Project Number: 02-001-020

Applicability of microbial phytase to increase phosphorus availability in soy protein-based diets for largemouth bass (*Micropterus salmoides*): a reevaluation of effects on production performance, phosphorus retention, and physiological responses.

LAYMAN'S REPORT

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SUMMARY OF RESEARCH ACTIVITIES

Activities related to the research project were initiated in June, 2019 and were anticipated to be completed by June, 2020.

- Acquisition of microbial phytase enzyme (Nathuphos® E 10000 L) and other feed ingredients.
- Formulation of experimental diets.
- Acquisition of largemouth bass (LMB) fingerlings.
- Conditioning of fingerings in holding tanks with commercial feed.
- Manufacturing of experimental diets.
- Stocking of LMB juveniles into culture system tanks and conditioning.
- Commencement of the growth trial (July 30, 2019).
- Maintenance and monitoring of culture system and fish.
- Acquisition of research items for final sampling.
- Final sampling for growth trial (October 16, 2019).
- Data computation for performance metrics (Growth Trial)
- Sample preparation and submission for chemical analyses (Growth Trial; ongoing).
- Data computation and statistical analyses (Growth Trial; completed for available data).
- Grow out of advanced LMB juveniles for the digestibility trial.
- Stocking advanced LMB juveniles for digestibility trial.
- Conduction of digestibility trial (October 15, 2019 - December 13, 2019).
- Fecal sample preparation and submission for chemical analyses (Digestibility Trial)
- Student presentation of research results in the Aquaculture America Conference, February, 2020: https://www.was.org/MeetingAbstracts/ShowAbstract/156735

Pending analyses: We still have samples being prepared for chemical analyses, including whole-body samples for proximate and mineral composition and defleshed-skeleton samples for mineral composition analyses. Due to COVID-19 and a shelter-in-place order all research activities in the Aquaculture Research Center and other research facilities of Kentucky State University were interrupted since March 16th, 2020. **Due to continued restriction of access and research activities by the Kentucky State University, we were unable to perform additional analyses within the project period ending on July 31, 2020.**

ABSTRACT

Soy-protein feedstuffs comprise high-quality sources of amino acids to aquatic animals and are primary ingredients in aquaculture feeds. Most of the phosphorus (P) in these ingredients, however, occurs as phytate which cannot be broken down by monogastric animals including fish. Consequently, supplementation of inorganic P is a common practice in aquaculture so that feeds supply adequate amounts of available P to farmed fish. The phytate P, on the other hand, passes unassimilated through the digestive tract of fish being conveyed to receiving waters, which can contribute to eutrophication processes. Application of exogenous phytase (the phytate-degrading enzyme) in feeds has been shown to increase the availability of phytate-P to monogastric animals, thereby maximizing the nutritional value of feeds and minimizing the need for supplemental P. In view of the need to optimize plant-based diets for largemouth bass (LMB, *Micropterus salmoides*), an increasingly important food fish in north America, the aim of this study was to optimize the utilization of phytate-P in soy-protein-based diets for LMB. An eleven-week growth trial and a ten-week digestibility evaluation were conducted in the Aquatic Animal Nutrition Laboratory of Kentucky State University wherein LMB juveniles were fed soy-based diets supplemented with a commercial phytase product at levels ranging from 0 to 2000 units of phytase activity/kg. These studies were conducted under controlled conditions using recirculating aquaculture systems which maintained optimal water quality for LMB. After eleven weeks of feeding in the growth trial, LMB fed diets containing a minimum of 1000 phytase units/kg displayed higher concentrations of ash and P in whole-body compared to fish fed diets containing up to 759 units of phytase/kg. Fish fed increased levels of supplemental phytase also utilized dietary protein and P more efficiently than low-phytase-fed LMB. In addition, when a minimum of 1000 units of the enzyme was supplemented to the diet, P became more available to the fish as observed in the ten-week digestibility assessment. This study demonstrated that supplementation of exogenous phytase can substantially increase the availability of phytate-P to LMB allowing for a more complete utilization of nutrients in soy-based feedstuffs.

MATERIALS AND METHODS

Experimental fish

Feed-trained LMB fingerlings were purchased from a commercial producer (Mayer Fish Farm, Cox's Creek, KY) and transported to the Aquatic Animal Nutrition Laboratory (AANL) located in the Aquaculture Research Center (ARC), Kentucky State University, Frankfort, KY. Upon arrival, the fish were stocked in a 1500-L rectangular fiberglass tank operating as a recirculating aquaculture system wherein they were grown to adequate size for the growth trial. Once the growth trial started, the remaining fish in the tank were transferred to a 0.1-acre earthen pond where they were grown to adequate size for the digestibility trial. In both systems, fingerlings were fed a commercial feed (45% crude protein [CP] and 12% crude fat; AQUAXCEL®, Cargill Inc., Franklinton, LA, USA) thrice daily to apparent satiety using automatic belt feeders (RAS) or twice daily by manually broadcasting feed over the water surface (pond).

Experimental Diets

Following a completely randomized experimental design, seven experimental diets were designed to contain 42% CP and 12% lipid (Table 1). A basal diet (D1, - Control) was formulated to be deficient in phosphorus (P) and contain no supplemental phytase (PTS). Five additional diets (D2 through D6) were designed by supplementing PTS (as Nathuphos® E 10000 L; BASF Corporation, Florham Park, NJ) to the basal formulation at graded amounts to obtain final PTS activity levels of 250, 500, 1000, and 2000 units (U)/kg. A P-replete diet (D7, + Control) was designed by the supplementation of calcium phosphate monobasic (CaP) to the basal formulation and served as both positive control for dietary P and reference for fish performance. Calcium carbonate was supplemented to all except D7 to balance calcium levels. All experimental diets were manufactured at the AANL. To facilitate distribution of the PTS product in the diets, an aliquot of Nathuphos® E 10000 L was firstly diluted in deionized water (DIW) to obtain a solution containing 2000 PTS U/mL. Aliquots from this solution corresponding to the target levels of dietary PTS were diluted into 1000 mL of DIW before application in each diet. Diet ingredients were weighed using a digital scale and mixed for a total of ~ 40 min in a Hobart A200T 20-quart mixer (Hobart Corp., Troy, OH). Coarse ingredients were mixed first (15 min) followed by the addition of the PTS solutions (+ 15 min), lipid (+ 10 min), and finally DIW to convey the mixture appropriate consistency for pelleting. Next, each diet was screw-pressed through a 3.2 mm die

plate using a commercial food processer (Hobart 4732A; Hobart Corp., Troy, OH). Diet temperature at die exit during pelleting ranged from 41.7 - 66.2 °C (mean ± SD = 50.7 ± 4.7 °C, n = 97) and was maintained below the recommended maximum exposure temperature for Nathuphos® E 10000 L (~ 90 °C). After dried to less than 10% moisture under forced air at room temperature, diet strands were broken into adequate-size pellets and sieved to remove fines. Diets were stored under -20°C until fed.

The analyzed composition of the experimental diets is presented in Table 2. Values of CP and crude lipid of the diets were close to the formulated levels and very similar across diets. Except for D7, which was supplemented with inorganic P (as CaP) and contained 1.15% total P, all remaining diets were P-deficient containing ~ 0.6% total P and ~ 0.3 non-phytate P. Levels of yttrium and other macro- and micro-minerals were fairly constant across diets. Analyzed PTS activity was close to target values and ranged from 95 U/kg in D1 to slightly over 2000 U/kg in D6. Although D7 was manufactured with the same ingredients as D1 and did not receive supplemental PTS, no PTS activity was detected in it.

Experimental Conditions

The growth trial was conducted indoors under controlled conditions in the AANL. Once adequate size for the trial was attained, 420 LMB juveniles (~ 10 g/fish) were stocked in 21, 110-L aquaria (20 fish/aquarium) which operates as a recirculating aquaculture system. This system operates under continuous mechanical and biological filtration, UV sterilization, and aeration through air diffusers to provide optimal conditions for the fish.

Each experimental diet was randomly assigned to triplicate aquaria. Fish in each aquarium were fed manually one of the assigned diets twice daily to apparent satiety for a total of eleven weeks. Apparent satiety was judged arbitrarily based on the feeding activity of the fish at each feeding.

Water quality was monitored routinely following standard procedures and maintained within adequate ranges for LMB. Temperature (mean ± standard deviation = 26.6 ± 0.6 ºC), dissolved oxygen (7.4 ± 0.7), pH (8.1 ± 0.2), and salinity (1.2 ± 0.7) were monitored daily using a YSI-Pro Plus multiparameter instrument (Yellow Springs Incorporated, Yellow Springs, OH, USA). Total ammonia nitrogen (TAN; 0.19 ± 0.07), and nitrite nitrogen (0.09 ± 0.04) were monitored weekly using a spectrophotometer (HACH DR 2800, HACH, Loveland, CO, USA).

Fish that were grown for the digestibility evaluation were transferred from the pond to an indoor recirculating aquaculture system with a mean weight of 78.6 ± 0.9 g/fish. The system was comprised of 20, 180-L polyethylene tanks with the same configuration as the system described above. Twenty fish were stocked in each tank and were conditioned for one week while being fed the same commercial feed used during the pond grow-out period. As for the growth trial, temperature (25.6 ± 1.0 ºC), dissolved oxygen (7.1 ± 0.4), pH (7.9 ± 0.3), salinity (2.2 ± 0.1), TAN (0.16 ± 0.06), and nitrite nitrogen (0.06 ± 0.02) were maintained within adequate ranges for the species.

Data Acquisition

All chemical analyses for dietary ingredients, experimental diets, whole fish, defleshed skeletons (pending), and fecal samples were carried out by the Agricultural Experiment Station Chemical Laboratories (ESCL, https://aescl.missouri.edu/index.html) at the University of Missouri, Columbia, MO; and followed standard analytical procedures (AOAC, 2006).

At the commencement of the growth trial, ~20 LMB from the original population were euthanized with an overdose of tricaine methanesulfonate (Tricaine-S; Western Chemical, Inc. Ferndale, WA) and stored at -20ºC pending chemical analyses of baseline fish. Upon conclusion of the growth trial, LMB were sampled after 24 hours of fasting. Fish in each aquarium were anaesthetized with Tricaine-S (100 mg/L), group weighed and counted for computing production performance metrics. Two fish were anesthetized and bled from the caudal vasculature using 1.0 mL syringes with 22-gauge needles and the blood was placed in 1.3 mL heparinized tubes. After centrifugation for 10 min at 4.0°C, the resulting plasma was stored at -20°C pending analysis of alkaline phosphatase enzyme (pending). Six fish were euthanized and stored at -20°C pending whole-body composition analyses. Three fish were euthanized and total lengths and weights were recorded prior to excision and weighing of livers and intraperitoneal fat (IPF). The resulting data was used to compute condition factor (K) and somatic indices including HSI (hepatosomatic index) and IPFI (IPF index). All remaining fish from each aquarium were euthanized and stored at -20°C pending whole-body proximate and mineral analyses (four fish) and defleshed-skeleton mineral analyses (remaining fish).

During the digestibility trial (10 weeks), fish in each tank were fed one of the randomly assigned experimental diets to apparent satiety once daily (8:00 h). Triplicate tanks were used for diets D1 to D6 and diet D7 was assigned to two tanks. Once the digestibility trial was completed,

fish in all tanks were conditioned to D7 and a third fecal sample from this diet was collected. During feeding on fecal collection days, a 10-min interval between tanks was applied to synchronize fecal collection at ~ 5.5-h post-feeding. Fecal collection procedures followed previously established protocols (Gaylord and Gatlin 1996; Rossi et al., 2017). Fecal samples were collected once a week using the stripping method in which the release of fecal material was accomplished by manually pressing the lower portion of fish's abdomen. Individual fish were gently netted out of the tanks and fecal material stripped into clean bowls before being transferred to plastic boats and dried overnight at 65°C using a convention oven. Stripped fish were placed in an intensely aerated tank before being returned to their respective tanks. Dry fecal samples from the various collections were pooled by tank and stored at -20°C pending analyses at ESCL.

Statistical Analyses

All statistical procedures were carried out using Statistical Analysis System software (SAS Institute, Cary, NC) and statistical significance was considered at $P < 0.05$. After validation of normality and homoscedasticity, the resulting data for each response variable were subjected to a one-way analysis of variance to detect significant treatment effect (s). When a treatment effect was detected, Tukey's HSD test and Dunnett's test were used to identify differences among treatments. Orthogonal linear and polynomial contrasts were performed in selected data to detect differences between treatments or trends (linear or quadratic) in fish's response to dietary phytase, respectively.

RESULTS

Growth Trial

Production performance

Largemouth bass displayed a quadratic response to dietary PTS in terms of feed intake ($P = 0.032$), but no significant trends were found for survival, growth, and feed conversion efficiency (Table 3). No dietary treatment effects on survival (78 – 98%) was detected ($P > 0.05$). Fish fed the D6 containing 2040 units/kg PTS activity was the only group displaying growth comparable ($P > 0.05$) to fish fed D7 (+ Control). Significantly lower feed intake was observed in LMB fed D2, D3, and D4 relative to D7-fed groups. Similarly, lower feed efficiency ratio (FER) was observed in LMB fed D1 (-Control) and D2 relative to D7-fed groups ($P < 0.05$).

Condition factor (K), somatic indices, whole-body composition and nutrient retention

Data for K, HSI and IPFI (not shown) were highly variable and no treatment effects were found. Whole-body concentrations of ash and P were significantly affected by PTS supplementation (Table 4). Increasing PTS activity from 95 to 2040 U/kg of diet augmented whole-body ash from 2.29% to 2.92% and phosphorus from 0.38% to 0.51%. Whole-body ash (3.88%) and phosphorus (0.64%) were highest in LMB fed the positive control diet (D7). Similar responses were also observed for protein and P retention efficiencies. As dietary PTS activity increased from 95 to 2040 U/kg, protein retention efficiency increased from 27.3% to 31.6% ($P < 0.05$); a level similar to that observed in D7-fed groups (Fig. 1). Likewise, phosphorus retention efficiency increased from 34.6% to a maximum of 63.6% surpassing that supported by D7 (47.5%), Fig. 2.

Defleshed-skeleton mineral composition

These analyses were not performed during the project period due to the COVID-19 shelter-in-place order determined by Kentucky State University. These critical analyses will be completed when funding to cover related costs are available.

Digestibility Trial

Phytase or inorganic P supplementation significantly affected the dietary availability of P to LMB. Significantly higher P availability was observed for the positive control diet (D7; 54.5%) and the diets with PTS activities of 948 (D5; 50.7%) and 2040 (D6; 52.7%) U/kg compared to diets D1 through D4 with PTS activities ≤ 759 U/kg and P availability ≤ 40% (Fig. 3).

CONCLUSIONS

This study showed that supplemental microbial phytase (Nathuphos® E 10000 L) can increase P availability in diets containing increased levels of soy proteins to LMB, corroborating findings in other species (NRC, 2011). Results of the digestibility trial showed a 25% increase in available P when a minimum of 1000 units of phytase/kg was present in the diet. The augment in gastrointestinal tract absorption of P as a result of phytase-mediated partial dephytinization of the diets (i.e., breakdown of phytate molecules) leading to accretions in ash and phosphorus concentrations in LMB fed D6 with 2040 PTSU/kg. Results for D5 which contained 948 PTSU/kg

are forthcoming and anticipated to be comparable to the former given the similarities in availability values observed in the digestibility study (Fig. 3). We also anticipate that results on mineral concentrations in defleshed-skeleton of the LMB will provide supporting evidence about the positive effects of supplemental phytase.

Notwithstanding the fact that within treatment variance might have precluded the detection of statistically significant trends in final weight of LMB in this study, supplemental phytase at 2000 units/kg in the diet significantly improved fish growth. Based on the mineral levels found in the fish, except for D7 which was supplemented with CaP, all diets were clearly deficient in P for optimum tissue mineralization. These observations are in agreement with our recent findings indicating that a minimum of 0.7% total non-phytate P is required for optimum tissue mineralization of LMB (Miller et al., 2020). Concurrently, this allowed a robust evaluation of supplemental phytase which was found to significantly improve the availability of phytate P to the LMB fed soy-based diets.

About two-thirds of the P in conventional solvent-extracted soybean meal (SBM) occurs as phytic acid which is unavailable to fish. Meanwhile, SBM is currently the number-one protein ingredient in aquafeeds and our studies have shown that it can contribute over 60% of total protein in LMB feeds. Hence, supplementing commercial feeds with 1000 - 2000 phytase units/kg can substantially reduce the output of P to receiving waters and the need for supplemental inorganic P. This work demonstrated the efficacy of commercial microbial phytase as an additive to improve the nutritional value of soy-protein-based feeds for LMB.

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Table 1. Ingredient composition of the negative control diet (D1). Dry matter basis.

Ingredients	Concentration
Conventional SBM	20.0
Soy protein concentrate	20.0
Corn protein concentrate	3.0
Wheat gluten	3.0
Wheat flour	8.0
Dextrinized corn starch	8.0
Calcium phosphate monobasic	0.0
Calcium carbonate	0.75
Others*	57.25

* Others include: fish meal, black soldier fly meal, binder, fish and vegetable oils, vitamin and mineral (P-free) premixes, calcium carbonate, yttrium oxide, amino acids, and cellulose.

Table 2. Analyzed composition and phytase activity (%, otherwise noted; dry matter basis) of the experimental diets (summarized).

Diet	D1 (-Control)	D2	D3	D4	D5	D6	D7 (+Control)
Phytase activity (units/kg)	0	250	500	750	1250	2000	0
Dry matter	90.5	89.1	89.4	89.5	89.9	91.1	89.8
Crude protein	43.5	43.7	43.6	43.5	44.2	44.0	44.4
Crude lipid	11.4	11.4	11.3	10.7	10.8	11.4	11.3
Crude fiber	3.6	3.8	4.4	3.9	4.5	3.8	3.4
Phytic acid	1.04	0.89	0.87	0.96	0.92	0.92	1.07
Elements							
Total phytate phosphorus	0.30	0.25	0.25	0.27	0.26	0.26	0.30
Total non-phytate phosphorus*	0.28	0.28	0.32	0.33	0.39	0.32	0.85
Phytase activity (units/kg)	95	345	519	759	948	2040	n.d.

PTSU = phytase units; n.d. = not detected; * = by difference.

Table 3. Production performance metrics of largemouth bass fed the experimental diets for 11 weeks.

Diet	PTS U/kg	Survival %	FI % BW/day	FW g	FER
D1 (- Control)	95	88	2.80	39.2*	0.68*
D2	345	98	2.65*	37.7*	0.69*
D3	519	90	2.69*	42.2*	0.73
D4	759	78	2.72*	40.5*	0.74
D5	948	97	2.79	42.7*	0.73
D6	2040	90	2.86	46.6	0.77
D7 (+ Control)	n.d.	87	3.00	55.4	0.84
Statistics (Pr > F)					
Polynomial contrasts (D1 to D6)					
Linear		0.855	0.131	0.087	0.217
Quadratic		0.641	0.043	0.569	0.979
ANOVA (All diets)		0.169	0.174	0.012	0.019

PTS = phytase; FI = feed intake; FW = final weight; FER = feed efficiency ratio; n.d. = not detected. Data is presented as mean ± SE. Means ± SE within a column with an asterisk (*) differ significantly from D7 per Dunnett's test.

Table 4. Whole-body concentrations of ash and phosphorus of largemouth bass fed the experimental diets for 11 weeks - selected treatments.

Diet	PTS U/kg	Ash %	Phosphorus %
D1 (- Control)	95	2.29 ± 0.03^{c}	0.38 ± 0.01^{c}
D6	2040	2.92 ± 0.04^{b}	0.51 ± 0.02^{b}
D7 (+ Control)	n.d.	3.88 ± 0.18^{a}	0.64 ± 0.01^{a}
ANOVA (Pr > F)	-	< 0.001	< 0.001

PTS = phytase. Data is presented as mean ± SE. Means ± SE within a column with different superscript letters differ significantly per Tukey's HSD test.

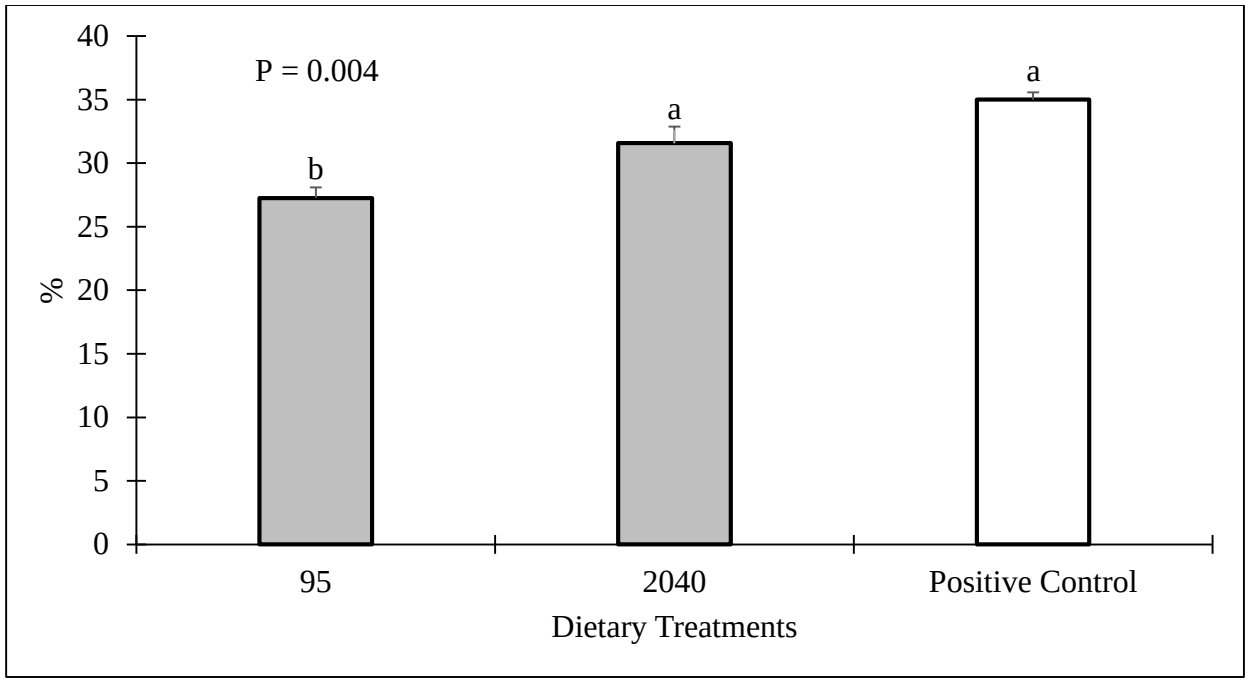

Figure 1. Protein retention efficiency (selected treatments) of largemouth bass as affected by dietary phytase activity (95 and 2040 PTSU/kg) and supplemental phosphorus (Positive Control). Data is presented as mean ± SE (error bars). Means ± SE with different superscript letters differ significantly per Tukey's HSD test.

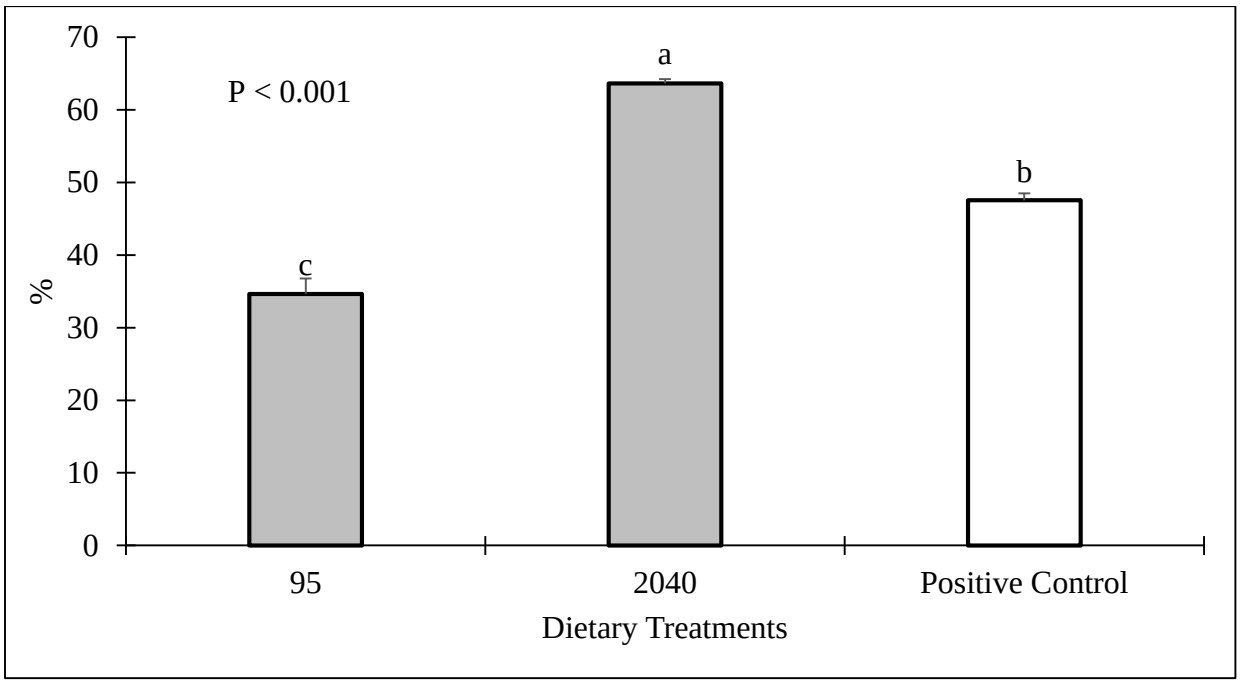

Figure 2. Phosphorus retention efficiency (selected treatments) of largemouth bass as affected by dietary phytase activity (95 and 2040 PTSU/kg) and supplemental phosphorus (Positive Control). Data is presented as mean ± SE (error bars). Means ± SE with different superscript letters differ significantly per Tukey's HSD test.

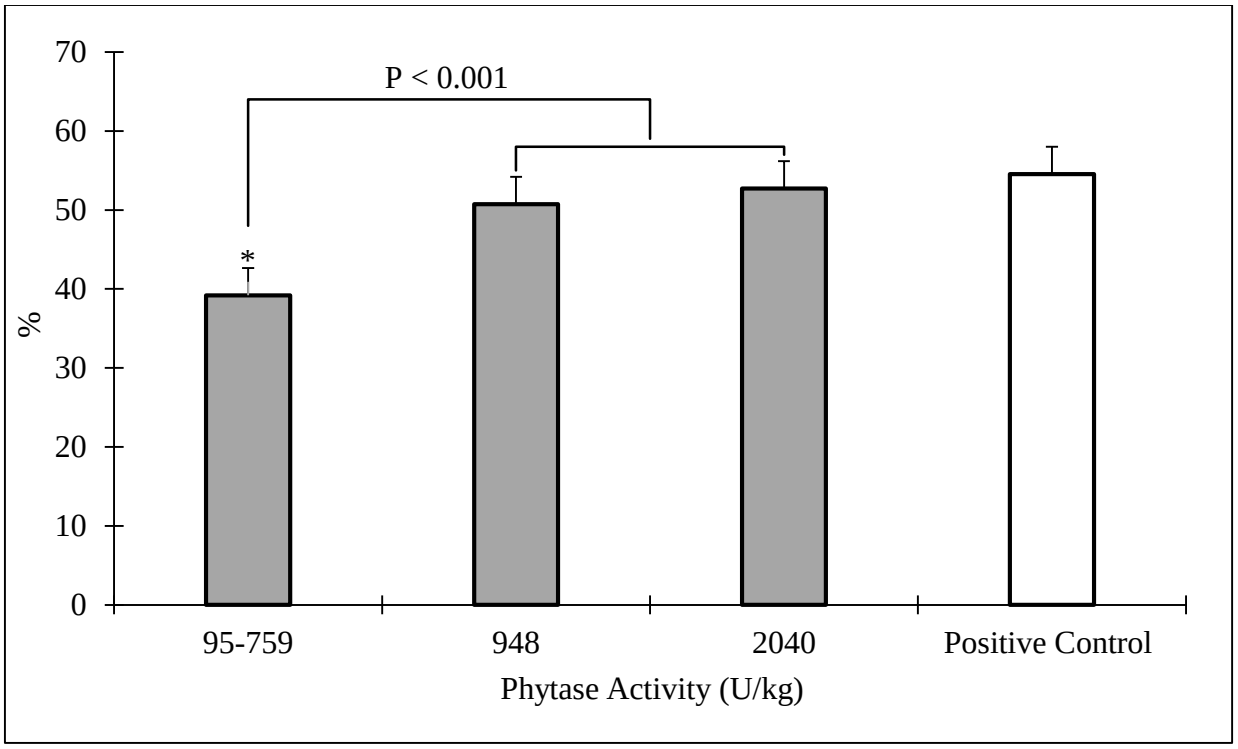

Figure 3. Apparent phosphorus availability (%) of the experimental diets to largemouth bass as affected by dietary phytase activity (95 to 2040 PTSU/kg) and supplemental phosphorus (Positive Control [D7]). Data is presented as mean ± SE (error bars). Brackets denote diet groups subjected to linear orthogonal contrasts. Means ± SE with an asterisk (*) differ significantly from the Positive Control per Dunnett's test.