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Assessment of total dietary phosphorus requirement of juvenile largemouth bass, *Micropterus salmoides*, using soybean meal-based diets: Effects on production performance, tissue mineralization, physiological parameters in plasma and intestine and expression of head-kidney genes

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Abstract

A 9-week feeding trial was conducted to evaluate effects of supplemental phosphorus (P) in soybean meal (SBM)-based diets for largemouth bass (LMB). Six isonitrogenous (~400 g/kg crude protein) and isolipidic (~130 g/kg crude lipid) diets containing non-phytic acid phosphorus (nPA-P) ranging from 4.1 to 10.5 g/kg were fed twice daily to triplicate groups of 20 feed-trained LMB (9.5g). Upon conclusion of the feeding trial, regression analyses showed that growth, feed efficiency and survival of LMB were unaffected by diet. Intraperitoneal fat index and whole-body lipid content of LMB decreased linearly as dietary nPA-P increased from 4.1 to ~7.0 g/kg, while protein retention efficiency increased quadratically over the dietary range of nPA-P. Regressions on whole-body P and skeletal mineral (P, Ca, Mg and Zn) concentrations of LMB revealed optimal levels when dietary nPA-P was ~7.0 g/kg. Increasing trends of MDA concentration and ALT activity in plasma and GP_x activity in intestine in response to dietary nPA-P were observed. Head-kidney genes responded to dietary treatments and expression of GH, IGF-1 and TGF-β1 were highest and that of TNF-α was lowest when dietary nPA-P was 6.9 g/kg. Based on our results, practical diets for juvenile LMB should contain a minimum of 7.0 g/kg total nPA-P.

KEYWORDS

antinutritional factor, eutrophication, mineral, phytate, phytic acid, plant feedstuffs

1 | INTRODUCTION

One of the current major topics of research is the continued development and evaluation of alternative protein feedstuffs to fishmeal in aquaculture feeds. While several protein feedstuffs have been utilized in the diet of aquatic animals, the use of plant-based ingredients in commercial feeds has increased rapidly in recent years (Tacon & Metian, 2015). Among the plant-protein feedstuffs currently available for use in feeds, SBM has been the leading ingredient (Troell et al., 2014) because of a number of favourable characteristics including wide availability at competitive prices, high protein content comprised by a reasonably well-balanced amino acid profile and relatively high nutritional value (Daniel, 2018; Gatlin et al., 2007).

Nevertheless, contrasting with the high protein availability of SBM is its low availability of phosphorus (P) to monogastric animals, including fish, which has been shown to be as low as 220 g/kg of total P (Sugiura et al., 1998). Such low availability of P is due to the inability of fish to degrade phytic acid (PA), the hexaphosphate

of myo-inositol ($C_6H_{12}O_{24}P_6$) that comprises the main form of P in plant-based ingredients including rapeseed meal (50–75 g PA/kg), SBM (10–15 g PA/kg) and sesame meal (24 g PA/kg; Francis et al., 2001). As much as 660 g/kg of SBM P is reportedly in the form of PA with negligible availability to fish (Riche & Brown, 1996; Sugiura et al., 1998). In turn, PA-P in feeds passes unassimilated through the gastrointestinal tract of aquatic animals and contributes to eutrophication processes when conveyed to receiving waters (Correll, 1998).

Phosphorus is generally considered the most important mineral required by fish (Brown et al., 1993). It is a primary component of skeletal tissue and is vital for a variety of metabolic processes such as genetic coding, energy transformations and permeability of cellular membranes. Dietary importance of P is compounded by low concentrations in water and PA limiting its availability, which leads to increased levels of inorganic P supplementation in aquaculture feeds containing plant-based feedstuffs. Knowledge of dietary P requirements of farmed fish is, therefore, critical for the optimization of P levels in feeds such that fish's dietary requirements are met without over-supplementation.

Dietary P requirements of various fish species have been reported (NRC, 2011; Prabhu et al., 2013). Nevertheless, to the best of our knowledge, the P requirement for largemouth bass (LMB), *Micropterus salmoides*, is still lacking in the literature. Largemouth bass have been shown to efficiently digest plant-based diets (Sampaio-Oliveira & Cyrino, 2008) and SBM proteins (Masagounder et al., 2009; Portz & Cyrino, 2004), as well as to tolerate high levels of SBM in practical diets (Cochran et al., 2009; Tidwell et al., 2005). Therefore, as the use of plant-based ingredients such as SBM in LMB feeds gradually increase and persist, a reference level for dietary P will ease the development of more precise and environmentally friendly formulations.

This study was conducted to assess the optimum dietary level (s) of total non-PA-P (nPA-P) necessary to support optimum production performance and tissue mineralization of LMB fed a SBM-based diet. Effects of gradually increasing levels of dietary nPA-P on physiological parameters in plasma and intestine and on the expression of head-kidney genes were also assessed.

2 | MATERIALS AND METHODS

2.1 | Fish

Feed-trained LMB fingerlings were purchased from F&L Anderson Farms, Lonoke, AR, USA and transported to the Aquatic Animal Nutrition Laboratory (AANL) at the Aquaculture Research Center, Kentucky State University, Frankfort, KY, USA. Upon arrival, the fish were acclimated to local water conditions and stocked into two 1,500-L rectangular fibreglass holding-tanks operating as a recirculating aquaculture system (RAS). In this system, fish were fed thrice daily with a commercial feed containing 500 g/kg crude protein (CP) and 140 g/kg crude fat (AQUAXCEL™ STARTER 5014, Cargill Animal Nutrition, Albany, NY, USA) until adequate size for the feeding trial was attained.

2.2 | Experimental diets

The formulation and ingredient composition of the experimental diets are presented in Table 1. A SBM-based (basal) diet was formulated to contain 420 g/kg crude protein (CP), 120 g/kg crude lipid, an estimated 13.8 MJ/kg digestible energy, and ~4.0 g/kg nPA-P. This basal level of nPA-P was anticipated to be deficient for adequate growth and/or skeletal mineralization of LMB based on established P requirement levels for other freshwater, scaled fish species (NRC, 2011). Five additional test diets were formulated by supplementing calcium phosphate monobasic (CaP; 265 g/kg P) at incremental levels of 5.5 g/kg to the basal diet to obtain a final range of total dietary nPA-P of 4.0 to ~11.0 g/kg. The inclusion of CaP into the basal diet was performed at the expense of both cellulose (as Alphacel) and calcium carbonate; the latter used to balance dietary calcium across diets.

The diets were produced in the AANL. Digital scales (Mettler Toledo ICS 435 and Mettler AT 261 DeltaRange, Mettler-Toledo Inc., Lexington, KY, USA) were used to weigh out ingredients. Resulting combined ingredients were mixed in a Hobart A200T 20-quart mixer (Hobart Corporation, Troy, OH, USA) for 15 min followed by the addition of deionized water (DIW) until adequate consistency for pelleting was attained. Diets were then screw-pressed through a 3.2 mm diet plate using a commercial food processing machine (Hobart 4732A). Resulting diet strands were separated by hand and subsequently dried at room temperature under forced air until a moisture content of <100 g/kg. Next, dry-diet strands were broken into pellets using a food chopper, sieved to remove fines and stored at −20°C until fed.

Chemical composition analyses of the experimental diets (Table 2) were carried out by the Agricultural Experiment Station Chemical Laboratories (ESCL) at the University of Missouri - Columbia, MO, USA (https://aescl.missouri.edu/index.html) following standard analytical protocols (AOAC, 2006). Crude protein and crude lipid values were similar to formulated values ranging from 405 to 414 g/kg and 125 to 128 g/kg, respectively. Concentration of acid-insoluble ash in the basal diet was 2.49 g/kg and was lower than expected representing less than 3% of total ash content. Concentration of nPA-P in the diets was obtained by subtracting PA-P (5.95 g/kg) determined in the basal diet from the total P content determined in each diet. Resulting nPA-P levels were close to formulated values at 4.1, 6.0, 6.9, 8.4, 9.5 and 10.5 g/kg. Concentrations of Ca, magnesium (Mg) and zinc (Zn) were similar across diets except for noticeable lower levels of Mg and Zn in D4. As expected, closely identical amino acid concentrations were found across diets. Lysine and methionine levels were within reported requirement values for LMB (Dairiki et al., 2007; Rossi et al., 2018), while levels of the other

TABLE 1 Formulation and ingredient composition of the experimental diets

	Diet (Formulated nPA-P level, g/kg)					
	Basal (4.0)	D2 (5.5)	D3 (7.0)	D4 (8.5)	D5 (10.0)	D6 (11.0)
Ingredients	g/kg, dry matter basis					
Calcium phosphate monobasic[a]	0.0	5.0	10.5	16.0	21.5	27.0
Calcium carbonate[a]	20.0	17.8	15.3	13.5	11.0	8.4
Alphacel[a]	15.4	12.6	9.6	5.9	2.9	0.0
Menhaden meal[b]	50.0	50.0	50.0	50.0	50.0	50.0
Poultry by-product meal[c]	100.0	100.0	100.0	100.0	100.0	100.0
Conventional soybean meal[c]	400.0	400.0	400.0	400.0	400.0	400.0
Soy-protein concentrate[c]	34.0	34.0	34.0	34.0	34.0	34.0
Corn-protein concentrate[c]	34.0	34.0	34.0	34.0	34.0	34.0
Wheat gluten[c]	34.0	34.0	34.0	34.0	34.0	34.0
Wheat flour[c]	100.0	100.0	100.0	100.0	100.0	100.0
Dextrinized corn starch[a]	60.0	60.0	60.0	60.0	60.0	60.0
Carboxymethyl cellulose[a]	15.0	15.0	15.0	15.0	15.0	15.0
Menhaden oil[b]	40.0	40.0	40.0	40.0	40.0	40.0
Soybean oil[d]	51.0	51.0	51.0	51.0	51.0	51.0
Vitamin premix[b,f]	6.0	6.0	6.0	6.0	6.0	6.0
Stay C (35% vitamin C)[c]	3.0	3.0	3.0	3.0	3.0	3.0
Choline chloride[c]	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix (low P)[a,g]	20.0	20.0	20.0	20.0	20.0	20.0
Lysine HCl[e]	2.0	2.0	2.0	2.0	2.0	2.0
L-methionine[e]	3.6	3.6	3.6	3.6	3.6	3.6
Glycine[e]	10.0	10.0	10.0	10.0	10.0	10.0

Abbreviation: nPA-P, non-phytic acid phosphorus.

[a]MP Biomedicals, 29525 Fountain Pkwy, Solon, OH 44139, USA.

[b]Omega Protein, Inc., 610 Menhaden Rd, Reedville, VA 22539, USA.

[c]Rangen Inc., 115 13th Ave S, Buhl, ID 83316, USA.

[d]Walmart Supercenter, 301 Leonardwood Rd, Frankfort, KY 40601, USA.

[e]Ajinomoto North America, Inc., 4020 Ajinomoto Drive, Raleigh, NC 27610, USA.

[f]DSM Nutritional Products, Inc., Parsippany, NJ, USA. Provided the following vitamins (mg/kg of dry matter, otherwise noted): retinol (A, 9920.6 IU), cholecalciferol (D3, 661.4 IU), tocopherol (E, 529.1 IU), cobalamin (B12, 0.036), biotin (B7, 0.5), menadione (K, 5.5), thiamin (B1, 48.6), riboflavin (B2, 79.4), pantothenic acid (B5, 158.7), pyridoxine (B6, 38.1), Niacin (B3, 330.7) and folic acid (B9, 13.2).

[g]Provided the following macrominerals (g/kg, dry matter basis): calcium (1.6), phosphorus (0.6), magnesium (0.5), sodium (1.4), potassium (2.7), chloride = (3.9) and sulphur (3.9); and microminerals (mg/kg, dry matter basis): iron (52.5), aluminum (0.5), iodine (6.0), copper (6.7), manganese (11.9), cobalt (13.0), zinc (35.7), selenium (0.4) and chromium (1.3).

essential amino acids were mostly in excess of reference values for other freshwater carnivorous teleosts (NRC, 2011).

2.3 | Experimental conditions

Handling and utilization of experimental fish in this study were performed in accordance with the animal welfare protocols of Kentucky State University and complied with local and international guidelines (FAO, 2004).

The feeding trial was conducted in the AANL. Twenty feed-trained juvenile LMB [mean ± standard deviation (*SD*) = 9.5 ± 0.04 g/fish] were selected manually and stocked into each of 18, 110-L glass aquaria operating as an indoor RAS. To ensure proper water quality, the RAS was connected to a propeller-washed bead filter, UV sterilization and biological filtration units. Each experimental diet was randomly assigned to three aquaria, and the fish were fed to apparent satiation twice daily (~ 8:30 a.m. and 3:30 p.m.) for nine weeks. Water quality parameters (mean ± *SD*) including dissolved oxygen (5.99 ± 0.68 mg/L), temperature (26.17 ± 0.83 °C), pH (7.30 ± 0.23) and salinity (1.79 ± 0.24 g/L) were monitored daily using an YSI Professional Plus 6050000 meter (YSI Incorporated, Yellow Springs, OH, USA). Total ammonia nitrogen (0.27 ± 0.04 mg/L) and nitrite-nitrogen

TABLE 2 Chemical composition of the experimental diets

	Diet (Formulated nPA-P level, g/kg)					
	Basal (4.0)	**D2 (5.5)**	**D3 (7.0)**	**D4 (8.5)**	**D5 (10.0)**	**D6 (11.0)**
	g/kg, dry matter basis (otherwise noted)					
Proximate composition						
Crude protein	407.0	405.0	408.0	407.0	413.0	414.0
Crude lipid	126.0	128.0	125.0	126.0	127.0	127.0
Fibre	34.0	26.0	32.0	24.0	22.0	22.0
Ash	104.0	104.0	107.0	109.0	112.0	112.0
Acid-insoluble ash	2.49	-	-	-	-	-
Mineral composition						
Non-phytic acid phosphorus[a]	4.1	6.0	6.9	8.4	9.5	10.5
Total phosphorus	10.0	12.0	12.9	14.3	15.4	16.4
Calcium	23.3	24.9	23.4	21.8	24.2	23.1
Magnesium	2.7	2.7	2.6	1.4	2.7	2.8
Zinc (mg/kg)	4.1	6.0	6.9	8.4	9.5	10.5
Amino acid composition						
Alanine	19.5	19.4	20.3	19.8	19.0	19.0
Arginine	24.1	23.9	24.8	24.5	24.5	24.6
Aspartate	33.6	33.6	34.6	34.1	35.8	34.7
Cysteine	6.2	6.0	6.1	6.2	6.2	6.1
Glutamate	70.9	69.5	70.5	69.9	72.0	70.8
Glycine	32.2	32.0	33.8	32.8	30.9	32.0
Histidine	8.7	8.8	9.0	8.8	9.3	9.0
Hydroxylysine	0.8	0.9	1.0	0.9	1.0	0.9
Hydroxyproline	4.7	4.1	4.9	4.6	3.5	4.9
Isoleucine	16.9	16.4	16.6	16.6	17.0	16.4
Lanthionine	0.2	1.0	0.8	1.0	0.9	0.8
Leucine	30.2	29.9	30.6	30.3	30.7	29.3
Lysine	22.4	21.6	22.4	22.0	22.6	22.6
Methionine	8.9	8.9	9.2	9.0	9.4	8.8
Ornithine	0.3	0.4	0.4	0.4	0.3	0.3
Phenylalanine	18.4	18.5	18.8	18.8	19.1	18.5
Proline	26.9	25.7	26.2	26.2	25.2	25.6
Serine	16.9	17.5	17.3	17.4	17.2	16.8
Taurine	1.5	1.9	1.8	1.8	1.8	1.7
Threonine	13.8	13.9	14.1	13.9	14.3	13.7
Tryptophan	3.4	4.4	4.3	4.2	4.3	4.6
Tyrosine	12.2	12.9	13.3	13.3	13.6	12.9
Valine	19.0	19.0	19.2	19.2	19.1	18.4

Note: Dietary moisture (g/kg; basal to D6, respectively) = 92.5, 91.1, 91.0, 90.7, 90.0, 90.1.

Abbreviation: nPA-P, non-phytic acid phosphorus.

In acid-insoluble ash, dashes in D2 through D6 denote data not available.

[a]Non-phytic acid phosphorus = total phosphorus − 5.95 g/kg phytic acid phosphorus in the basal diet (mean of four samples analysed in triplicate).

(0.20 ± 0.07 mg/L) were tested weekly using reagent kits and a spectrophotometer (HACH DR 2800, HACH, Loveland, CO, USA). A central regenerative air blower supplied air to all aquaria through ceramic diffusers. Syphoning of settled solids and partial water exchanges using dechlorinated city water were carried out daily to maintain adequate water quality and prevent element load in the

recirculating water. Artificial lighting controlled by a central timer provided a 12-hr photoperiod throughout the feeding trial.

2.4 | Sampling, data acquisition and calculations

Anaesthesia and euthanasia of fish were performed as needed in this study by using tricaine methanesulfonate (Tricane-S; Western Chemical, Inc., Ferndale, WA, USA) at approximately 100 and 250 mg/L, respectively. During all sampling procedures, random selection was performed in the collection of fish for the various analyses.

2.4.1 | Production performance and condition indices

At the conclusion of the feeding trial, fish from each aquarium were anesthetized, group weighed and counted for computation of production performance metrics. Three fish from each aquarium were individually weighed and measured for total length as well as weight of excised intraperitoneal fat (IPF), liver, and intestine for computing condition factor (K) and somatic indices including IPF index (IPFI), hepatosomatic index (HSI), and intestinosomatic index (ISI).

Metrics and respective formulae used to evaluate production performance, K and somatic indices and nutrient retention efficiency of LMB in response to dietary nPA-P are presented below (Rossi & Davis, 2012; Yadav et al., 2020; Yamamoto et al., 2020):

- Average final weight = [total group weight (g) ÷ number of fish];
- Weight gain (% of initial weight) = [(final weight - initial weight) ÷ initial weight (g)] ×100;
- Thermal growth coefficient (TGC) = [(final weight$^{1/3}$- initial weight$^{1/3}$ (g)) ÷ (# days fed × culture water temperature (°C))] × 1,000;
- Feeding rate [% of body weight (BW)/day) = [dry feed fed (g) ÷ ((initial boy weight × final body weight (g)$^{1/2}$)) ÷ days on feed] × 100;
- Feed efficiency ratio (FER) = [(final weight - initial weight) ÷ dry feed consumed (g)];
- Survival (%) = [final population ÷ initial population] ×100;
- Condition factor (K) = [final weight (g) ÷ length (cm)^3] × 100;
- Somatic indices (IPFI, HSI, and ISI; %) = [intraperitoneal fat, liver, or intestine weight (g) ÷ final weight (g)] × 100;
- Nutrient retention efficiency [protein (PRE) and minerals; %] = [((final body weight (g) × final body nutrient (%)) – (initial body weight (g) × initial body nutrient (%))) ÷ nutrient intake (g)] × 100;

2.4.2 | Whole-body and skeletal composition analyses

At the beginning of the feeding trial, approximately thirty-five fish from the original population were euthanized and kept frozen (−20°C) as a composite sample for subsequent whole-body chemical analyses of baseline fish.

At the conclusion of the feeding trial, four fish from each aquarium were euthanized and stored at −20°C for whole-body composition analyses. Subsequently, the frozen fish were sliced, homogenized in a food chopper and refrozen at −20°C overnight. A portion of the frozen homogenate was sliced to increase surface area, then dried in triplicate at 120°C for three hours (AOAC, 1990) using a FREAS Mechanical Convection Oven 645 (Thermo Fisher Scientific, Waltham, MA, USA) to determine whole-body moisture content. These samples were then combined with the remainder of the homogenate (dried following the same procedures), finely ground, bagged and stored at −20°C pending additional analyses (CP, lipid, ash, P, Ca) at ESCL following standard analytical procedures (AOAC, 2006).

Remaining fish from each aquarium as well as sampled fish with intact skeleton were frozen at −20°C for subsequent mineral composition analysis of skeleton. For the preparation of skeleton samples, frozen fish were thawed out, eviscerated as needed and filleted, then boiled in DIW for ~30 min according to the procedures of Davis and Robinson (1987). Following boiling, remaining flesh was manually removed and the defleshed skeletons were dried, finely ground and stored frozen. Mineral composition analyses (P, Ca, Mg and Zn) of the dry skeletons were carried out at ESCL (AOAC, 2006).

2.4.3 | Physiological parameters

Two fish from each aquarium were anesthetized, and blood was drawn from the caudal vasculature using 1-ml syringes with 24-gauge needles. Collected blood was placed in 1.5 ml heparinized tubes and centrifuged (Eppendorf Centrifuge 5,430 R, Hauppauge, NY, USA) for 10 min (6,000 × *g*, 4°C) and the resulting plasma stored at −80°C pending physiological analyses. These fish were then dissected and intestines stored at −80°C for subsequent physiological analyses. Intestine samples were incorporated with ice-cold physiological saline (10 volumes w/v) and then were homogenized using a Bead Mill 24 (Thermo Fisher Scientific, Pittsburgh, PA, USA). Thereafter, samples were centrifuged for 20 min (4,000 × *g*, 4°C) and the supernatants were stored frozen at −20°C pending physiological analyses (Habte-Tsion et al., 2016). Activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and superoxide dismutase (SOD) and concentrations of glucose (GLU) and malondialdehyde (MDA) in plasma, as well as activities of glutathione peroxidase (GP_x) and catalase (CAT) in intestine of LMB were analysed colorimetrically using commercial kits according to manufacturer protocols. All colorimetric analyses were carried out using a Synergy HTX multi-mode microplate reader (BioTek Instruments, Inc., Vinooski, VT, USA).

2.4.4 | Head-kidney gene expression

Head kidneys were collected from the same fish sampled for the physiological parameters and were stored at −80°C pending gene

TABLE 3 Production performance metrics of largemouth bass fed the experimental diets for nine weeks. Mean ± SE

	Dietary nPA-P level (g/kg, dry matter basis)								
	4.1	6.0	6.9	8.4	9.5	10.5	Model	R^2	Pr > F
IW (g)	9.57 ± 0.03	9.53 ± 0.03	9.57 ± 0.03	9.53 ± 0.03	9.40 ± 0.15	9.5 ± 0.00	–	–	–
FW (g)	47.4 ± 3.0	50.9 ± 3.1	51.1 ± 2.4	56.7 ± 1.2	51.8 ± 3.1	55.4 ± 1.9	SOP	0.30	0.165
WG (% of initial)	380 ± 35	425 ± 42	411 ± 39	464 ± 10	451 ± 29	472 ± 14	SOP	0.31	0.062
TGC	2.53 ± 0.18	2.73 ± 0.17	2.67 ± 0.18	2.87 ± 0.03	2.80 ± 0.12	2.90 ± 0.06	SOP	0.26	0.109
FER	0.62 ± 0.02	0.65 ± 0.04	0.62 ± 0.03	0.68 ± 0.02	0.70 ± 0.01	0.70 ± 0.03	SOP	0.31	0.059
Survival (%)	97 ± 1.7	98 ± 1.7	95 ± 2.9	95 ± 2.9	100 ± 0.0	98 ± 1.7	SOP	0.08	0.546

Abbreviations: FER, feed efficiency ratio; FW, final weight; IW, initial weight; nPA-P, non-phytic acid phosphorus; SE, standard error; SOP, second-order polynomial (quadratic); TGC, thermal growth coefficient; WG, weight gain. In IW, dashes denote data not available.

expression analyses. Expression of selected genes in the head kidney of LMB was assessed using real-time polymerase chain reaction (PCR) analysis, as detailed in Habte-Tsion et al. (2015). Following extraction of total RNA from head kidneys, Nanodrop OneC (Thermo Scientific) was used to determine RNA quantity and quality. Next, a Prime-Script™ RT reagent kit (Takara) was used to synthesize complementary DNA (cDNA), as specified in the manufacturer's instructions. First, a pre-mix of respective RNA, PrimeScript™ RT enzyme mix I, 5× PrimeScript™ buffer and RNase-free distilled water was prepared. Then, oligo dT primers (50 μM) were added to reverse transcribe the RNA at 37°C for 15 min and subsequently inactivation at 85°C for 5 s. Target genes included growth hormone (GH), insulin-like growth factor 1 (IGF-1), fatty acid synthase (FASN), copper (Cu)/Zn-superoxide dismutase (SOD), tumour necrosis factor alpha (TNF-α) and transforming growth factor (TGF-β1). All gene-specific primers were designed according to the partial cDNA sequences of the target genes for *M. salmoides* (GH: forward, 5'- AGGAGCAGCGTCAACTCAAC-3'; reverse, 5'-TGTGTCTCGTGCTTGTCGAT -3'; accession # DQ666528) or by using published sequences (Habte-Tsion et al., 2020), and were synthesized by invitrogenTM (Life technologies, USA). Expression of target mRNA levels was determined through real-time PCR using PrimeScript™ Reagent Kit (Takara), according to standard protocols. Briefly, cDNA (2.0 μl) was reacted with 10.0 μl SYBR® Premix Ex Taq II (2×), 0.8 μl forward primer (10 μM), 0.8 ml reverse primer (10 μM), 0.4 ml ROX™ reference dye or dye II (50×) and 6.0 μl RNase-free DIW in a final reaction volume of 20 μl. A 7500 Real-Time PCR System (Applied Biosystems) was used to carry out real-time PCR utilizing the following thermocycling conditions for the target genes. The first was the initial denaturation step for 30 s at 95°C. Next, forty cycles were conducted of each of the following parameters in order: 95°C for 5 s, 60°C for 34 s and 95°C for 30 s, 95°C for 3 s and 60°C for 30 s. In order to verify that a single PCR product was generated, a melting curve analysis was performed over a range of 50–95°C. The threshold cycle number (CT) was determined for each sample using 7500 Software version 2.0.4 (Applied Biosystems). Concentrations of the target genes were based on the CT, and the expression levels were normalized to those of a housekeeping gene (β-actin).

After ensuring the primers were amplified with an efficiency of approximately 100%, expression data were analysed using 2-ΔΔCT method. Gene-specific standard curves were used to determine cDNA concentration in each sample. Standard curves were generated based on 10-fold serial dilutions for endogenous control and target genes.

2.5 | Statistical analysis

Statistical analysis was performed using Statistical Analysis System software (SAS; Version 9.4, SAS Institute Inc., Cary, NC, USA), and statistical significance was considered at $p \leq .05$. All resulting data were subjected to regression analyses to assess LMB responses to supplemental P and determine the optimum level of dietary nPA-P. For each response variable, the most parsimonious regression model was screened according to Vedenov and Pesti (2008). Screened models in this study included linear (L), second-order polynomial (quadratic; SOP), linear-broken line (LBL), quadratic broken-line (QBL) and four-parameter saturation kinetics (4-SK). Each aquarium ($n = 3$) represented an experimental unit for all assessed response metrics except those quantified in plasma/intestine and head kidney for which the individual fish was considered the experimental unit ($n = 6$).

3 | RESULTS

3.1 | Production performance, condition factor and somatic indices

Production performance metrics of LMB are presented in Table 3. Over the nine weeks of feeding, fish grew to a final mean weight of 47.4–56.7 g and survival was unaffected by diet averaging 97.2% overall. Analyses on growth, feeding rate and FER revealed no statistically significant dietary effects, though upward trends in weight gain ($p = .062$) and FER ($p = .059$) were noticeable as dietary nPA-P increased.

TABLE 4 Condition factor and somatic indices of largemouth bass fed the experimental diets for nine weeks. Mean ± *SE*

	Dietary nPA-P level (g/kg, dry matter basis)								
	4.1	6.0	6.9	8.4	9.5	10.5	Model	R^2	Pr > F
K	1.36 ± 0.02	1.30 ± 0.03	1.25 ± 0.02	1.28 ± 0.03	1.29 ± 0.04	1.29 ± 0.03	SOP	0.27	0.093
IPFI (%)	1.96 ± 0.12	1.75 ± 0.24	1.41 ± 0.15	1.52 ± 0.14	1.38 ± 0.19	1.44 ± 0.16	LBL	0.40	0.021
HSI (%)	1.83 ± 0.08	1.78 ± 0.11	1.38 ± 0.07	1.53 ± 0.08	1.48 ± 0.15	1.56 ± 0.11	SOP	0.22	0.150
ISI (%)	2.97 ± 0.18	2.74 ± 0.12	2.47 ± 0.12	2.75 ± 0.12	2.75 ± 0.23	2.65 ± 0.11	SOP	0.19	0.198

Abbreviations:HSI, hepatosomatic index; IPFI, intraperitoneal fat index; ISI, intestinosomatic index; K, condition factor; LBL, linear broken-line model; nPA-P, non-phytic acid phosphorus; SE, standard error; SOP, second-order polynomial model (quadratic).

Dietary nPA-P did not affect ($p > .05$) K and most somatic indices of LMB except IPFI (Table 4). As dietary nPA-P increased from 4.1 to 6.9 g/kg, IPFI displayed a linear decline from 1.96 ± 0.12% to 1.41 ± 0.15% and then plateaued.

3.2 | Whole-body and defleshed-skeleton composition and nutrient retention efficiency

Dietary nPA-P did not influence whole-body moisture and crude protein of LMB ($p > .05$), whereas concentration of whole-body Ca and estimated retention efficiencies of Ca and nPA-P were affected (Table 5). Whole-body Ca displayed a linear ascend to 11.6 ± 0.2 g/kg at 6.9 g/kg dietary nPA-P and then remained nearly constant. Retention of dietary Ca by the fish increased in response to P supplementation to 41.6 ± 4% at 8.4 g/kg nPA-P, whereas retention of dietary nPA-P lessened from 91.9 ± 4% to 61.7 ± 2% within the dietary range of nPA-P. Lipid deposition in the fish descended linearly from 88.4 g/kg to 75.0 g/kg when dietary nPA-P increased from 4.1 to ≥7.3 g/kg (Figure 1a).Whole-body concentrations of ash (Figure 1b) and P (Figure 1c) also responded to supplemental P which was required to sustain levels similar to those observed in baseline fish. According to the 4-SK model, whole-body ash and P of LMB were optimal (95% of maximum response) when the diet contained 7.0 and 7.1 g/kg nPA-P, respectively. Retention of dietary protein by the LMB trended upwards in response to dietary P and was highest (34.1 ± 0.2%) when dietary nPA-P was 9.5 g/kg (Figure 1d).

Mineralization of LMB bones assessed by defleshed-skeleton levels of P, Ca, Mg and Zn in this study showed that supplementation of P to the basal diet was required to maintain skeletal mineral concentrations of the growing fish similar or above baseline levels (expect Zn; Figure 2a–d). According to the 4-SK model, the optimum dietary level of nPA-P necessary to optimize P, Ca and Mg concentrations in the LMB skeleton was 6.6, 7.0 and 7.1 g/kg, respectively (Figure 2a–c). Likewise, the quadratic trend observed for Zn indicated that maximum skeletal deposition of this element was achieved when dietary nPA-P was 7.0 g/kg (Figure 2d).

3.3 | Physiological parameters

Supplementation of P to the deficient basal diet had different impacts on the physiological parameters assessed in plasma and intestine of the LMB (Table 6). Glucose concentration and SOD activity

TABLE 5 Whole-body composition, calcium and nPA-P retention efficiencies of largemouth bass fed the experimental diets for nine weeks. Mean ± SE

		Dietary nPA-P level (g/kg, dry matter basis)								
	Baseline	4.1	6.0	6.9	8.4	9.5	10.5	Model	R^2	Pr > F
Moisture (g/kg)	733	707 ± 5	709 ± 7	705 ± 4	710 ± 3	700 ± 2	707 ± 2	SOP	0.25	0.827
Crude protein (g/kg)	176	171 ± 3	173 ± 2	179 ± 3	174 ± 2	180 ± 1	174 ± 1	SOP	0.28	0.194
Calcium (g/kg)	10.9	8.5 ± 0.3	9.2 ± 0.9	11.6 ± 0.2	11.7 ± 0.7	11.7 ± 0.7	12.9 ± 0.1	LBL	0.65	<0.001
Calcium retention (%)	n/a	24.0 ± 2	25.5 ± 4	34.7 ± 3	41.6 ± 4	38.3 ± 3	44.4 ± 1	SK	0.74	<0.001
nPA-P retention (%)	n/a	91.9 ± 4	71.9 ± 10	74.7 ± 6	69.7 ± 6	63.4 ± 4	61.7 ± 2	SOP	0.55	0.003

Abbreviations: LBL, linear broken-line model; nPA-P, n/a, not applicable; non-phytic acid phosphorus; SE, standard error; SK, four-parameter saturation kinetics model; SOP, second-order polynomial (quadratic).

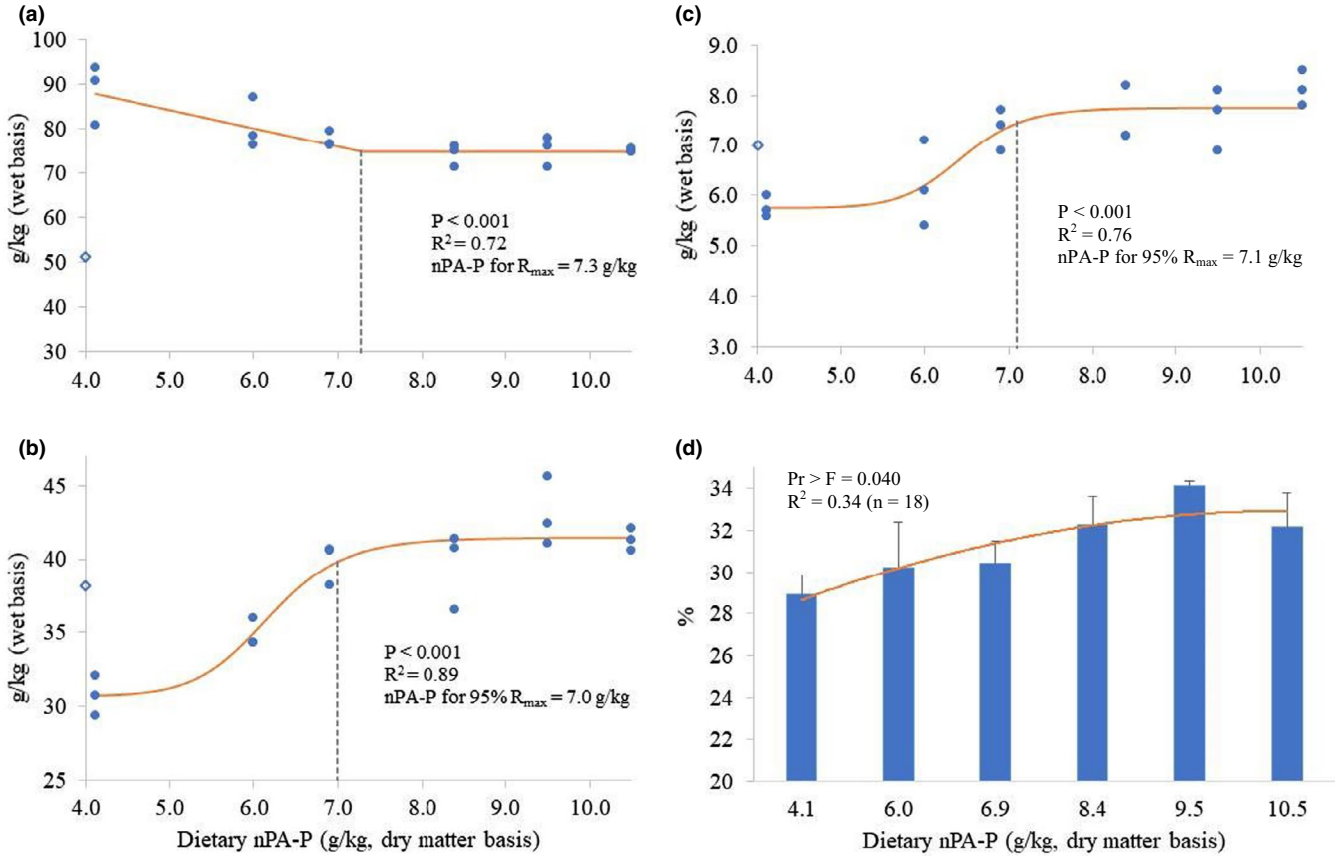

FIGURE 1 Whole-body concentrations of lipid (a), ash (b) and phosphorus (c); and protein retention efficiency (d) of largemouth bass fed the experimental diets for nine weeks. Dashed lines denote dietary nPA-P (non-phytic acid phosphorus) supporting maximum response [R_{max}; linear broken line model (a)] or 95% of R_{max} [95%R_{max}; four-parameter saturation kinetics model (b and c)]. In a, b and c, open diamond on *Y*-axis denotes level in baseline fish. In d, error bars denote standard error. Some data points overlap ($n = 18$)

in plasma, as well as intestinal CAT activity were unaffected by diet ($p > .05$). A reducing trend in plasma ALP activity as dietary nPA-P increased from 4.1 to 8.4 g/kg was noticeable, though the response was not statistically significant ($p = .063$). Plasma activity of ALT increased linearly over the dietary range of nPA-P while ascending plasma MDA concentrations plateaued at nPA-P levels ≥8.4 g/kg. Likewise, after a linear ascend, GP_x activity in intestine plateaued at dietary nPA-P levels ≥6.9 g/kg.

3.4 | Head-kidney gene expression

Results from gene expression analyses are presented in Table 7. Expression of Cu/Zn-SOD gene was unaffected by diet ($p > .05$) while statistically significant effects were observed for GH, IGF-1, FASN, TNF-α and TGF-β1. Expressions of GH and IGF-1 genes were upregulated as dietary nPA-P increased from 4.1 to 6.9 g/kg, then declined. Expression of FASN gene remained unaffected up to 8.4 g/kg dietary nPA-P, then displayed a linear descend to a relative expression of 0.45 ± 0.07-fold change. Expression of TNF-α gene was gradually downregulated from 1.63 ± 0.20 to 0.56 ± 0.08-fold change as dietary nPA-P increased from 4.1 to 6.9 g/kg, then was upregulated. Conversely, expression of TGF-β1 peaked between 6.9 and 8.4 g/kg dietary nPA-P, then decreased to a relative expression (0.58 ± 0.09-fold change) similar to that observed in basal diet-fed fish.

4 | DISCUSSION

This study provides information regarding impacts of dietary P deficiency on the physiology of LMB and, to our knowledge, the first reference level of total dietary P for practical feeds. Our attempt to further refine the dietary requirement by determining the availability of P in the basal diet using larger juvenile LMB and acid-insoluble ash (AIA) as the marker yielded unreliable data, which were not used. Besides possible limitations of AIA as a marker in digestibility studies (Li et al., 2008; Morales et al., 1999; Tacon & Rodriguez, 1984), the low dietary concentration of AIA and the limited amount of faecal material collected by stripping were considered compounding effects. Despite this, as retention of nPA-P by the LMB was nearly 92% at basal level and 75% at 6.9 g/kg dietary nPA-P, most of the PA-unbound P was retained up to requirement level supporting the conclusion that the estimated dietary

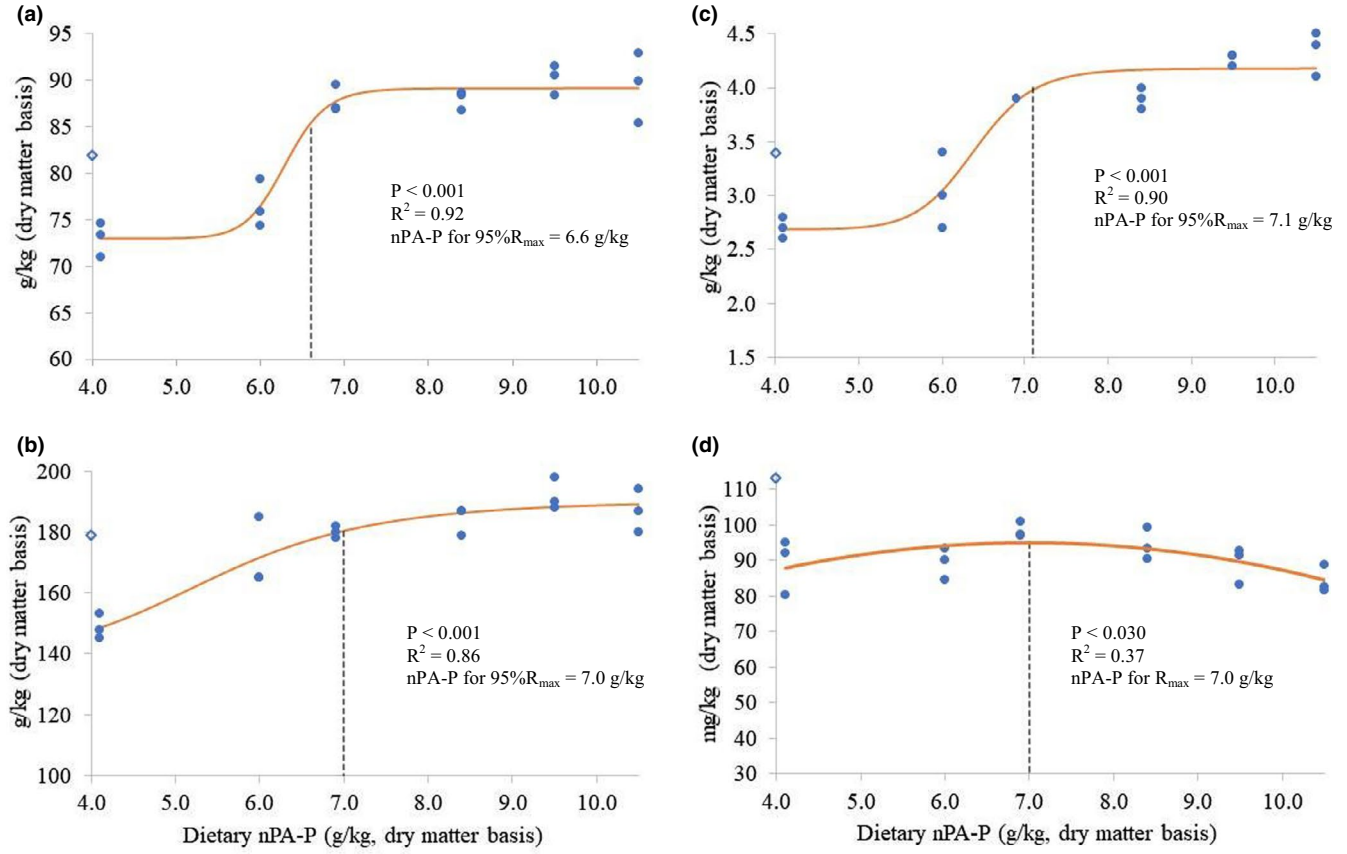

FIGURE 2 Skeletal concentrations of phosphorus (a), calcium (b), magnesium (c) and zinc (d) of largemouth bass fed the experimental diets for nine weeks. Dashed lines denote dietary nPA-P (non-phytic acid phosphorus) supporting 95% of maximum response [95%R_{max}; four-parameter saturation kinetics model (a, b and c)] or maximum response [R_{max}; second-order polynomial model (d)]. Open diamond on *Y*-axis denote level in baseline fish. Some data points overlap ($n = 18$)

TABLE 6 Plasma and intestine physiological parameters of largemouth bass fed the experimental diets for nine weeks. Mean ± SE

	Dietary nPA-P level (g/kg, dry matter basis)								
	4.1	6.0	6.9	8.4	9.5	10.5	Model	R^2	Pr > F
ALP (U/ml)	8.98 ± 0.54	6.18 ± 0.58	5.87 ± 0.73	4.58 ± 0.41	9.26 ± 0.60	5.06 ± 1.09	SOP	0.15	0.063
ALT (μU/ml)	33.03 ± 1.1	36.86 ± 1.5	37.22 ± 0.6	37.23 ± 1.4	41.43 ± 1.7	40.54 ± 0.8	L	0.39	<0.001
GLU (nmol/μl)	5.60 ± 0.80	5.46 ± 0.62	5.13 ± 1.15	5.13 ± 1.11	4.46 ± 0.53	4.18 ± 0.50	SOP	0.11	0.161
MDA (nmol/ml)	5.96 ± 0.99	5.87 ± 0.31	6.16 ± 0.09	8.14 ± 0.20	7.66 ± 0.13	8.04 ± 0.15	SK	0.84	<0.001
SOD (U/mg)	2.29 ± 0.66	2.70 ± 0.71	2.47 ± 0.85	3.36 ± 1.08	3.34 ± 0.77	2.64 ± 0.56	SOP	0.06	0.341
GP_x (U/mg)*	1.38 ± 0.05	1.46 ± 0.02	1.48 ± 0.05	1.47 ± 0.01	1.49 ± 0.02	1.49 ± 0.03	LBL	0.19	0.030
CAT (mU/ml)*	2.32 ± 0.07	1.79 ± 0.25	2.24 ± 0.34	2.52 ± 0.35	2.49 ± 0.33	2.42 ± 0.38	SOP	0.04	0.515

Abbreviations: *, determined in the intestine; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CAT, catalase; GLU, glucose; GP_x, glutathione peroxidase; L, linear model; LBL, linear broken-line model; MDA, malondialdehyde; nPA-P, non-phytic acid phosphorus; SE, standard error; SK, four-parameter saturation kinetics model; SOD, superoxide dismutase; SOP, second-order polynomial model (quadratic).

requirement of 7.0 g/kg total nPA-P is a reasonable reference value for practical formulations.

Although slight improvements in weight gain and FER were noticeable when nPA-P increased from 4.1 to 6.0 g/kg, the overall growth and feed utilization efficiency of LMB were not influenced ($p > .05$) by the incremental supplementation of P to the basal diet. These findings are in agreement with observations in other studies of inconsistent responses of fish to dietary P. For instance, rainbow trout, *Oncorhynchus mykiss*, fed diets containing P from 7.5 to 21.9 g/kg showed no significant effects of P supplementation (Bureau & Cho, 1999). In red drum, *Sciaenops ocellatus*, Davis and Robinson (1987) found no significant effects of dietary P levels ranging from 2.6 to 13.1 g/kg on growth and FCR after eleven weeks of feeding. Conversely, significant effects on fish production performance resulting from P

TABLE 7 Head-kidney gene expression (fold changes) of largemouth bass fed the experimental diets for nine weeks. Mean ± SE

	Dietary nPA-P level (g/kg, dry matter basis)								
	4.1	6.0	6.9	8.4	9.5	10.5	Model	R^2	Pr > F
GH	0.74 ± 0.09	0.97 ± 0.22	1.23 ± 0.12	0.74 ± 0.16	0.62 ± 0.16	0.59 ± 0.12	SOP	0.22	0.020
IGF-1	1.09 ± 0.22	1.22 ± 0.19	2.19 ± 0.25	1.67 ± 0.31	0.98 ± 0.26	0.83 ± 0.26	SOP	0.26	0.007
FASN	0.92 ± 0.21	0.92 ± 0.14	0.92 ± 0.13	1.02 ± 0.24	0.58 ± 0.08	0.45 ± 0.07	LBL	0.27	0.006
Cu/Zn-SOD	1.01 ± 0.16	0.99 ± 0.11	1.20 ± 0.13	1.22 ± 0.14	1.25 ± 0.12	0.68 ± 0.11	SOP	0.15	0.073
TNF-α	1.63 ± 0.20	1.00 ± 0.16	0.56 ± 0.08	0.64 ± 0.21	0.81 ± 0.23	1.18 ± 0.15	SOP	0.43	<0.001
TGF-β1	0.59 ± 0.11	1.13 ± 0.10	1.29 ± 0.12	1.19 ± 0.19	0.74 ± 0.09	0.58 ± 0.09	SOP	0.54	<0.001

Abbreviations: Cu/Zn-SOD, copper/zinc-superoxide dismutase; FASN, fatty acid synthase; GH, growth hormone; IGF-1, insulin-like growth factor-1; LBL, linear broken-line; nPA-P, non-phytic acid phosphorus; SE, standard error; SOP, second-order polynomial model (quadratic); TGF-β1, transforming growth factor-beta1; TNF-α, tumour necrosis factor alpha.

supplementation to deficient diets have been found. Shao et al. (2008) fed black seabream, *Sparus macrocephalus*, diets with graded levels of available P (1.8 to 10.7 g/kg) and found that weight gain and feed efficiency were improved as dietary P rose to 5.4 g/kg. Wang et al. (2005) also found that juvenile Japanese flounder, *Paralichthys olivaceus*, fed diets with total P ranging from 3.3 to 21.2 g/kg exhibited higher weight gain and feed efficiency in the 5.1 g/kg P treatment.

In this study, LMB retained dietary protein more efficiently as dietary P additively increased. Similarly, effects of P supplementation on protein efficiency ratio optimized in Japanese flounder at 5.1 g/kgtotal P and in black seabream, *Sparus macrocephalus*, at 5.4 g/kg available P have been observed (Shao et al., 2008; Wang et al., 2005). In contrast, Chaimongkol and Boonyaratpalin (2002) observed no effects of P supplementation on the protein retention efficiency of seabass, *Lates calcarifer*. Therefore, efficiency of dietary protein utilization in fish in response to dietary P appears to concur with responses on growth and feed efficiency, which are less pronounced on a time-dependent manner when compared to effects on tissue mineralization from a P-deficient diet.

Increased fat deposition is a typical sign of P deficiency, and it has been observed in various species. Sakamoto and Yone (1980) found that increased fat deposition in red seabream, *Chrysophrys major*, was associated with dietary P deficiency. In carp, Takeuchi and Nakazoe (1981) found that diets low in P corresponded with higher concentrations of crude fat in both carcass and viscera. Likewise, increased whole-body lipid was observed in rainbow trout fed P-deficient diets by Skonberg et al. (1997) while Elangovan and Shim (1998) found higher fat content in juvenile tiger barb, *Barbus tetrazona*, fed diets low in available P (1.7 and 3.7 g/kg). In the current study, dietary P deficiency affected both IPFI and whole-body fat content of LMB, both of which were highest in fish fed the basal diet containing 4.1 g/kg nPA-P, corroborating previous findings.

Since skeletal mineralization is primarily driven by dietary minerals and P is a major element in bones, its supplementation to the basal diet was expected to increase mineralization of LMB tissues which would be detected both in whole-body and skeleton. In turn, positive effects of supplemental P on the whole-body concentrations of ash, P and Ca were observed and the mineralization of LMB skeleton quantified by levels of P, Ca, Mg and Zn was optimized when dietary nPA-P was ~7.0 g/kg (i.e., 6.6, 7.0, 7.1 and 7.0 g/kg, respectively). These findings are supported by observations in other teleosts including rainbow trout (Sarker & Satoh, 2009), Nile tilapia (Sarkar et al., 2004), sunshine bass, *Morone chrysops* × *M. saxatilis* (Brown et al., 1993), seabass (Chaimongkol & Boonyaratpalin, 2002) and black seabream (Shao et al., 2008), demonstrating the importance of satisfying dietary P requirements.

Alkaline phosphatase is a membrane-bound enzyme that mediate the hydrolysis of phosphate esters in alkaline medium. In our study, ALP results were near significant ($p = .063$), and a decreasing trend in ALP activity in plasma of LMB was noticed as dietary nPA-P increased from 4.1 to 8.4 g/kg. These results corroborate observations of reduced ALP activities in seabream, *Sparus macrocephalus*, in response to increased dietary P (Shao et al., 2008), but disagree with findings in channel catfish (Eya & Lovell, 1997) and rainbow trout (Shearer & Hardy, 1987) showing ALP activities to increase or be unaffected by supplemental P in deficient diets, respectively. In addition, the positive response of plasma ALT to dietary nPA-P in our study might be associated with increased amino acid metabolism. Along with other transaminases, ALT plays a key role in amino acid metabolism (Wu, 2013) and its activity was positively correlated with protein retention efficiency by the LMB in our study.

Malondialdehyde is an end product of lipid hydroperoxide degradation and lipid peroxidation whose concentration is often used as an indicator of oxidative stress and damage (Ates et al., 2018). In fish, studies have shown that dietary P can affect MDA concentration in tissues. For instance, MDA concentration in the hepatopancreas of Jian carp decreased as dietary P increased from 1.7 to 5.5 g/kg, then increased in response to higher levels of P (Feng et al., 2013). A similar trend was observed in muscle of grass carp, *Ctenopharyngodon idella*, by Wen et al. (2015). Conversely, although MDA concentration in plasma was also affected by dietary P in our study ($p < .001$), MDA responded positively to dietary P up to 8.4 g/kg nPA-P, which might have resulted from increased lipid metabolism in liver and other tissues evidenced by a decline in lipid deposition. Despite the observed increase in plasma MDA indicating increased oxidation in tissues, activities of SOD in plasma and CAT in intestine of LMB were unaffected. However, similarly to the positive responses observed in Jian carp by Feng et al. (2013), our results showed that a minimum

of 6.0 g/kg nPA-P was necessary in the diet of LMB to maximize intestinal GP_x activity.

While effects of P supplementation on the expression of the genes assessed in our study appear to be limited in the literature, effects of dietary P deficiency on the expression of genes encoding for complements 3 & 4 (C3 & C4), immunoglobulin *M* (IgM), anti-inflammatory cytokines and antimicrobial peptides (Chen et al., 2017) have been observed. The kidney plays a key role in the mechanisms by which animals regulate absorption of Ca and P, including fish (Lock et al., 2007). Therefore, it was the organ of choice in our attempt to investigate the effects of dietary P on the expression of genes regulating growth, lipogenesis, antioxidant- and immune-related responses of LMB. Despite no effects were observed in Cu/Zn-SOD gene expression, GH, IGF-1, FASN, TNF-α and TGF-β1 genes were responsive to dietary nPA-P. GH is a protein hormone that has major influence on metabolism (protein, lipid and carbohydrate), and it prompts body growth by stimulating IGF secretion in multiple tissues including the kidney (Sakamoto & Hirano, 1993). Based on our findings, both skeletal mineralization and expressions of GH and IGF genes in head kidney respond positively to supplemental P until the dietary requirement is met. FASN is a complex multifunctional enzyme that catalyses the de novo synthesis of fatty acids (Chirala & Wakil, 2004; Zuo et al., 2017). In our study, the expression of head-kidney FASN gene remained fairly constant between dietary nPA-P levels of 4.1 and 8.4 g/kg, then was downregulated. Similar effects of dietary P were reported by Ji et al. (2017) who observed a downregulation of FASN gene in the liver of bighead carp, *Aristichthys nobilis*, when dietary P surpassed the requirement for maximum growth and feed efficiency. Although the mechanism by which adequate supply of P downregulates FASN in fish tissues appears to be unclear, it might be associated with a normalization of oxidative phosphorylation (Sugiura et al., 2004) allowing lipogenic substrates to be adequately oxidized for energy instead of being converted to lipids, and deposited lipids to be oxidized through β-oxidation. The observed changes in whole-body lipid of LMB in response to dietary P in our study (Figure 1a) support this hypothesis. TGF-β are cytokines that affect cell growth and differentiation, wound healing, extracellular matrix regulation and immune function; while TNF-α is pro-inflammatory. Based on the observed expressions of head kidney TNF-α and TGF-β1 genes, it appears that an adequate supply of dietary P can enhance the physiological status of LMB by down- and upregulating pro- and anti-inflammatory cytokines, respectively.

In conclusion, based on the findings of this study, a minimum of 7.0 g/kg total dietary nPA-P is required to support adequate production performance, tissue mineralization and physiological status of LMB. This estimate of total nPA-P requirement is similar to requirement values reported for other carnivorous freshwater species and can be used as a reference for balancing P in commercial feeds for LMB.

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DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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