosphorus	1.31	1.15	1.01	0.75	0.43	1.23
Available phosphorus	0.91	0.63	0.44	0.33	0.03	
Calcium	1.85	1.64	1.28	0.99	0.68	3.63
Magnesium	0.25	0.24	0.28	0.27	0.27	0.16
Potassium	0.87	1.10	1.35	1.60	1.90	1.53
Zinc	15.82	14.22	12.59	12.12	12.40	17.81
Ca/P ratio	1.41	1.43	1.27	1.30	1.59	2.95
Phytate	0.40	0.52	0.57	0.43	0.40	
Native phytase (FTU/g)	<100	<100	<100	<100	<100	<100

#Micro mineral mix contains per kilogram: Vit. A (20, 000 IU), Vit. D3 (5, 000 IU), Vit. E (300 mg), Vit K3 (10 mg), Vit. B1 (20 mg), Vit. B2 (25 mg), Vit. C (300 mg), Niacin (120 mg), Ca Pantothenate (60 mg), Vit. B6 (10 mg), Vit. B12 (0.05), Folic acid (5 mg), Biotin (1 mg), Choline chloride (5 mg), Inositol (50 mg), Manganese (30 mg), Iron (35 mg), Zincn (45 mg), Copper (3 mg), Iodine (5 mg), Cobalt (2 mg), Lysine (85 mg), Selenium (0.15 mg), Antooxidant (80 mg), Methionine (100 mg).

* Ascarboxymethyl cellulose

content of the tube was mixed and centrifuged for 30 minutes at 3000 g. 1 ml of the supernatant was transferred to another test tube and 1.5 ml of 2, 2'- Bipyridine solution added. The absorbance was measured at 519 nm against distilled water.

Determination of phytase activity

"Phytase activity in feed sample was determined by Engelen et al. [30] with slight modifications according to the relative method of BASF [29] using methods by Engelen et al. [30]. 100 g of each sample were incubated with sodium phytate as substrate, which was dissolved with acetic buffer. The incubation was stopped by adding acidic molybdate/vanadate reagent to produce a colored complex with the phosphate formed. The enzyme phytase liberates inorganic phosphate from the substrate sodium phytate during incubation and the intensity of the yellow color of the vanadomolybdophosphorus complex is a measure of the amount of phosphate liberated and was measured at a wavelength of 415 nm.

Growth and nutrient utilization

Growth performance was monitored biweekly with the following parameters measured:

Weight gain=Final weight–initial weight

Feed Conversion Ratio (FCR): Feed Intake (g)/Fish weight gain (g)

Specific Growth Rate (%): In (W2-W2) / (t2-t1), where W2 and W1 are weights on day t2 and t1, respectively.

Where, W2=final weight, W1=initial weight, t2=time at the end of experiment, t1=time at end of experiment

Survival rate (%) =Initial number of fish-Mortality/Initial number

of fish × 100

Bone mineral analysis

At the beginning and end of each feeding trial, bones of fish sacrificed for each experiment were investigated for calcium, phosphorus, magnesium, iron and zinc analysis described as follows:

Intact spinal, skull, pectoral, dorsal and caudal bones were removed from each group of fish and pooled for bone mineral analysis. Whole fish flesh was stripped off from the vertebrae (spinal) and the adhering flesh removed by light brushing and rinsing in distilled water. The vertebrae were oven-dried dried for 2 hours at 110°C and then pulverized, dried again, and weighed. The dried samples were ashed at 550°C for 6 h. Ash weight was calculated as a percentage of dry, fat-free bone weight. For mineral estimation, the ash was digested in a boiling nitric acid and perchloric acid mixture (2:1) according to the method by AOAC [28]. After appropriate dilution, calcium, magnesium, potassium, zinc content was estimated by atomic absorption spectrophotometer using flame atomic absorption spectrophotometer model Buck 205, Buck Scientific, USA, while phosphorus was estimated spectrophotometrically using molybdovanadate method [28].

Determination of phosphorus retention efficiency

Phosphorus retention efficiency was determined by method of Cao et al. [31] as,

Phosphorus retention efficiency=Phosphorus retained/Phosphorus fed

Phosphorus retained= $(FBW \times F_p) - (IBW \times I_p)$

Phosphorus fed=Dp × FI

Where, FBW=Final Body Weight; IBW=Initial Body Weight;

F_P=Phosphorus in final carcass; I_P=Phosphorus in initial carcass; D_P=Phosphorus in diet

Statistical analysis

All data were subjected to one-way analysis of variance at significance level of 0.05. Individual differences in treatment means were determined by Duncan new multiple range test [32]. Interactions between dietary levels of phytase and experimental diets were determined using multiple regression analysis (factorial). Data was treated as mean+S.D. A dose-response data or growth model [33] of graded phytase inclusion was determined by the linear equation below.

$$Y=a_0+a_1X$$

Results

Mineral composition of diet

With the exception of S0P0 diet (fish meal based), there was a highly significant improvement in phosphorus levels from 1.15 ± 0.01% in S1P0 to 1.21 ± 0.01% in S1P4. Diet S2P4 showed a significant increase (P<0.05) in phosphorus (0.86 ± 0.01%) compared to S2P3 (0.76 ± 0.01%), S2P2 (0.83 ± 0.01%). There were no significant phytase effects (P<0.05) between diets S4P4, S4P0, and S4P1; S4P0, S4PI, S4P3; and between diets S3P4, S3P1, and S3P0, although phosphorus level for S3P1 (0.76 ± 0.01%) was higher than diets S3P0 (0.75 ± 0.01%) and S3P4 (0.75 ± 0.01%) (P>0.05). Diets with 100% soya bean had the lowest values of phosphorus, which ranged between (0.40 ± 0.02%) in S4P2 to 0.45 ± 0.01% in S4P3. Phytase significantly (P<0.05) affected available phosphorus with significant increase from S1P0 (0.63 ± 0.01%) to S1P4 (1.03 ± 0.02%), which was not significantly different (P>0.05) from S1P1 (1.03 ± 0.02%), but higher than S0P0 (0.91 ± 0.01%). S1P4 recorded highest available level, while S4P3 showed the lowest (0.01 ± 0.01%). Diet S3P1 (0.42 ± 0.02%) had the highest available phosphorus level compared to S3P0 (0.33 ± 0.01%), S3P2 (0.21 ± 0.01%), S3P3 (0.24 ± 0.01%), and S3P4 (0.31 ± 0.02%), which was not significantly

different from S3P0 (P>0.05). There were no also differences between diets S4P1 (0.23 ± 0.01%) and S4P4 (0.16 ± 0.01%); and between S3P2, S4P1, S3P3 (P>0.05). Phytase supplementation significantly improved calcium levels in all soyabean diets compared to control diets, except S0P0, without phytase (P<0.05). Diets S1P1 (1.70 ± 0.01%) and S1P3 (1.70 ± 0.01%), which were higher than S1P0 (1.64 ± 0.01%), S1P2 (1.59 ± 0.01%), and S1P4 (1.56 ± 0.01%) (P<0.05). Phytase improved significantly calcium levels from 0.99 ± 0.01% (S3P0) to 1.20 ± 0.01% (S3P4) (P<0.05). There was no significant difference between S4P4 (0.72 ± 0.01%) and S4P3 (0.72 ± 0.01%) (P>0.05). Calcium levels was highest in S0P0 (1.85 ± 0.01%) and lowest in S4P3 (0.72 ± 0.01%). Phytase supplementation increased significantly zinc levels in diets with 25%, 50%, and slightly in 75% soya bean meal (S3P1). Values increased from 14.22 ± 0.01% (S1P0) to 15.01 ± 0.01% (S1P4). There was a significant increase in zinc levels from 12.59 ± 0.02% (S2P0) to 12.89 ± 0.01% (S2P4). Dietary supplementation in 75% soya bean showed a statistically significant increase (P<0.05) in zinc levels for S3P1 (12.48 ± 0.01%) compared to S3P0 (12.12 ± 0.02%), S3P2 (12.19 ± 0.01%), S3P3 (11.85 ± 0.01%), and S3P4 (12.30 ± 0.01%). Dietary copper was significantly improved with phytase addition to all soya bean diet (P<0.05). Mineral composition of diet based on full fat soyabean is shown in Table 2.

Growth and nutrient utilization

Final weight (Table 3) ranged from 373.84 ± 43.78 g (S4P0) to 1526.56 ± 64.78 g (S0P0). There were, however, no statistically significant effects (P>0.05) of phytase on final weight for diets S0P0, S1P0, S1P1, S1P2, S1P3, and S1P4; S2P0, S2P1, and S2P2; S2P1, S2P2, S3P0, S3P2, and S3P3; S4P0, S4P1, S4P2, S4P3, and S4P4. Final weight of fish fed 250FTU/g recorded the highest value compared with fish fed 0FTU/g, 500FTU/g, 750FTU/g, and 1000FTU/g. Fish fed low phosphorus diet (100% soya bean) showed reduced total weight compared to high (0%, 25%, 50%) and medium phosphorus diet. Phytase increased significantly total weight of fish fed 25% soya bean diets (S1) from

Table 2: Mineral composition of experimental diet based on full fat and supplemented with phytase.

Treatments	Phosphorus (%)	Available Phosphorus (%)	Calcium (%)	Magnesium (%)	Potassium (%)	Zinc (ppm)
S0P0	1.31 ± 0.01[m]	0.91 ± 0.01[j]	1.85 ± 0.01[q]	0.25 ± 0.01[bc]	0.87 ± 0.01[a]	15.82 ± 0.02[s]
S1P0	1.15 ± 0.01[k]	0.63 ± 0.01[h]	1.64 ± 0.01[n]	0.24 ± 0.02[b]	1.10 ± 0.01[c]	14.22 ± 0.01[n]
S1P1	1.17 ± 0.01[k]	1.03 ± 0.02[k]	1.70 ± 0.01[p]	0.26 ± 0.01[bcd]	1.08 ± 0.01[bc]	14.87 ± 0.01[q]
S1P2	1.21 ± 0.01[l]	0.60 ± 0.01[h]	1.59 ± 0.01[m]	0.27 ± 0.01[cde]	1.09 ± 0.01[bc]	14.41 ± 0.01[o]
S1P3	1.17 ± 0.01[k]	0.66 ± 0.01[i]	1.70 ± 0.01[p]	0.26 ± 0.01[bcd]	1.06 ± 0.01[b]	14.70 ± 0.01[p]
S1P4	1.21 ± 0.01[l]	1.03 ± 0.02[k]	1.56 ± 0.01[l]	0.27 ± 0.01[cde]	1.07 ± 0.01[b]	15.01 ± 0.01[r]
S2P0	1.01 ± 0.01[j]	0.44 ± 0.01[fg]	1.28 ± 0.01[i]	0.28 ± 0.02[def]	1.35 ± 0.01[e]	12.59 ± 0.02[k]
S2P1	0.90 ± 0.01[i]	0.46 ± 0.01[g]	1.35 ± 0.01[k]	0.28 ± 0.01[def]	1.34 ± 0.01[e]	12.79 ± 0.01[l]
S2P2	0.83 ± 0.01[g]	0.43 ± 0.02[f]	1.66 ± 0.01[o]	0.36 ± 0.01[h]	1.28 ± 0.02[d]	12.44 ± 0.01[i]
S2P3	0.76 ± 0.01[e]	0.37 ± 0.01[e]	1.36 ± 0.01[k]	0.28 ± 0.01[ef]	1.34 ± 0.01[e]	12.90 ± 0.01[m]
S2P4	0.86 ± 0.01[h]	0.68 ± 0.01[i]	1.32 ± 0.01[j]	0.28 ± 0.01[def]	1.36 ± 0.01[e]	12.89 ± 0.01[m]
S3P0	0.75 ± 0.01[e]	0.33 ± 0.01[d]	0.99 ± 0.01[e]	0.27 ± 0.01[cde]	1.60 ± 0.01[h]	12.12 ± 0.02[e]
S3P1	0.76 ± 0.01[e]	0.42 ± 0.02[f]	1.06 ± 0.01[f]	0.28 ± 0.01[def]	1.60 ± 0.01[h]	12.48 ± 0.01[j]
S3P2	0.72 ± 0.01[d]	0.21 ± 0.01[c]	1.05 ± 0.01[f]	0.31 ± 0.01[g]	1.50 ± 0.01[f]	12.19 ± 0.01[f]
S3P3	0.78 ± 0.01[f]	0.24 ± 0.01[c]	1.17 ± 0.01[g]	0.29 ± 0.01[fg]	1.58 ± 0.01[h]	11.85 ± 0.01[d]
S3P4	0.75 ± 0.01[e]	0.31 ± 0.02[d]	1.20 ± 0.01[h]	0.29 ± 0.01[efg]	1.56 ± 0.01[g]	12.30 ± 0.01[g]
S4P0	0.43 ± 0.01[bc]	0.03 ± 0.01[a]	0.68 ± 0.01[b]	0.27 ± 0.01[cde]	1.90 ± 0.01[j]	12.40 ± 0.01[h]
S4P1	0.44 ± 0.01[bc]	0.23 ± 0.01[c]	0.65 ± 0.01[a]	0.28 ± 0.01[def]	1.75 ± 0.01[i]	11.82 ± 0.01[c]
S4P2	0.40 ± 0.02[a]	0.15 ± 0.01[b]	0.82 ± 0.01[d]	0.29 ± 0.01[efg]	1.96 ± 0.01[l]	11.73 ± 0.02[a]
S4P3	0.45 ± 0.01[c]	0.01 ± 0.01[a]	0.72 ± 0.01[c]	0.29 ± 0.01[efg]	1.92 ± 0.01[k]	12.49 ± 0.01[j]
S4P4	0.42 ± 0.01[ab]	0.16 ± 0.01[b]	0.72 ± 0.01[c]	0.20 ± 0.01[a]	1.96 ± 0.01[l]	11.76 ± 0.01[b]

Mean values with the same alphabet superscript in the same column are not significantly different at the 0.05 level (2-tailed)

Table 3: Growth parameters and nutrient utilization of *Clarias gariepinus* fed experimental diet (full fat).

Trt	Initial weight	Final weight (g)	Weight gain (g)	SGR (%)	FCR	Survival rate (%)	Ca/P ratio	Analysed Phytase Activity (FTU/g)
S0P0	309.09 ± 14.38	1526.56 ± 64.78[g]	1219.47 ± 47.57[f]	1.90 ± 0.01[ef]	1.29 ± 0.01[a]	92.31 ± 0.00[cd]	1.41 ± 0.00[bc]	<100
S1P0	312.99 ± 0.06	1351.26 ± 7.55[fg]	1043.78 ± 0.29[ef]	1.74 ± 0.01[cdef]	1.38 ± 0.08[a]	100.00 ± 0.00[d]	1.43 ± 0.00[bcd]	<100
S1P1	330.47 ± 8.74	1413.18 ± 102.09[fg]	1082.71 ± 110.82[ef]	1.72 ± 0.03[cdef]	1.39 ± 0.06[a]	98.08 ± 2.72[d]	1.45 ± 0.00[bcd]	580
S1P2	308.77 ± 16.06	1252.15 ± 10.01[def]	943.39 ± 26.07[de]	1.43 ± 0.11[cde]	1.57 ± 0.22[ab]	100.00 ± 0.00[d]	1.33 ± 0.01[as]	720
S1P3	370.97 ± 17.65	1433.75 ± 110.61[fg]	1082.78 ± 121.28[ef]	1.67 ± 0.09[cdef]	1.47 ± 0.11[a]	96.16 ± 5.44[d]	1.45 ± 0.00[bcd]	890
S1P4	294.81 ± 52.13	1337.42 ± 3.80[efg]	1042.62 ± 48.33[ef]	1.73 ± 0.12[cdef]	1.37 ± 0.13[a]	98.08 ± 2.72[d]	1.29 ± 0.02[a]	1510
S2P0	304.78 ± 13.85	1290.05 ± 30.28[def]	985.27 ± 16.43[de]	1.66 ± 0.04[cdef]	1.34 ± 0.08[a]	86.54 ± 2.72[c]	1.27 ± 0.02[a]	<100
S2P1	270.65 ± 10.34	1096.13 ± 82.38[cd]	825.49 ± 72.03[cd]	1.66 ± 0.03[cdef]	1.40 ± 0.01[a]	94.23 ± 2.72[cd]	1.50 ± 0.04[de]	270
S2P2	284.98 ± 25.05	1133.55 ± 110.37[cde]	848.57 ± 85.32[cd]	1.69 ± 0.04[cdef]	1.38 ± 0.01[a]	86.54 ± 8.16[c]	2.00 ± 0.02[h]	580
S2P3	314.79 ± 35.94	1428.10 ± 244.04[fg]	1113.31 ± 208.10[ef]	1.66 ± 0.06[cdef]	1.29 ± 0.08[a]	100.00 ± 0.00[d]	1.80 ± 0.03[g]	1260
S2P4	294.05 ± 19.85	1277.64 ± 23.77[def]	983.60 ± 43.62[de]	1.69 ± 0.06[cdef]	1.34 ± 0.18[a]	94.23 ± 2.72[cd]	1.54 ± 0.00[ef]	1260
S3P0	312.41 ± 13.59	1040.13 ± 142.81[c]	727.72 ± 129.22[c]	1.39 ± 0.22[cd]	1.67 ± 0.12[ab]	98.08 ± 2.72[d]	1.30 ± 0.00[a]	<100
S3P1	251.59 ± 68.82	1366.47 ± 89.64[fg]	1114.88 ± 20.82[ef]	2.04 ± 0.00[g]	1.30 ± 0.06[a]	73.08 ± 10.88[b]	1.41 ± 0.02[b]	330
S3P2	342.73 ± 25.84	1093.83 ± 110.35[cd]	751.10 ± 136.19[c]	1.67 ± 0.13[cdef]	1.74 ± 0.22[ab]	96.15 ± 0.00[d]	1.46 ± 0.00[bcde]	660
S3P3	311.63 ± 5.52	999.23 ± 92.29[c]	687.60 ± 86.78[c]	1.80 ± 0.06[cdef]	2.40 ± 0.18[b]	94.23 ± 2.72[cd]	1.49 ± 0.02[cde]	1050
S3P4	327.72 ± 34.65	992.13 ± 15.21[c]	664.41 ± 19.44[c]	1.38 ± 0.10[cd]	2.36 ± 0.23[b]	94.23 ± 2.72[cd]	1.60 ± 0.02[f]	1340
S4P0	310.44 ± 32.31	373.84 ± 43.78[a]	63.41 ± 11.48[a]	0.22 ± 0.1[a]	16.71 ± 1.43[e]	75.00 ± 2.72[b]	1.59 ± 0.04[f]	<100
S4P1	317.25 ± 41.10	635.44 ± 54.15[ab]	318.19 ± 13.05[b]	0.83 ± 0.04[b]	3.30 ± 0.37[c]	92.31 ± 0.00[cd]	1.48 ± 0.04[bcde]	330
S4P2	293.01 ± 13.91	488.40 ± 22.60[ab]	195.40 ± 8.70[ab]	0.61 ± 0.00[b]	4.43 ± 0.06[d]	96.16 ± 5.44[d]	2.07 ± 0.13[h]	640
S4P3	309.04 ± 12.0	526.81 ± 60.99[ab]	217.77 ± 48.99[ab]	0.63 ± 0.07[b]	4.49 ± 0.54[d]	94.23 ± 2.72[cd]	1.61 ± 0.01[f]	830
S4P4	312.77 ± 22.65	458.48 ± 26.87[ab]	145.56 ± 49.30[ab]	0.90 ± 0.78[b]	4.97 ± 0.48[d]	55.19 ± 1.90[a]	1.73 ± 0.00[g]	930
Sig.	>0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	

Mean values with the same alphabet superscript in the same column are not significantly different at the 0.05 level (2-tailed)

Table 4: Effects of phytase on growth and nutrient utilization in *Clarias gariepinus* fed soyabean based diet.

Phytase (FTU/g)	Initial weight	Final weight (g)	Weight gain (g)	SGR	FCR	Survival rate (%)	Ca /P
0	309.94 ± 13.80	1116.37 ± 428.04	807.93 ± 428.67	1.46 ± 0.48	4.48 ± 6.47	90.38 ± 9.64	1.40 ± 0.12[a]
250	292.49 ± 46.32	1127.8 ± 336.33	835.32 ± 344.72	1.55 ± 0.60	1.85 ± 0.91	89.42 ± 11.21	1.46 ± 0.04[a]
500	307.37 ± 28.45	991.98 ± 322.57	684.61 ± 316.63	1.42 ± 0.39	2.28 ± 1.34	94.71 ± 6.48	1.71 ± 0.35[b]
750	326.61 ± 31.76	1096.97 ± 413.94	775.36 ± 400.46	1.25 ± 0.66	2.41 ± 1.38	96.15 ± 3.56	1.59 ± 0.14[ab]
1000	307.33 ± 30.20	1016.42 ± 371.84	709.04 ± 381.61	1.42 ± 0.46	2.51 ± 1.60	85.43 ± 18.84	1.54 ± 0.17[ab]
Total	308.80 ± 31.45	1072.12 ± 364.99	764.62 ± 365.09	1.42 ± 0.51	2.79 ± 3.37	91.18 ± 11.24	1.53 ± 0.21

1351.26 ± 7.55fg (S1P0) to 1433.75 ± 110.61g (S1P3). Fish fed S1P2 recorded the lowest (943.39 ± 26.07 g). Fish fed S2P1 (825.49 ± 72.03 g) had the lowest weight gain for diets based on 50% soya bean meal (S2) with phytase increasing weight gain from 985.27 ± 16.43 g (S2P0) to 1113.31 ± 208.10 g (S2P3). S2P4 fish was not significantly different from S2P1 fish in weight gain (P>0.05). Phytase inclusion in 75% soya bean diet (S3) resulted in highest weight gain for S3P1 fish (1114.88 ± 20.82 g), which was statistically different from S3P2 (751.10 ± 136.19 g), S3P3 (687.60 ± 86.78 g), and S3P4 (664.41 ± 19.44 g) (P<0.05). There was, however, no significant difference between S3P1, S0P0, S1P0, S1P1, S1P3, S1P4, and S2P3 fish (P>0.05). Weight gain increased significantly with phytase addition to diet based on 100% (S4) soya bean meal (P<0.05). Fish fed S4P4 had the lowest weight gain compared to S4P1, S4P2, and S4P3.Weight gain of fish fed 100% was, however, significantly lower compared to fish fed 0% (S0), 25% (S1), 50% (S2) and 75% (S3) soya bean meal (Table 4). Figure 1 showed weight gain by fish fed soya bean diet (full fat) supplemented with phytase with the lowest values recorded 100% soya bean. There was also a strong, but negative, relationship (correlation) between final weight of fish and calcium/phosphorus ratio (Table 5) in diet (r=-0.439, P<0.05), while regression coefficient (Table 5 and Figures 2-4) was positive (r=0.193, P<0.05) between final weight and Ca/P ratio. Relationship (correlation) between weight gain and Ca/P ratio (Table 5) showed a
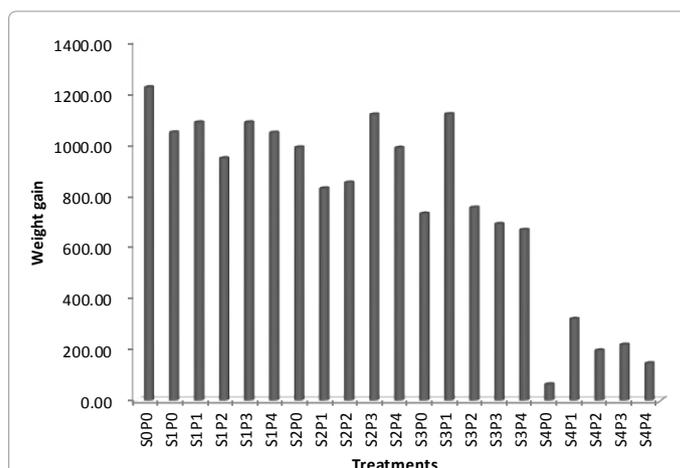
Figure 1: Weight gain of fish fed phytase-treated diet based on full-fat soyabean meal.
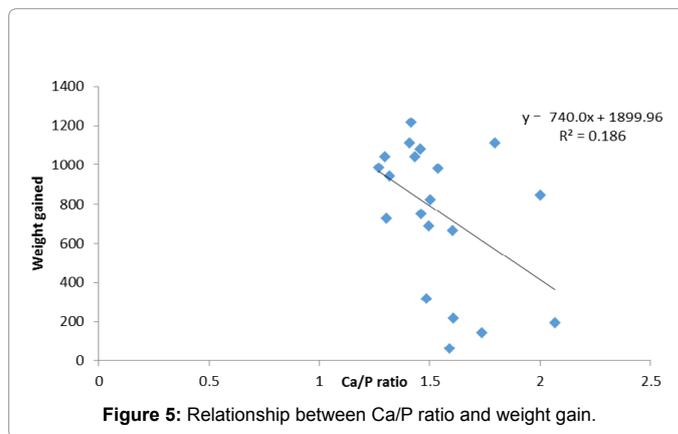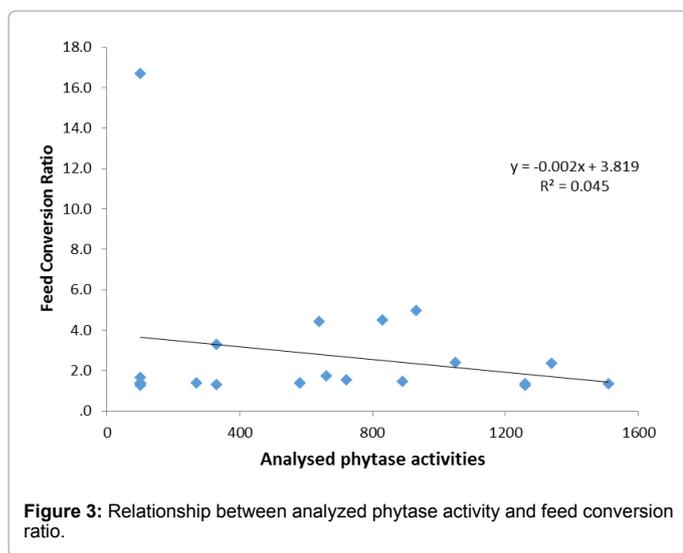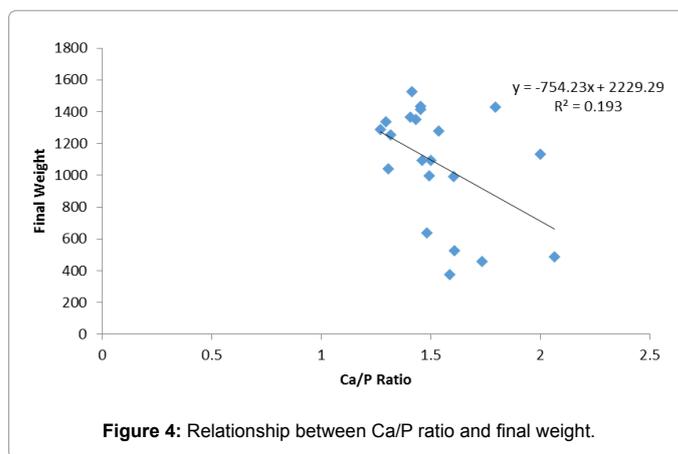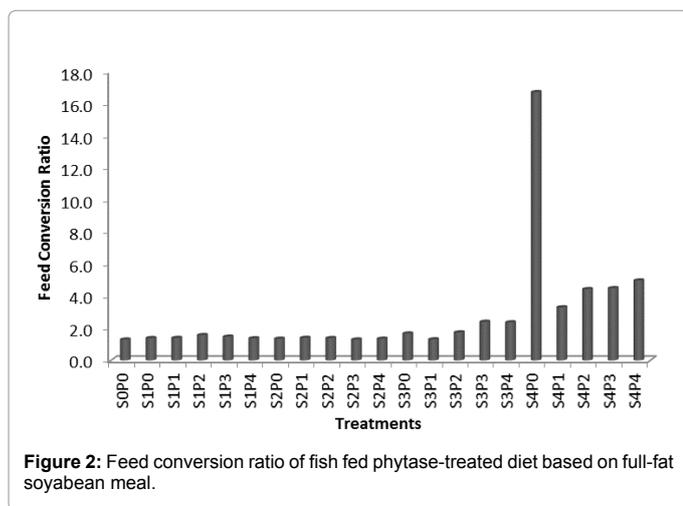
negative correlation (r=-0.431), while regression coefficient (Table 5, Figure 5) was positive (r²=0.186, P<0.05) (P<0.05) with Ca/P ratio. Fish fed 250 FTU/g phytase had the highest weight gain (P>0.05), compared

Table 5: Relationship between calcium-phosphorus ratio, growth and nutrient utilization parameters.

Parameters	a (Intercept)	b (Slope)	r² (Regression coefficient)	r (Correlation coefficient)	Sig.	P
Final weight	2229.287	-754.226	0.193	0.439	0.004	<0.05
Weight gained	1899.961	-740.001	0.186	0.431	0.004	<0.05
Feed conversion ratio	-2.537	3.471	0.048	0.219	0.163	>0.05
Specific growth rate	1.928	-0.330	0.019	0.138	0.385	>0.05
Survival rate	106.691	-10.110	0.037	0.191	0.225	>0.05

The independent variable is the Ca-P ratio

The dependent variables are the Growth and Nutrient Utilization Parameters

Figure 2: Feed conversion ratio of fish fed phytase-treated diet based on full-fat soyabean meal.

Figure 4: Relationship between Ca/P ratio and final weight.

Figure 3: Relationship between analyzed phytase activity and feed conversion ratio.

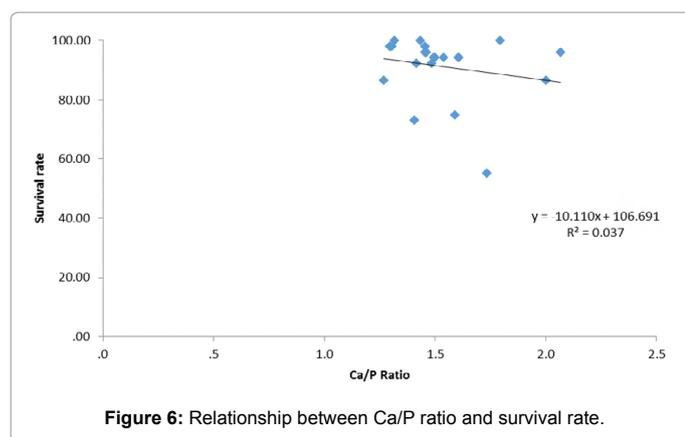
Figure 5: Relationship between Ca/P ratio and weight gain.

to other phytase levels (Table 4). There was a statistically significant difference (P<0.05) in the feed conversion ratio of fish fed experimental diets (Table 3), which ranged between 1.29 ± 0.01 (S0P0) and 16.71 ± 1.43 (S4P0). There was no significant difference (P>0.05) between FCR values of fish fed S4P2 (4.43 ± 0.06), S4P3 (4.49 ± 0.54), and S4P4 (4.97 ± 0.48). The lowest FCR value for fish fed 75% soya bean with phytase was recorded for S3P1 (1.41 ± 0.01), while the highest was S3P3 (2.40 ± 0.18) (P<0.05). There is no significant difference between FCR of fish fed S3P1, S0P0, S1P0, S1P1, S1P1, S1P3, S1P4, S2P0, S2P1, S2P2, S2P3, S2P4 (P>0.05). Feed conversion by fish fed soya bean (full fat) diet

with phytase is shown in Figure 2. In Table 4, phytase addition to diet at 250 FTU/g showed the lowest FCR value (1.85 ± 0.91), compared to 0 FTU/g (4.48 ± 6.47), 500 FTU/g (2.28 ± 1.34), 750 FTU/g (2.41 ± 1.38), and 1000 FTU/g phytase (2.51 ± 1.60) (P>0.05). Correlation (r=0.219, P>0.05) and regression (R²=0.048, P>0.05) are positive between FCR and Ca/P ratio. Specific growth rate was highest for fish fed S3P1 and the lowest in fish fed S4P0. Correlation and regression coefficients between SGR and Ca/P ratio were (r=-0.138, P>0.05) and (r²=0.019, P>0.05), respectively. Calcium/phosphorus ration was significantly affected by phytase supplementation in experimental diets (P<0.05). Values ranged from 1.27 ± 0.02 (S2P0) to 2.07 ± 0.13 (S4P2). Survival rate (Table 3) of fish showed a significant decrease in value with increasing levels of soya bean in basal diet (P<0.05). A significant decrease in survival rate was observed when phytase was supplemented in diet based on 25% and 75% soya bean with phytase (P<0.05). Fish fed S3P1 showed the lowest survival of fish (73.08 ± 10.88%) compared

Table 6: Growth and nutrient utilization in juvenile *Clarias gariepinus* suboptimal phytase diet based on soyabean meal (full fat).

Trt	Initial weight (g)	final weight (g)	weight gain (g)	SGR (%)	FCR	Survival rate (g)	Ca/P
S0P0	309.09 ± 14.38	1526.56 ± 64.78^g	1219.47 ± 47.57^f	1.90 ± 0.01^a	1.29 ± 0.01^{ef}	92.31 ± 0.00	1.41
S$_3$D	383.14 ± 2.92^a	606.81 ± 99.29^a	223.68 ± 102.21^a	0.55 ± 0.21^a	7.34 ± 3.50^a	93.0 ± 2.0^a	2.95
S$_3$P$_{50}$	372.71 ± 38.47^a	581.68 ± 40.50^a	208.98 ± 78.98^a	0.54 ± 0.21^a	7.03 ± 2.68^a	96.5 ± 1.5^a	1.61
S$_3$P$_{100}$	367.19 ± 67.81^a	555.55 ± 237.36^a	188.36 ± 169.55^a	0.44 ± 0.30^a	10.70 ± 7.97^a	95.0 ± 3.0^a	1.78
S$_3$P$_{150}$	383.16 ± 49.92^a	518.64 ± 155.01^a	135.73 ± 104.74^a	0.33 ± 0.20^a	13.45 ± 9.22^a	95.0 ± 4.95^a	1.76
S$_3$P$_{200}$	356.94 ± 30.67^a	493.61 ± 48.42^a	137.68 ± 19.17^a	0.39 ± 0.01^a	9.33 ± 0.69^a	98.0 ± 0.0^a	1.98
Sig	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	

Mean values with the same alphabet superscript in the same column are not significantly different (P>0.05) (2-tailed). n=2

Figure 6: Relationship between Ca/P ratio and survival rate.
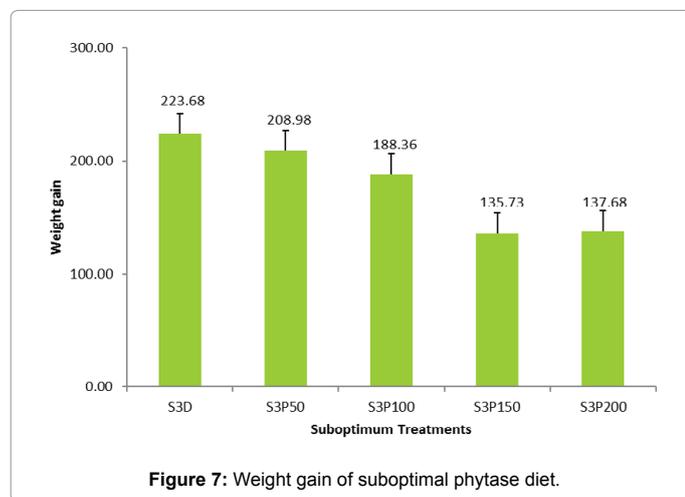
Figure 7: Weight gain of suboptimal phytase diet.

to S3P0 (98.08 ± 2.72%), S3P2 (96.15 ± 0.00%), S3P3 (94.23 ± 2.72%) and S3P4 (94.23 ± 2.72%). Fish fed 100% soya bean diet supplemented with 250, 500 and 750 FTU/g phytase had significantly higher values (P<0.05) of survival compared to control diet (S4P0). Regression analysis (Figure 6) showed a negative relationship between Ca/P ratio and survival rate of fish (P>0.05). When fish were fed suboptimal phytase (less than 250 FTU/g) diet between 50 FTU/g and 200 FTU FTU/g in 75% soya bean meal diet, there were significant reduction in growth and nutrient utilization parameters (Table 6) compared to phytase-adequate diet with phytase at 250 FTU/g suggesting optimum is 250 FTU/g. There was a significant increase in Ca/P ratio in diets based on soya bean meal, which increased from 1.27 ± 0.02 in diet S2P0 to 1.59 ± 0.04 in S4P0. Addition of phytase resulted in a significant reduction in Ca/P ratio of diets of fish fed 25% soya bean (P<0.05) from

1.43 ± 0.00 (S1P0) to 1.29 ± 0.02 (S1P4) (P<0.05). Diet of fish fed S3P1 had the lowest Ca/P ratio (1.41 ± 0.02), compared to Ca/P ratios of S3P2 (1.46 ± 0.00), S3P3 (1.49 ± 0.02), and S3P4 (1.60 ± 0.02). Ca/P is of S3P1 is not different (P>0.05) from S0P0, S1P0, S1P1, and S1P3) (P>0.05). Fish fed diets supplemented with 250 FTU/g (1.46 ± 0.04) phytase had the lowest Ca/P ratio compared to diets with 500 (1.71 ± 0.35), 750 (1.59 ± 0.14), and 1000 FTU/g (1.54 ± 0.17) (P<0.05) (Table 4 and Figure 7).

Bone mineralization

There was significant reduction (P<0.05) in bone phosphorus (Table 7) in diet without phytase from 4.50 ± 0.01% in S1P0 to 2.33 ± 0.01% in S4P0. Values of phosphorus, however, increased significantly in 25% soya bean diet for fish fed S1P1-S1P3 and in 50% soya bean for S2P1 and S2P2 fish. In fish fed 75% soya bean diet with phytase addition, there was no difference between S3P0 and S3P4 fish (P>0.05). However, there were significant increase in bone phosphorus when fish were fed 100% soya bean diet with phytase from 2.33 ± 0.01% in S4P0 to 2.89 ± 0.01% in S4P4 (P<0.05). S0P0 fish had the highest phosphorus (5.74 ± 0.01%). Table 8 shows that phosphorus was highest in 250 FTU/g compared to other phytase levels. Fish fed experimental diet showed significant differences in bone calcium (P<0.05). Levels of calcium decreased significantly in soya bean diet with phytase from 2.33 ± 0.01% in fish fed S1P0 to 1.41 ± 0.01% in S4P0. Addition of phytase significantly increased calcium levels in fish fed 25% and 100% soya bean (P<0.05). Values of calcium also increased in fish fed 75% soya bean, which was, however, not significant (P>0.05). The lowest recorded bone calcium for experimental fish was in fish fed S4P0 (1.41 ± 0.01%), while fish fed S0P0 had the highest level of bone calcium (2.89 ± 0.01%). Bone zinc improved significantly in all diets compared to diets without phytase. Like phosphorus and calcium, values were higher for 250 FTU/g in fish fed 75% soya bean, compared to 0, 500, 750 and 1000 FTU/g phytase (Table 8, suggesting 250 FTU/g is optimum for both growth and bone mineralization in juvenile *Clarias gariepinus*. Phosphorus retention was highest in fish fed 75% soya bean with 250 FTU/g (Table 7) and was highest (0.73 ± 0.23) compared to 0 FTU (0.53 ± 0.54), 500 FTU (0.58 ± 0.25), 750 FTU (0.57 ± 0.20), and 1000 FTU (0.58 ± 0.15) (Table 8).

Discussion

Mineral composition of the diet

Phytase addition to fish diet has been reported to increase dietary mineral retention in fish [8,34-36]. In this experiment, significant effect of phytase on mineral availability was observed. Although phosphorus in 25% soya bean and 50% soya bean (full fat) were sufficient for fish in meeting the requirement for the mineral (0.67-0.82%) as determined by Nwanna et al. [37], the significant increase in dietary phosphorus by phytase in the former could be related to phytate hydrolysis and

Table 7: Bone mineralization and phosphorus retention efficiency of fish fed phytase-treated experimental diet based on soyabean meal (full fat).

Treatment	Phosphorus (%)	Calcium (%)	Magnesium (%)	Iron (ppm)	Zinc (ppm)	Phosphorus retention efficiency (%)
Initial fish	2.21 ± 0.01	2.59 ± 0.01	0.17 ± 0.01	7.29 ± 0.01	28.77 ± 0.01	
S0P0	5.74 ± 0.01[q]	2.89 ± 0.01[k]	0.25 ± 0.01[h]	5.89 ± 0.01[r]	27.41 ± 0.01[d]	0.62 ± 0.01[i]
S1P0	4.50 ± 0.01[j]	2.33 ± 0.019[gh]	0.22 ± 0.019[g]	5.54 ± 0.02[p]	24.71 ± 0.01[b]	0.62 ± 0.01[hi]
S1P1	5.68 ± 0.01[p]	2.85 ± 0.01[jk]	0.18 ± 0.01[ef]	4.49 ± 0.01[l]	28.81 ± 0.01[f]	0.53 ± 0.01[f]
S1P2	5.30 ± 0.01[n]	2.50 ± 0.01[i]	0.20 ± 0.01[fg]	6.45 ± 0.01[t]	29.34 ± 0.01[i]	0.54 ± 0.01[f]
S1P3	5.13 ± 0.01[m]	2.72 ± 0.01[j]	0.26 ± 0.01[h]	5.10 ± 0.01[n]	35.03 ± 0.01[p]	0.45 ± 0.01[e]
S1P4	4.14 ± 0.01[i]	2.53 ± 0.01[i]	0.25 ± 0.01[h]	5.23 ± 0.01[o]	28.21 ± 0.01[e]	0.70 ± 0.01[k]
S2P0	4.89 ± 0.01[l]	2.19 ± 0.01[fg]	0.11 ± 0.01[a]	6.32 ± 0.01[s]	32.19 ± 0.01[m]	0.78 ± 0.01[m]
S2P1	5.28 ± 0.01[n]	2.49 ± 0.01[hi]	0.20 ± 0.01[fg]	4.15 ± 0.01[j]	32.16 ± 0.01[m]	0.72 ± 0.01[kl]
S2P2	5.60 ± 0.01[o]	2.49 ± 0.35[hi]	0.25 ± 0.01[h]	7.98 ± 0.01[u]	29.28 ± 0.04[h]	0.92 ± 0.01[o]
S2P3	4.69 ± 0.01[k]	2.05 ± 0.01[ef]	0.15 ± 0.01[cd]	3.09 ± 0.01[g]	22.79 ± 0.01[a]	0.82 ± 0.01[n]
S2P4	3.78 ± 0.01[h]	1.65 ± 0.01[bc]	0.17 ± 0.01[de]	2.08 ± 0.01[e]	34.28 ± 0.04[o]	0.74 ± 0.01[l]
S3P0	3.42 ± 0.01[g]	2.23 ± 0.01[g]	0.19 ± 0.01[ef]	4.23 ± 0.01[k]	31.19 ± 0.01[j]	1.07 ± 0.01[p]
S3P1	3.26 ± 0.01[e]	1.97 ± 0.01[e]	0.15 ± 0.01[cd]	2.31 ± 0.01[f]	32.62 ± 0.01[n]	1.08 ± 0.03[p]
S3P2	3.13 ± 0.01[d]	1.88 ± 0.01[de]	0.14 ± 0.01[bcd]	3.35 ± 0.01[h]	27.44 ± 0.01[d]	0.60 ± 0.01[gh]
S3P3	3.42 ± 0.01[g]	1.98 ± 0.01[e]	0.17 ± 0.01[de]	4.61 ± 0.01[m]	36.59 ± 0.01[s]	0.35 ± 0.01[c]
S3P4	3.37 ± 0.01[f]	2.25 ± 0.01[g]	0.31 ± 0.01[i]	5.74 ± 0.01[q]	31.73 ± 0.01[l]	0.46 ± 0.01[e]
S4P0	2.33 ± 0.01[a]	1.41 ± 0.01[a]	0.12 ± 0.01[ab]	0.67 ± 0.01[a]	31.38 ± 0.01[k]	-0.44 ± 0.01[a]
S4P1	3.42 ± 0.01[g]	1.74 ± 0.01[cd]	0.14 ± 0.01[abc]	3.51 ± 0.01[i]	37.29 ± 0.01[q]	0.59 ± 0.01[g]
S4P2	2.66 ± 0.01[b]	1.64 ± 0.01[bc]	0.14 ± 0.01[bcd]	1.05 ± 0.01[b]	38.09 ± 0.01[r]	0.26 ± 0.01[b]
S4P3	2.64 ± 0.01[b]	1.56 ± 0.01[b]	0.25 ± 0.01[h]	1.61 ± 0.01[d]	29.04 ± 0.01[g]	0.67 ± 0.01[j]
S4P4	2.89 ± 0.01[c]	1.50 ± 0.01[b]	0.11 ± 0.01[a]	1.22 ± 0.01[c]	27.37 ± 0.01[c]	0.42 ± 0.01[d]
Total	4.06 ± 1.10	2.13 ± 0.45	0.19 ± 0.06	4.03 ± 1.99	35.57 ± 23.16	0.59 ± 0.31
Sig	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

Mean values with the same alphabet superscript in the same column are not significantly different at the 0.05 level (2-tailed)

Table 8: Effect of phytase on bone minerals and phosphorus retention efficiency in fish fed soybean diet.

Phytase	Phosphorus (%)	Calcium (%)	Magnesium (%)	Iron (ppm)	Zinc (ppm)	Phosphorus retention efficiency
0 FTU	4.17 ± 1.25[a]	2.21 ± 0.50	0.18 ± 0.06	4.53 ± 2.16	29.38 ± 3.01[a]	0.53 ± 0.54
250 FTU	4.41 ± 1.16[b]	2.26 ± 0.47	0.17 ± 0.03	3.61 ± 0.89	32.71 ± 3.22[a]	0.73 ± 0.23
500 FTU	4.17 ± 1.38[a]	2.13 ± 0.42	0.18 ± 0.05	4.70 ± 2.88	31.03 ± 4.43[a]	0.58 ± 0.25
750 FTU	3.97 ± 1.06[ab]	2.07 ± 0.44	0.21 ± 0.05	3.60 ± 1.46	30.86 ± 5.44[a]	0.57 ± 0.20
1000 FTU	3.54 ± 0.50[c]	1.98 ± 0.45	0.21 ± 0.08	3.56 ± 2.08	30.39 ± 2.97[a]	0.58 ± 0.15
Sig	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

breakdown, which made phosphorus available (Table 2) in these diet [5,6,8] in excess of requirement [14]. While dietary phosphorus declined in diet with 50% full fat soya bean with increasing phytase addition from 250 FTU/g to 1000 FTU/g (Table 2), values of available phosphorus increased with 1000 FTU/g phytase. Increase in available phosphorus in both diets could be due to high phytate (Table 1) in 25% (0.52%) and 50% soya bean (0.57%) without phytase, compared to phytate levels in 75% (0.43%) and 100% (0.40%) full fat soya bean diet. The magnitude of response to phytase was greater in ingredients containing high level of phytate for basal diet with 25% and 50% soya bean with phytase supplementation compared to 75% and 100% full fat soya bean diet [6,38]. Available phosphorus levels (Table 2) in 75% full fat soya bean with 250 FTU/g (S3P1) was highest compared to values in other diets (S3P0, S3P2, S3P3, S3P4), which indicated that phytase at that level significantly increased phytate hydrolysis [6] and thus increasing the level of available phosphorus from 0.33 ± 0.01% in control S3P0 to 0.42 ± 0.02%, which was above the requirement (0.33%-0.40%) for catfish [13,39]. Values of available phosphorus in 100% soya bean diet (full fat) with phytase showed a significant increase and could be due increased phytate utilization even at lowest level of

dietary phosphorus compared to other dietary soya bean levels [6,40]. Although, levels of calcium were above the requirement of 0.45% for catfish [24], calcium increased with phytase in all diet except in 25% soya bean. Increase in calcium could be due to phytase effect, which released calcium from calcium-phytate complex and the presence of high phytate in these diets [6,38], thus making it available in the diet [23]. Phytase has little effect in diet when phytate content is less than 2.0 g/kg diet [6]. Increase in dietary zinc is associated with phytase-induced breakdown of phytate-zinc complex [6], thus improving utilization of phytate-bound minerals, especially phosphorus and zinc.

Growth and nutrient utilization

Phytase has been extensively used in animal nutrition to enhance growth and feed and nutrient utilization [19,37,38,41]. Phytase enhanced apparent digestibility of minerals and their deposition in the fish [20]. It increased mineral utilization through the breakdown of phytate-mineral complex [23], which resulted in increased growth and feed efficiency of the fish [7,37] through improvement in phosphorus bioavailability [23]. Improved growth performance and nutrient

utilization were observed in this research investigating the possibility of substituting entirely or almost entirely level of fish meal in fish diet [7]. Phytase addition at 250, 500, 750, and 1000 FTU/g to 25% full fat soya bean meal recorded positive weight gain (Table 3) compared to diet without phytase (25% soya bean, no phytase). When fish meal was substituted with 50% soya bean (full fat), positive effect was observed with 750 FTU/g compared to other diets (S2P1, S2P2, S2P4), and was comparable to weight gain of fish fed control diet (S0P0). At higher level of fish substitution by soya bean (full fat), phytase inclusion at 250 FTU/g was better than at 0, 500, 750, and 1000 FTU/g.

Weight gain at 250 FTU/g in 75% soya bean (full fat) was better than at lower fish meal substitution (25% and 50%) by soya bean (full fat) with phytase addition at any level, and performed as fish fed control (100% fish meal). This could be explained by fact that phytase is effective in hydrolyzing phytate at low dietary phosphorus and low available phosphorus level [6,7,23]. The total and available phosphorus in 75% soya bean diet (full fat) without phytase are much lower (0.75 ± 0.01% and 0.33 ± 0.01%) than at 25% (1.15 ± 0.01% and 0.63 ± 0.01%) and 50% (1.01 ± 0.01% and 0.44 ± 0.01%). Denbow et al. [42] tried seven levels of supplemental phytase at 0, 200, 400, 600, 800, 1000, and 1200 FTU/g in the semi purified diet of broilers containing 0.20, 0.27 or 0.34% available phosphorus. They observed improved body weight gain and feed intake (P<0.01) at all available phosphorus levels, but the magnitude of response was maximum at low available phosphorus level. Lim et al. [40] supplemented microbial phytase at 500 FTU/g in a corn-soya bean meal based broiler diet (0.25%, 0.35% and 0.45% available phosphorus) and observed significant (P<0.01) improvements in body weight gain. They reported enhanced benefits of phytase in low available phosphorus diet, but no significant (P<0.01) effect in diets containing higher level of phytate phosphorus. Johnston and Southern (2000) used eight levels of phytase supplementation at 0, 200, 400, 600, 800, 1000, and 1200 FTU/g) in low phytate phosphorus broiler diet and reported improvements in body weight gain, and that increased levels of phytase supplementation had little effect on growth performance (P<0.01). Phytase supplementation at 500 FTU/g to low available phosphorus (0.30%) diets significantly (P<0.05) improved the growth performance of broilers. This was also observed in improved growth performance in fish fed with phytase (250 FTU/g) soya bean diet (75%) with low basal available phosphorus (0.33 ± 0.01 g) compared with fish fed 25% and 50% soya bean with available phosphorus of 0.63 ± 0.01% and 0.44 ± 0.01%, respectively (Table 2). Additionally, fish fed 75% soya bean with 250 FTU/g showed the best growth performance and nutrient utilization, which was reported to be best by Castro et al. who reported 75% soya bean with phytase (4,000 FTU/g, Ronozyme) as best for Rainbow trout. In the study of Li and Robinson, fish fed the diets containing 250 FTU/g phytase or above consumed more feed, gained more weight, and had a lower feed conversion ratio in comparison to fish fed the basal diet containing no microbial phytase. In Li et al., phytase supplementation at 250 FTU/g in catfish diet increased feed intake, body weight gain, feed efficiency, and bone ash and phosphorus concentration. In the present research, 250 FTU/g at 75% soya bean and at all levels of soya bean (irrespective of soya bean levels, Table 4), had the highest specific growth rate, weight gain, and lowest feed conversion compared with others levels of soya bean diet (full fat). Feed conversion declined with increasing phytase as seen from the regression graph between FCR and analyzed phytase activity (Figure 3), which means weight gain is optimum or highest with low phytase. In Li and Robinson, channel catfish fed the diets containing 250 FTU/g or above consumed more feed, gained more weight, and had a lower feed conversion ratio in comparison to

fish fed the basal diet containing no microbial phytase. Robinson et al. reported that phytase at 250 FTU/g could effectively replace dicalcium phosphate supplement in the diet of channel catfish without affecting growth, feed efficiency or bone P deposition. This was also observed in this experiment in which diets with 250 FTU/g at 75% soya bean (S3P1) had better growth performance (Table 2) compared to positive control, S_3D (Table 6), suggesting phytase at this level can effectively replace inorganic phosphorus in *Clarias gariepinus* diet. Fish fed suboptimal phytase diet (Table 6) showed poor growth performance compared to diet with optimal level of phytase at 250 FTU/g (Table 3), which could be due to low phosphorus availability and high Ca/P ratios in the diet (Table 6) compared to S3P1 and S0P0 (Table 3).

Fish fed 100% soya bean diet (full fat) showed the poorest growth response compared to other soya bean levels, although there were significant improvement in weight gain compared to control (S4P0). The low growth in 100% soya bean fish could be related to unbalanced amino acid profile of soya bean compared to fish meal [43] limiting its total substitution of fish meal as well as the high level of fat, which was beyond the maximum level that can be used in fish feed [24]. Fish fed 100% soya bean also showed the lowest feed intake, specific growth rate, protein efficiency, and the highest feed conversion. 100% soya bean diet (full fat) also showed a significantly higher calcium/phosphorus ratio, which depresses phytate hydrolysis and reduce growth [23]. The Ca:P of 75% soya bean meal supplemented with 250 FTU/g (1.41:1) is comparable to that of 100% fish meal diet (1.41:1), suggesting optimum level in soya bean diet (full fat) for *Clarias gariepinus* fish diet. In Table 4, phytase at 250 FTU/g gave the highest growth response with optimum Ca/P of 1.46 compared to phytase at 0 FTU/g (Ca/P=1.40), 500 FTU/g (Ca/P=1.71), 750FTU/g (Ca/P=1.59) and 1000 FTU/g (Ca/P=1.54) (Table 6). In Kim et al. [44], growth performance and nutrient utilization in weaning pig fed phytase-supplemented-canola-soya bean diet with high phytate and Ca/P of 1.46 was significantly higher than diets without phytase. Cao et al. [7] recommended a range of Ca:P between 1.1:1 and 1.4:1 for optimum phytase activity in fish feed. The high Ca:P in S2P1 (1.54), S2P2 (2.00) and S2P4 (1.54) as well as the high level in 100% soya bean with phytase, in the diet could be responsible for the poor weight gain of fish fed these diets compared to S0P0, S2P0, S2P3, S3P1 and S3P4. Ca:P ratios are crucial to phytase efficacy as reviewed by Lei and Stahl [22], since high level chelate with phytate and compete with active site of phytase, which reduces phytase efficacy and phosphorus availability [1]. Qian et al. reported that reducing Ca:P ratios from 2.0 to 1.2:1 in phytase supplemented diets (700 and 1050 FTU/kg) of pig, increased phytase efficacy by the order of 16%. Liu et al. observed that reducing Ca:P ratios from 1.5 to 1.0:1 in phytase (500 FTU/kg) supplemented low phosphorus diets increased P absorption in the small intestine of pigs. Similarly, Qian et al. found that increasing Calcium (5.61-10.20 g/kg) and Ca:P ratios (1.1-2.0:1) depressed weight gain of broilers.

Bone mineralization

The effect of phytase supplementation in diet fish has been shown improve bone mineralization in several studies [1,7,9] Channel catfish fed phytase-supplemented diets had higher concentrations of ash, calcium, phosphorus and manganese in their bones than the fish fed on a control diet. Bone mineralization in Common carp was improved by phytase addition in the diet [37,41].

In this study, phytase supplementation improved bone parameters, depending on the level of soya bean in the diet. Bone phosphorus, calcium, iron, and zinc (Table 7) were improved in fish fed 25 and 50% soya bean (full fat) with phytase supplementation at 250-750

FTU/g and 250-500 FTU/g, respectively, compared to basal controls in these diets [5,45]. Increased mobilization of phosphorus store in soft tissue such as the flesh from fish fed these diets improved bone mineralization in these fish without depleting their store in the flesh, suggesting adequate phosphorus intake, contrary to who reported that insufficient phosphorus intake leads to the mobilization of phosphorus from the bone and transfer to soft tissues and metabolic processes [46]. According to Baruah et al. [5], dietary supplementation of microbial phytase at 500 FTU/g in *Labeo rohita* (Hamilton) juveniles' diet significantly increased bone sodium, calcium, potassium, manganese and iron content by 15, 12.1, 17.4, 20.4 and 40.7%, respectively. Robinson et al. [14] reported that phytase of 250 FTU/g could effectively replace dicalcium phosphate supplement in the diet of channel catfish without affecting growth, feed efficiency or bone phosphorus deposition. In this study, fish fed 75% soya bean (full fat) with 250 FTU showed significant reduction in bone phosphorus and calcium, which could be due to inadequate supply [36,46] fat in the diet. Reduction in bone phosphorus and calcium in 75% soya bean with 250 FTU/g could be due to inhibition of their absorption by fat in the diet for whole body retention and bone mineralization. However, in other diets (25%, 50% and 100% soya bean), phytase at 250 FTU/g improved significantly bone phosphorus compared to controls without phytase. Reqz et al. reported that soyabean oil affected bone characteristics (bone length and volume) in animals. There was, however, significant increase in iron and zinc, indicating phytase effectively released zinc from zinc-phytate complex [23] for retention in whole body and bone mineralization. Improvement of zinc in diet and bone by supplementing phytase at 250 FTU/g could be the reason why it produced the greatest growth response in fish fed 75% soya bean meal diet compared to other phytase supplemented soya bean diet, and could effectively replace fish meal at that level of soya bean in the diet of juvenile *Clarias gariepinus*. Zinc exhibits a greater effect on growth than growth hormone and the nutrition of zinc for monogastric animals is similar physiologically to that of man. Most zinc deficiency is attributable to the abundance of dietary phytate, a natural compound found in all plant seeds and some roots and tubers. Fish fed 100% soya bean showed the greatest positive and consistent response to increasing phytase with the highest phosphorus at 250 FTU/g as observed in 25% and 50% soya bean, which could explained by low phosphorus level that enhances phytase response [40], and improving bone parameters. Irrespective of soyabean levels, fish fed diets with 250 FTU/g phytase showed the highest bone phosphorus and calcium compared to 500, 750, and 1000 FTU/g (Table 8).

In conclusion, the research has demonstrated that phytase at 250 FTU/g was optimum for juvenile *Clarias gariepinus* as observed by improvement in growth (Figure 1), bone mineralization (Table 8) and phosphorus retention efficiency (Table 7) compared to other levels, and can replace dicalcium phosphate [14] however, the effect of Ca/P, from the research, has also shown the negative effect its negative effects at high levels as seen from the relationships between weight gain and total weight. Hence, it is recommended that, since the optimum phytase diet, which produced the best growth at 75% soya bean diet at 250 FTU/g (Ca/P for S3P1=1.41), which also gave the highest growth response (Ca/P=1.46) compared to phytase at 0 FTU/g (Ca/P=1.40), 500 FTU/g (Ca/P=1.71), 750 FTU/g (Ca/P=1.59) and 1000 FTU/g (Ca/P=1.54), Ca/P in the diet of juvenile *Clarias gariepinus* should range between 1.41-1.46 for optimum phytase activity. Available phosphorus should be as low as possible (<0.33%).

Acknowledgement

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