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Dietary available phosphorus requirement for tambaqui, *Colossoma macropomum*, juveniles based on growth, haematology and bone mineralization

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Abstract

An economical and efficient approach to reduce the impact of P discharge by aquaculture industry is to adjust the P level in fish feeds to the precise nutrient requirement at different growth stages in a digestible nutrient basis. However, P requirement seems to be species specific and affected by several physiological, dietary and environmental factors. Based on the importance of tambaqui (*Colossoma macropomum*) to Latin American aquaculture, we designed a 63-day trial to evaluate the effect of available P (AP) levels on growth, nutrient digestibility, haematology and blood biochemical parameters, carcass proximate composition and bone mineralization. Quadruplicate groups of tambaqui juveniles (144 ± 2.0 g) were fed five isonitrogenous (278 g kg^{-1} digestible protein) and isocaloric (13.5 Mj DE kg^{-1}) diets containing graded AP levels (3.0, 5.6, 7.5, 9.1 and 11.0 g kg^{-1}) following a completely randomized design. Dicalcium phosphate (DCP) was used as the main P source. No mortalities and signs of P deficiency were observed among the dietary treatments. A remarkably high P digestibility was observed in all plant-based diets with a tendency of decreasing P digestibility with the increase in total P levels. Tambaqui seems to be able to grow well without inorganic P supplementation during the trials; however, this species required 7.0 g AP kg^{-1} diet for proper bone mineralization. P supplementation had a limited effect on haematology and blood biochemistry of tambaqui. Ecological implications of natural feeding habit and evolutionary position of this species are further discussed, and new hypothesis are drawn based on our results.

KEY WORDS: digestibility, growth, mineral, phytic-P, plant diets, tambaqui, welfare

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Introduction

Phosphorus (P) is one of the most important nutrients in fish nutrition due to its role for maintaining proper growth, bone mineralization, reproduction and nucleic acids biosynthesis. Furthermore, phosphorus is a component of cell membrane phospholipids and is the main molecule in energy-rich compounds, that is ATP, which are central molecules in the intermediary metabolism (Roy & Lall 2003; Uyan *et al.* 2007; NRC 2011). Additionally, feed utilization and stress tolerance is significantly affected by P supplementation (Vielma *et al.* 2002; Yang *et al.* 2006; Ye *et al.* 2006), and this last parameter is regarded as an essential factor for the development of aquaculture (Sukumaran *et al.* 2008).

Besides all the metabolic function played by phosphorus, studies have emphasized the importance of reducing P levels in fish diets aiming to improve sustainable aquaculture production. However, farmed fish could show severe clinical problems in long-term period if this strategy is used without the knowledge of P metabolism or species-specific P requirement (Sugiura *et al.* 2004). Thus, the knowledge

of the implications of P restriction is essential for the adequate growth and welfare of farmed fish.

The majority of P requirement studies in fish use growth, bone mineralization and metabolic changes as response criteria. However, few studies have included parameters of fish welfare, such as haematology and immune parameters, as response criteria of P adequacy in requirement studies. For instance, a positive effect of P supplementation on antibody production and resistance to *Edwardsiella ictaluri* infection were observed in channel catfish (Eya & Lovell 1998), while no effect of P supplementation or deficiency on immune response of *Coregonus lavaretus* has been reported (Jokinen *et al*. 2003). Similarly, inconclusive effects of dietary P supplementation on haematology and blood chemistry have been reported for Indian major carp, *Catla catla* (Sukumaran *et al*. 2008).

Tambaqui (*Colossoma macropomum)* is a freshwater Neotropical fish with great potential for Latin American aquaculture (Guimarães & Martins 2015). This species is the second most farmed species in Brazilian aquaculture due to its high growth rates, adaptation to intensive culture systems and the fillet quality. Despite the importance of tambaqui for the Latin American countries, and especially for Brazil, very limited information on the nutrient requirements for this specie is available. Additionally, this information is of utmost importance as the future of tambaqui farming depends on the production of more efficient and species-specific diets to reduce production cost and maintain an environmentally sustainable production.

Very limited studies have been conducted with Neotropical fish species, with emphasis to tambaqui. To the best of our knowledge, there are just two studies with Pacu (*Piaractus mesopotamicus*), a Neotropical fish from the same family as tambaqui (Signor *et al*. 2011; Diemer *et al*. 2014), and until now, there is no report with tambaqui. Therefore, we designed a feeding and a digestibility trial to determine the available phosphorus (AP) requirement for tambaqui using as response criteria not only growth and body composition, but also some fish welfare parameters.

Materials and methods

To determine the available P requirement for tambaqui, a series of two trials were performed to determine the effect of graded AP levels on growth response, nutrient digestibility, body proximate composition, haematology and blood biochemical parameters of tambaqui. All experimental procedures were approved by the Animal Ethics Committee of the Universidade Federal de Goiás (protocol 149/10-CEUA).

Diet preparation

Diets were manufactured using conventional plant ingredients to contain the same protein and digestible energy levels according to previous data for tambaqui (Macedo-Viegas *et al*. 1996; Almeida *et al*. 2011). The digestible nutrient values were calculated according to the ADC values obtained for tambaqui in our laboratory (M.F. Sena, I.G. Guimarães, J.G. Araújo, D.M.C. Pádua & J.H. Stringhini, in prep.). Dicalcium phosphate was used as the inorganic P source. An unsupplemented diet with no adding dicalcium phosphate was formulated as the control diet, and by adding dicalcium phosphate, the following dietary available P levels were obtained: 5.6, 7.5, 9.1 and 1.1 g kg^{-1} (Table 1). The unsupplemented diet contained 3.0 g kg^{-1} total P. Although no data on optimum Ca : P ratio for growth and bone development are available for tambaqui, all diets were formulated to contain the Ca : P ratio recommended for freshwater fish by NRC (2011) to reduce the effects of Ca : P imbalances on the results. Chromic oxide was included at 2.0 g kg^{-1} in all the diets as a digestibility marker for nutrient digestibility evaluation.

All ingredients were ground until sieve in a mesh diameter of 500 μm. Diets were mechanically mixed with water (250 g kg^{-1} of dry weight), and the moist mixture was extruded in a single-screw laboratory extrusion (PQ30, Inbramaq, Ribeirão Preto, Brazil). Diets were oven-dried until present moisture <100 g kg^{-1} and stored at −18 °C until further use.

Digestibility trial

Ninety tambaqui juveniles, initial weight 100 ± 2.1 g, were randomly stocked into ten 60-L aquaria. This set of aquaria was used for the feeding procedure and was connected to a recirculated water system. Five conic-bottomed 300-L aquaria were used to collect faeces by sedimentation following a modified procedure of the Guelph system described previously by Guimarães *et al*. (2008). Both systems were connected to a biological filter and water temperature was thermostatically controlled and individually aerated using air stones.

Faeces collection was performed according to Guimarães *et al*. (2008) with slight modifications proposed by Mota *et al*. (2015) for collecting faeces with tambaqui. Briefly, all fish were fed the control diet for 15 days; then,

Table 1 Percentage and proximal composition of experimental diets

Ingredients	Available phosphorus levels (g kg^{-1})				
	3.0	5.6	7.5	9.1	11.0
Soybean meal	440.0	440.0	443.5	443.5	425.0
Corn	160.0	155.0	115.0	87.5	70.0
Broken rice	100.0	100.0	90.0	70.0	60.0
Cottonseed meal	90.0	90.0	90.0	90.0	
Corn gluten meal	70.6	70.6	73.6	79.1	
Soybean oil	58.1	70.1	87.1	103.1	114.1
Wheat middlings	20.0	20.0	20.0	20.0	
Corn starch	34.5	–	–	–	–
Dicalcium phosphate	–	30.0	59.0	88.0	120.0
Limestone	11.5	9.0	6.5	3.5	–
Vit./min.mix[3]	13.6	13.6	13.6	13.6	
Cr_2O_3[1]	1.0	1.0	1.0	1.0	1.0
NaCl[2]	0.7	0.7	0.7	0.7	0.7
Proximal composition[4] (dry matter basis)					
Digestible energy (Mj kg^{-1})	13.50	13.50	13.54	13.54	13.56
Digestible protein (g kg^{-1})	295.1	295.5	294.5	293.8	293.4
Crude protein (g kg^{-1})	302.4	301.8	301.1	300.4	299.7
Crude fiber (g kg^{-1})	43.3	43.3	42.8	42.4	40.3
Fat (g kg^{-1})	75.9	87.6	103.1	117.9	127.6
Ca (g kg^{-1})	5.9	12.3	18.5	24.4	30.9
Total P (g kg^{-1})	3.4	6.6	9.4	11.2	13.5
Avail. P (g kg^{-1})	3.0	5.6	7.5	9.1	11.0
Ca : Avail. P ratio	2.42	2.46	2.47	2.46	2.46

[1] Chromic oxide.
[2] Sodium chloride.
[3] The supplement provided the following per kg diet: Mannan-oligosaccharides (MOS) (min.) 120 mg kg^{-1}; Vitamin A (min.) 16 000 IU kg^{-1}; Vitamin D3 (min.) 4500 IU kg^{-1}; Vitamin E (min.) 250 IU kg^{-1}; Vitamin B2 (min.) 32 mg kg^{-1}; Vitamin B1 (min.) 32 mg kg^{-1}; Vitamin C (min.) 325 mg kg^{-1}; Niacin (min.) 170 mg kg^{-1}; Vitamin B6 (min.) 32 mg kg^{-1}; Biotin (min.) 10 mg kg^{-1}; Folic acid (min.) 10 mg kg^{-1}; Vitamin B12 (min.) 32 mcg kg^{-1}; Choline chloride (min.) 2.000 mg kg^{-1}; Manganese (min.) 50 mg kg^{-1}; Zinc (min.) 150 mg kg^{-1}; Iron (min.) 150 mg kg^{-1}; Copper (min.) 20 mg kg^{-1}; Cobalt (min.) 0.5 mg kg^{-1}; Iodine (min.) 1.0 mg kg^{-1}; Selenium (min.) 0.70 mg kg^{-1}.
[4] Analysed composition; Digestible/available values were calculated according to the ADC of nutrients in Table 4.

experimental diets were randomly assigned to the feeding aquaria; fish were fed during 7 days prior to the beginning of faecal collection (acclimatization period); the first faeces collection was carried out. Fish were fed twice daily until apparent satiation throughout the faeces collection period. The first groups of fish (five) were then transferred to collecting faeces aquaria, remaining for 8 h. During this procedure, the faeces were collected repeatedly at 30-min intervals, refrigerated, bulked by each group of fish and centrifuged to remove the excess of water. Faeces were oven-dried at 55 °C, ground and stored at −20 °C until chemical analysis. Fish were then returned to the feeding aquaria. On the consecutive day, the remaining groups (the other five groups of fish) were transferred and the faeces were collected. This procedure was repeated two times to obtain the amount of faeces needed to compound a replication (each group of fish), and test diets were reassigned to the feeding aquaria for the next round. The acclimatization and faecal collection process (round) were repeated two times to obtain quadruplicate measurements per test diet (each round was compounded by two replications).

During the experimental period, a 12-h light : 12-h dark photoperiod was maintained, dissolved oxygen content was approximately 6.1 mg L^{-1}; pH 6.7–7.8 and total ammonia–nitrogen was 0.038 and 0.057 mg L^{-1}. Temperature ranged from 28.5 to 30.9 °C.

Experimental procedure for the growth trial

The feeding trial was conducted in a recirculating system. A homogenous group of 140 tambaqui juveniles was selected by weight (144 ± 2 g) and randomly stocked into twenty 400-L aquaria. The experimental diets were randomly assigned to the tanks and fed to quadruplicate groups of fish for 63 days. Fish were fed until apparent satiation at 08:00, 12:00 and 16:00 h. During the feeding trial, water quality parameters were maintained in the optimum range for tambaqui rearing (pH 6.5 ± 0.2; dissolved oxygen 5.8 ± 0.7 mg L^{-1} and ammonia (NH_3) 0.19 ± 0.1 mg L^{-1}). Water temperature was heater-controlled and kept at 27.5 ± 0.4 °C. All tanks were maintained under natural photoperiod. Dissolved P level in the tap water was <0.004 mg L^{-1}, and accumulated faeces were removed by syphoning.

During the experiment, fish mortality was recorded. At the beginning and before each weighing sampling (every 21 days), fish were starved for 24 h and then weighed by group.

Haematology

At the end of the feeding trial, eight fish per treatment were randomly collected and anaesthetized with benzocaine at 67 mg L^{-1}. Blood samples were collected from the caudal vasculature using 3-mL syringes rinsed with anticoagulant (3% EDTA) for haematological assays. Red blood cell (RBC) and leucocyte (WBC) counts were determined by dilution and enumeration in a haemocytometer.

Haemoglobin (Hb) was determined by cyanometahaemoglobin colorimetric method using a commercial kit (Labtest Diagnóstica S.A., Belo Horizonte, Brazil). Haematocrit (Htc) percentage was determined by the microhaematocrit method. Total plasma protein (TPP) was measured using a manual Goldberg refractometer (Weiss & Wardrop 2011).

Mean corpuscular volume [MCV = (Htc × 10)/erythrocytes] and mean corpuscular haemoglobin concentration [MCHC = (Hb × Htc) × 100] were also calculated.

The albumin concentration (ALB) was determined by the bromocresol method using a commercial kit for colorimetric determination (Labtest Diagnóstica S.A.). The albumin : globulin ratio (A/G) was determined using ALB and TPP values [Globulin = TPP – ALB; A/G = ALB/Globulin]. Serum alkaline phosphatase activity and serum phosphate concentration were determined using colorimetric commercial kit (Labtest Diagnóstica S.A.).

Analysis and measurement

A group of 10 fish at the beginning of the experiment and two fish per aquarium at the end were collected and killed with high benzocaine concentration (193 mg L^{-1}). Fish were ground in a meat mincer, and samples were stored frozen (−18 °C) to determine the whole-body proximate composition. Eight fish per diet were dissected for vertebrae removal and determination of mineral content according to Roy *et al.* (2002). Proximate composition analysis and mineral content of feed ingredients, experimental diets, faeces and whole fish were performed according to the standard methods of AOAC (1995). Samples were dried to a constant weight at 105 °C to determine moisture. Protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method; lipid by ether extraction using Soxhlet; ash by combustion at 550 °C, crude fibre by fritted glass crucible method after treatment with H_2SO_4 and NaOH. Samples of fish vertebrae and experimental diets were analysed to determine calcium (Ca), magnesium (Mg), manganese (Mn) and zinc (Zn) concentrations by flame atomic absorption spectrophotometry on a Shimadzu AA-6800 (Shimadzu, Japan), while total P concentration was analysed by a colorimetric process, using the vanado-molybdate reagent. Chromic oxide was determined according to Bremer Neto *et al.* (2005), and gross energy content was determined in an adiabatic calorimetric bomb (Parr Instrument Company, Moline, IL, USA).

Calculations and statistical analysis

The following variables were calculated:

$$\text{Feed intake (FI)} = \text{Feed consumption (g)}/((\text{FW} + \text{IW})/2) \times t)$$

$$\text{Daily weight gain (DWG)} = (\text{FW} - \text{IW})/t$$

$$\text{Feed conversion ratio (FCR)} = \text{Dry feed fed in g}/\text{wet weight gain in g}$$

$$\text{Specific growth rate (SGR)} = (\text{Ln FW} - \text{Ln IW}) \times 100/t$$

$$\text{Protein efficiency ratio (PER)} = \text{Wet weight gain in g}/\text{protein intake in g}$$

where, FW is final body weight, IW is initial body weight, t is experimental duration in days.

Apparent digestibility coefficients (ADC) of nutrients and minerals were calculated according to Bureau & Hua (2006).

The growth trial followed a completely randomized design with five treatments and four replicates, while the digestibility trial consisted of five treatments (dietary P levels) and four replications (groups of fish in each round) arranged in a randomized block design. Each round of faecal collection with two replications was considered the block. All data were verified for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's F test). The concentration of dietary available P was the fixed factor in this study. Data were analysed using PROC GLM (SAS Institute, Inc., Cary, NC, USA) for a one-way analysis of variance (ANOVA), and GraphPad Prism 6.02 (Graphpad software, San Diego, CA) was used for graphs preparation. When the quadratic term in the model was statistically significant, the relationship between P level and the measured parameter was further evaluated using PROC NLIN (Robbins *et al.* 1979) to determine the response curve and estimate the optimum P requirement level in relation to the measured parameter. The models used to estimate the requirement were selected based on the least sum of squared differences between the values of the observed and predicted values of the dependent variable (Shearer 2000). For response variables that were significantly different and no model was able to be fitted, pairwise comparisons between treatments means were made using the Student–Newman–Keuls multiple range test. A significance level of $P \leq 0.05$ was used for all statistical analyses. All percentage data were arcsine-transformed before analysis.

Results

Growth performance of tambaqui juveniles fed diets containing graded levels of available P is shown in Table 2. Except for the FCR and PER, available P levels did not affect growth potential of tambaqui ($P > 0.05$); in fact, the highest P levels tended to reduce weight gain, but this was not significant. Fish fed diets containing 9.1 g kg^{-1} available P showed the highest FCR and PER, being different only from fish fed 3.0 (unsupplemented diet) and 7.5 g kg^{-1} available P ($P < 0.05$).

Dietary available P levels only affected the whole-body ash content ($P < 0.05$) of tambaqui (Table 3). A second-order polynomial model best fitted to the whole-body ash and vertebrae Mg content with a 95% optimum level estimated at 6.72 g kg^{-1} for both parameters ($P < 0.01$) (Fig. 1a,d, Table 3). The broken-line model best fitted to the vertebrae Ca and P content data ($P < 0.01$). Estimated requirements based on Ca and P vertebrae content were 9.5 and 7.0 g kg^{-1}, respectively (Fig. 1b,c). Ca : P ratio tended to decrease according to the increase in available P levels until reach a plateau at 1.39 (Table 3). No effect of available P supplementation on vertebrae Mn and Zn content was observed in this study ($P > 0.05$) (Table 3).

The effect of dietary P levels on ADC of nutrients is shown in Table 4. The increase in dietary P levels only affected the ADC of P ($P < 0.05$). ADC of P tended to decrease according to the increase in dietary P levels and reached the plateau at 8.8 g kg^{-1} diet.

Except for the haematometric indices, all other haematological parameters (RBC, Hb, Htc and WBC) of tambaqui were affected by the dietary AP levels (Fig 2) ($P < 0.05$). Fish fed diets containing the highest P level (11 g kg^{-1}) showed the highest RBC count (Fig 2a), Htc (Fig 2b) and Hb (Fig 2c), while fish fed 5.6 g AP kg $diet^{-1}$ showed the highest WBC count (Fig 2d). Except for Hb data, fish fed the unsupplemented P diet showed the lowest RBC and Htc values and WBC count.

Except for the serum albumin content, the other serum protein profile parameters of tambaqui were not affected by the dietary AP levels (Fig 3). A second-order polynomial regression best fitted to the serum albumin content (Fig 3b). The optimum available P level based on 95% of the maximum response was 7.0 g kg^{-1}.

No differences on serum Pi (inorganic phosphate) were observed when tambaqui was fed diets containing different available P levels (Fig 4a), while the serum alkaline phosphatase (ALP) activity tended to decrease up to ~ 7.5 g kg^{-1} and then increase until reach the maximum activity at 11.0 g kg^{-1} available P. The quadratic relationship best fitted to the ALP activity data (Fig 4b).

Discussion

This is the first study to report phosphorus requirement for a characin fish based on available phosphorus (AP) levels. To our surprise, tambaqui juveniles seems to grow well on plant-based diets containing low levels of available P (3.0 g kg^{-1}) over 63 days of feeding trial without showing any deficiency signs throughout the experimental period. The lack of effect of P supplementation on growth of fish has been reported for other aquacultured species, such as Pacu (*Piaractus mesopotamicus*) (Signor *et al.* 2011; Diemer *et al.* 2014), channel catfish (*Ictalurus punctatus*) (Eya & Lovell 1997), yellow catfish (*Pelteobagrus fulvidraco*) (Wang *et al.* 2016), rainbow trout (*Oncorhynchus mykiss*) (Skonberg *et al.* 1997; Bureau & Cho 1999), sea bass (*Lates calcarifer*) (Chaimongkol & Boonyaratpalin 2001) and Atlantic salmon (*Salmo salar*) (Vielma & Lall 1998). On the other hand, several studies have reported an improved growth performance when dietary P was supplemented for different

Table 2 Final weight (FW), daily weight gain (DWG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER) of tambaqui (*Colossoma macropomum*) fed diets containing graded levels of available P for 63 days

	Available P level				
	3.0	5.6	7.5	9.1	11
FW (g)	272.1 ± 2.8	300.3 ± 6.2	285.4 ± 4.9	280.5 ± 4.7	283.9 ± 4.9
DWG, g day^{-1}	2.11 ± 0.12	2.02 ± 0.09	2.16 ± 0.20	1.74 ± 0.27	1.96 ± 0.14
SGR, %	1.00 ± 0.05	0.97 ± 0.03	1.02 ± 0.06	0.86 ± 0.11	0.95 ± 0.04
FI, g day^{-1}	3.81 ± 0.13	3.91 ± 0.06	3.87 ± 0.24	3.57 ± 0.34	3.79 ± 0.21
FCR*	1.81 ± 0.05^{b}	1.94 ± 0.06^{ab}	1.79 ± 0.08^{b}	2.07 ± 0.13^{a}	1.93 ± 0.07^{ab}
PER*, %	1.81 ± 0.05^{a}	1.69 ± 0.05^{ab}	1.83 ± 0.07^{a}	1.59 ± 0.09^{b}	1.70 ± 0.07^{ab}

Means followed by different lower case letters within the same row indicate significant difference by SNK test (*$P < 0.05$).

Whole body[1] (g kg^{-1})	Dietary available P levels (g kg^{-1})				
	3.0	5.6	7.5	9.1	11
Moisture	675.8 ± 12.56	677.1 ± 8.58	673.2 ± 10.19	687.1 ± 8.33	673.8 ± 10.15
Crude protein	160.5 ± 7.65	162.2 ± 6.5	162.6 ± 6.8	156.9 ± 4.8	163.1 ± 5.9
Fat	115.5 ± 13.86	110.6 ± 6.86	115.4 ± 10.75	112.0 ± 6.88	113.7 ± 8.23
Ash[3]	12.15 ± 1.70	14.17 ± 1.3	18.20 ± 1.8	19.18 ± 1.42	17.01 ± 2.0
Vertebrae[2]					
P (g kg^{-1})[4]	28.5 ± 5.4	42.7 ± 3.2	51.4 ± 1.2	56.1 ± 3.7	54.9 ± 4.8
Ca (g kg^{-1})[4]	61.7 ± 0.6	63.5 ± 3.1	68.0 ± 2.9	74.6 ± 3.5	73.8 ± 3.4
Ca/P	2.23 ± 0.4^{a}	1.49 ± 0.1^{b}	1.32 ± 0.04^{c}	1.33 ± 0.09^{c}	1.35 ± 0.06^{c}
Mg (g kg^{-1})[3]	3.2 ± 0.6	4.2 ± 0.5	3.2 ± 0.3	4.3 ± 0.4	6.0 ± 0.7
Mn (μg kg^{-1})	6.0 ± 2.0	7.0 ± 4.0	8.0 ± 3.0	11.0 ± 2.0	8.0 ± 4.0
Zn (μg kg^{-1})	70.0 ± 20.0	50.0 ± 20.0	60.0 ± 40.0	60.0 ± 20.0	80.0 ± 40.0

[1] Values are means of two determination per fish, two fish per tank and four tanks per treatment.
[2] Values are means of one determination per fish, two fish per tank and four tanks per treatment.
[3] Quadratic regression: Ash = 9.66 + 3.031x + 0.2142x^2, R^2 = 0.84, $X_{0.95}$ = 6.72 g kg^{-1} ($P < 0.01$); Mg = 2.199 + 0.69x + 0.0488x^2, R^2 = 0.78, $X_{0.95}$ = 6.72 g kg^{-1} ($P < 0.05$).
[4] Broken-line model: Phosphorus (P) = 10.77 + 5.828 (7.0 − x), R^2 = 0.98, X_0 = 7.0 g kg^{-1}; Ca = 54.17 + 2.04 (9.5 − x), R^2 = 0.91, X_0 = 9.5 g kg^{-1}.

Table 3 Whole-body proximate composition and vertebrae mineral composition of tambaqui (*Colossoma macropomum*) fed diets containing graded levels of available phosphorus for 63 days

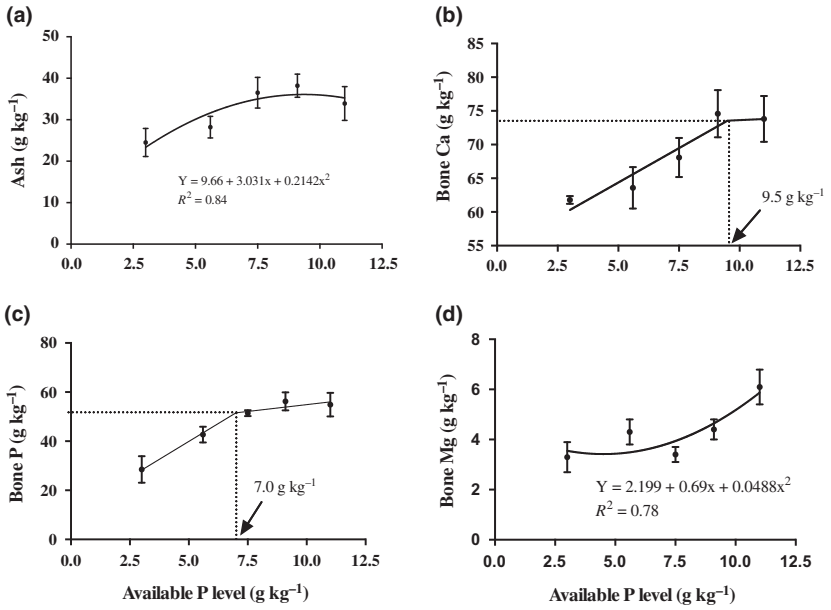

Figure 1 Whole-body ash (a), vertebrae calcium (b), vertebrae phosphorus (c) and vertebrae magnesium (d) of tambaqui (*Colossoma macropomum*) fed diets containing analysed dietary available P levels. Each point represents the mean of four replicates.

fish species (NRC 2011). These differences among studies could be a result of a combination of several factors such as the clearance of body P stores, feed formulation and tested species (Wang *et al*. 2016).

Recently, a meta-analytical study observed a poor relationship between growth rate and subclinical P deficiency among aquacultured fish from different taxa, which can be explained by the ability of fish to use flexible body P pools (presumably dominated by bone P) to meet metabolic demand (Benstead *et al*. 2014). According to these authors, rapidly growing and P-rich fishes tend to present higher P requirement to sustain higher growth rates and to create P-rich tissues as a flexible P body store. Therefore, requirement estimates based on whole body or vertebrae P may be more adequate than based on weight gain, which have been pointed out by other reports (Antony Jesu Prabhu *et al*. 2013; Wang *et al*. 2016). In this regard, tambaqui seems to require 7.0 g AP kg^{-1} diet to maintain proper bone

Table 4 Effect of dietary phosphorus levels on apparent digestibility coefficients (ADC) of dry matter (DM), crude protein (CP), gross energy (GE) and phosphorus (P) for tambaqui ($n = 4 \pm$ SD)

tP (g kg^{-1})	ADC DM	ADC CP	ADC GE	ADC P
3.4	90.3 ± 1.6	97.6 ± 0.5	92.4 ± 1.2	87.9 ± 2.3
6.6	90.5 ± 1.0	97.9 ± 0.2	92.8 ± 0.8	84.3 ± 0.9
9.4	89.5 ± 2.1	97.8 ± 0.4	92.7 ± 1.2	81.6 ± 4.0
11.2	88.9 ± 0.7	97.8 ± 0.1	92.7 ± 0.4	80.9 ± 1.8
13.5	88.5 ± 1.8	97.9 ± 0.4	92.9 ± 1.0	82.7 ± 2.9
ANOVA *P*-value	ns	ns	ns	0.01
Regression				
Quadratic r^2-value	–	–	–	0.97 (0.013)
Broken line r^2-value	–	–	–	0.98 (0.0025)

ns – not significant; values between parenthesis indicate the *P* value.

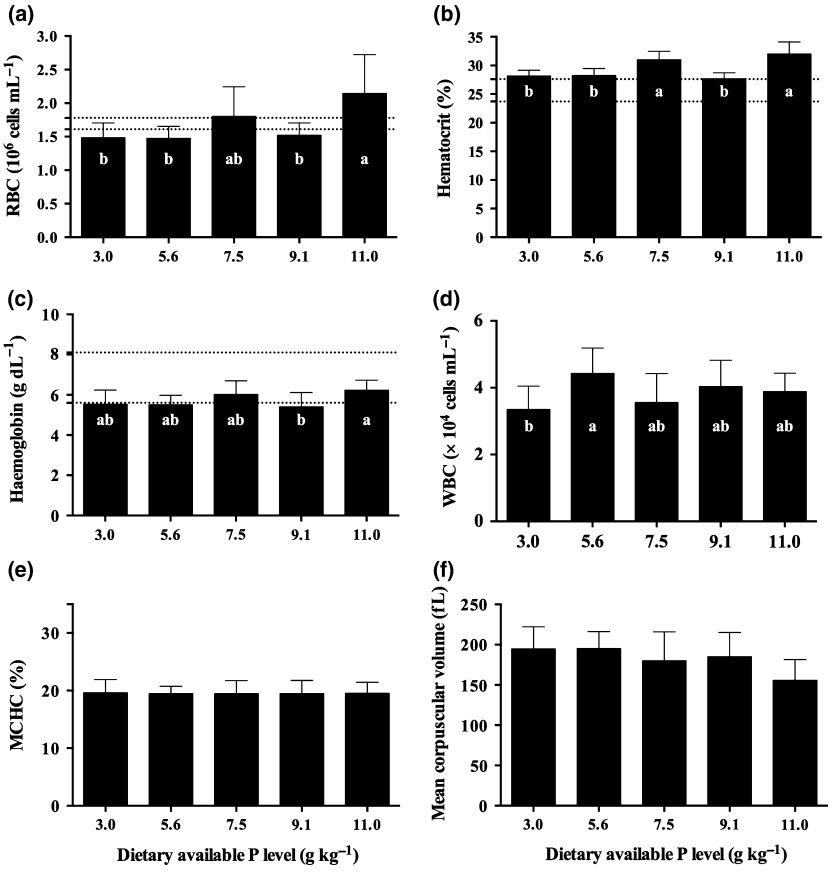

Figure 2 (a) RBC (red blood cell count), (b) Htc (haematocrit), (c) Hb (haemoglobin), (d) WBC (white blood cell count), (e) MCHC (mean corpuscular haemoglobin concentration) and (f) MCV (mean corpuscular volume) of tambaqui fed practical diets containing graded levels of available phosphorus. Values are means of two determinations per fish, two fish per tank and four tanks per treatment. Bars with different small letters are significantly different by Duncan multiple range test ($P < 0.05$). Space between the dotted lines indicates the reference interval for tambaqui (Ranzani-Paiva *et al*. 1998, 1999; Tavares-Dias & Sandrin 1998; Tavares-Dias *et al*. 1998).

mineralization. In addition to these results, we have observed a tendency of growth depression and poor feed utilization when AP levels above 7.5 g kg^{-1} were supplemented in tambaqui diets. Although this has not been well described in the aquaculture literature, there are compelling evidences in ecological studies for a growth depressing effect of P excess in vertebrates, fish included (Boersma & Elser 2006; Benstead *et al*. 2014). These growth-depressing effects may be driven by evolutionary adaptations of some fish species to environmental low P content, leading low trophic species (such as herbivores) to be less specialized in dealing with excess P intake than higher predators (Boersma & Elser 2006) and develop physiological mechanisms to efficiently use P from plant products. This may explain the growth performance data and the high P digestibility in plant-based diets of tambaqui in this trial. Furthermore, the trial length could have not been long enough to produce deficiency signs in this size range of fish.

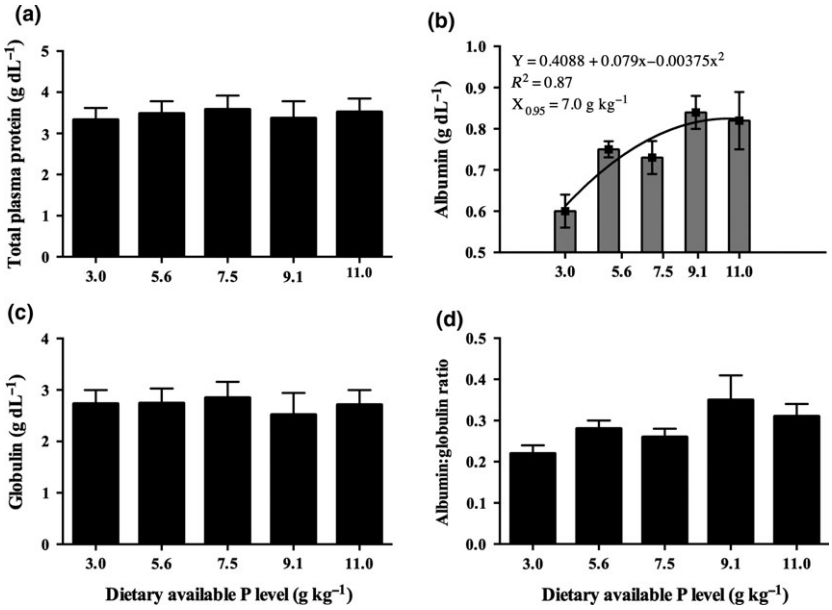

Figure 3 Total plasma protein (a), albumin (b), globulin (c) and albumin to globulin ratio (d) of tambaqui fed diets containing graded levels of available phosphorus. Values are means of two determinations per fish, two fish per tank and four tanks per treatment. Quadratic regression was significant at $P < 0.001$. $X_{0.95}$ indicates the optimum available P level based on 95% of the X_{max}.

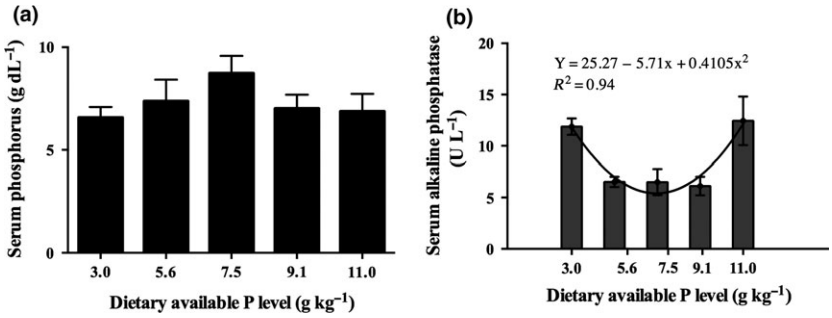

Figure 4 Serum phosphorus (a) and alkaline phosphatase activity (b) of tambaqui fed diets containing graded levels of available phosphorus. Values are means of two determinations per fish, two fish per tank and four tanks per treatment. Quadratic regression was significant at $P < 0.001$.

Haematology has been used as a tool to describe the physiological state of fish in response to the presence of toxic compounds, genetic variability and stress (Svobodová & Vysuková 1991). Additionally, nutritional status has been regarded as one of the factors that greatly affect haematology, and the effects of specific nutrients have been studied as a mean to counteract the detrimental effects of stress in intensive production systems (Barros *et al.* 2006).

Studies evaluating the effect of P supplementation on fish haematology and disease resistance are scarce (Oliva-Teles 2012). P supplementation only affected total leucocyte count of Indian major carp (*Catla catla*) (Sukumaran *et al.* 2008). However, except for haematometric indices, all other haematological parameters were affected by P supplementation in our study. The lowest RBC count, HTC and leucocyte count of tambaqui fed the unsupplemented P diets may be explained by two hypothesis: (i) the reduction in intracellular P content causes erythrocyte dysfunction in mammals which leads to a decline of 2,3-bisphosphoglycerate, shifting the oxyhaemoglobin dissociation curve to the left and causing hypoxia at the cellular level with cell death (Lichtman *et al.* 1971; Knochel 1977, 2000) and (ii) the reduction in cell membrane fluidity due to the lack of phosphate groups for phospholipids biosynthesis may have increased erythrocyte haemolysis leading to a lower RBC count, Hb level and Htc. In fact, blood samples of the groups fed the unsupplemented P diets showed a higher frequency of haemolysis than the other dietary groups. Although most of the effects of P deficiency on haematology are reported with higher vertebrates, it is most likely that the same effects occur in fish if we take in mind that fish erythrocytes contain 5–6 times more P than terrestrial animals (Mccay 1931).

Although fish fed the unsupplemented diet showed the lowest RBC and WBC counts, and HTC, these values are within the reference interval reported in the literature for tambaqui kept in intensive production systems (Ranzani-Paiva *et al.* 1998, 1999; Tavares-Dias & Sandrin 1998;

Tavares-Dias *et al*. 1998). Due to the lack of effect of AP supplementation on growth parameters, we may assume that tambaqui fed the diets with the lowest AP levels was under marginal deficiency which could be detected by the haematology data.

The results of serum ALP activity of tambaqui fed diets containing graded levels of AP were different from those reported for other fish species. On the other side, the results were similar to those observed with higher vertebrates. Most of the studies with fish have reported a positive quadratic effect between dietary P levels and ALP activity (Eya & Lovell 1997, 1998; Coloso *et al*. 2003), while a negative linear effect is observed in terrestrial animals (Boyd *et al*. 1983; Koch *et al*. 1984; Cashman & Flynn 1999). Although the optimum value for ALP activity based on the regression equation (6.95 g AP kg diet^{-1}) correlates well with the estimated requirement for optimum P retention in the vertebrae, several studies reported inconsistent results of ALP activity. Thus, ALP activity may not be an adequate parameter to estimate P requirement for fish, differently from terrestrial animals.

Differently from other fish species (Brown *et al*. 1993; El-Zibdeh *et al*. 1995; Rodehutscord 1996; Vielma & Lall 1998; Sugiura *et al*. 2000; Roy & Lall 2003; Cheng *et al*. 2005; Yang *et al*. 2006), dietary AP supplementation did not affect serum Pi concentration of tambaqui. On the other side, no effect of P deficiency or adequacy on total plasma P was observed in Atlantic salmon and rainbow trout (Skonberg *et al*. 1997; Baeverfjord *et al*. 1998) which is in accordance with our study. According to Skonberg *et al*. (1997), blood plasma P levels are insensitive as a response indicator of P status of fish because of three reasons: (i) blood P levels tend to reflect recent dietary intake more than the actual P status of the fish; (ii) different methods measure different fractions of P in the serum, plasma or whole blood; and (iii) non-nutritional factors, such as sampling time (after meal), handling of the samples, collection methods, temperature, salinity and various stresses on fish when or before sampling, also have profound effects on the blood P level.

Although no effects of P supplementation on plasma protein profile have been reported previously in fish, the quadratic relationship between AP levels and albumin observed in this study may be a result of (i) the increased need of the organism to transport phosphate leading to increased albumin synthesis, once phosphate can link to albumin to increase their transport (Bondi 1987), and (ii) as a way to adjust blood pH and osmoregulation due to the imbalance of phosphorus concentration. However, there are few reports on the mechanisms of phosphorus absorption and transport in fish, with emphasis to herbivorous species, leading to limited conclusions.

Quantitative P requirement has been recently summarized by NRC (2011), ranging from 3 to 15 g kg^{-1} of total P, and 3 to 9 g kg^{-1} available P for most fish species. Requirement as below as this observed for tambaqui were reported for subadult and fingerling channel catfish (*Ictalurus punctatus*) (Wilson *et al*. 1982; Eya & Lovell 1997), while reported AP requirement based on weight gain for Nile tilapia has been estimated to vary from 4.5 to 5.2 g kg^{-1} at different growth stages (Furuya *et al*. 2008a,b; Quintero Pinto *et al*. 2011). In our study, tambaqui seems to require around 7.0 g kg^{-1} of AP for higher vertebrae P retention and proper health status (based on serum albumin); however, when using growth data to estimate the requirement, this species seems to grow well on diets formulated with only plant products. Additionally, tambaqui seems to have a great ability to digest phytic-P from plant ingredients based on the digestibility trial of the diets. The unsupplemented diet showed the highest P digestibility (87.92%) than the other dietary P levels. Reports on tilapia and carp have observed much lower P ADC using all plant diets (NRC 2011). Although no studies have reported the ability of tambaqui to use phytate from plant products, some studies have reported the presence of gut-associated microbiota able to synthesize phytase in fish in addition to expression of endogenous phytase at the brush border membrane (Ellestad *et al*. 2002, 2003; Roy *et al*. 2008; Khan & Ghosh 2012; Kumar *et al*. 2012; Ray *et al*. 2012; Dan & Ray 2014), supporting the results of our study. Although only one study has determined the potential of phytate hydrolysis by fish endogenous phytase (around 50% in hybrid tilapia), there are compelling evidences that this may occur in other fish species and need to be further explored.

The high P digestibility associated with the growth data supports the hypothesis from ecology studies that some species may have adapted to low P intake and may be more susceptible to a growth-depressing effect of excess P. This may be the case of tambaqui, once seeds and other fibrous plants, and zooplankton compose the natural diet of wild tambaqui, leading the species to develop a gut-associated microbiota able to use phytic-P. However, this needs to be further studied to prove our hypothesis. Additionally, tambaqui seems to require low AP levels for growth (around 3.0 g kg^{-1} diet) and 7.0 g kg^{-1} for adequate bone mineralization.

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