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Different dietary protein levels for *Podocnemis unifilis* subadult farming: hematological and biochemical assessment

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ABSTRACT

This study evaluated the physiological responses of subadult yellow-spotted Amazon river turtle, *Podocnemis unifilis*, to incremental crude protein levels (29%, 32%, 35%, and 39% CP) as extruded commercial feeds. The hematological and plasma biochemical parameters of *P. unifilis* were analyzed at 60, 120, and 180 days of the feeding trial. The increase in total thrombocyte, leukocyte, lymphocyte, heterophil, and eosinophil numbers, besides high respiratory burst activity showed an improvement in animal immune defense response to incremental protein in diets, acting together to maintain the integrity of the tissues against antigens and infectious agents. Elevated subadult *P. unifilis* plasma total cholesterol and triglyceride levels observed after increasing the feeding time (until 180 days) with incremented protein levels in artificial diets during captivity are a nutritional warning, related to the condition of being less physically active. Based on these results, periodic physiological evaluations are particularly important to ensure the healthy and adequate nutritional conditions of captive-bred animals, such as maintaining lower stocking densities of animals to avoid stress and, if possible, providing them with fresh food in addition to extruded artificial feed.

KEYWORDS

Leukocytes; glucose; triglyceride; cholesterol; health status

Introduction

Podocnemis unifilis (Tröschel 1848), the yellow-spotted Amazon river turtle, is traditionally and widely used in Amazonian gastronomy, especially for its meat and eggs, and is a staple food for riverine populations (Duarte, Costa, and Andrade 2008). As a result, wild populations of the species are illegally and intensely exploited (Almeida and Abe 2009; Araújo et al. 2013a) and considered vulnerable in the IUCN Red List of Threatened Species (IUCN 2016).

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Podocnemis unifilis farming is regulated in Brazil, along with that of three other Amazonian freshwater species, *Podocnemis expansa, Podocnemis sextuberculata* (Schweigger, 1812), and *Kinosternon scorpioides* (Linnaeus, 1766) (IBAMA 2015).

Studies on feeding behavior, nutritional needs in captivity and the effects of feed on turtle health and productivity are essential for the development of turtle farming. However, the nutritional needs of *Podocnemis* species, such as suitable feed levels and composition for these animals in captivity (Duncan and Marcon 2009; Malvasio et al. 2003; Sá et al. 2004) remain poorly understood (Araújo, Palha, and Rosa 2013b; Portal et al. 2002; Fachín-Terán, Vogt, and Gomez 1995; Lara et al. 2012; Yoshioka et al. 2017, 2015). Currently, commercial balanced fish feed is the main source of protein and nutrients used for captive-bred *P. unifilis* (Almeida and Abe 2009), and levels of 28% to 30% crude protein are considered suitable for these turtles in captivity (Portal et al. 2002). Likewise, *P. expansa* hatchlings fed isocaloric plant diets with crude protein levels from 18% to 30% exhibited the best growth and weight gain with levels above 27% (Sá et al. 2004). For *P. unifilis* hatchlings, 36% of crude protein in feed has been recommended, based on feed tests with 28–55% crude protein levels and the response of selected physiological parameters (hematology and plasma biochemistry variables) (Yoshioka et al. 2017, 2015).

Hematological and biochemical parameters and peripheral blood cell counts are important and commonly used methods to diagnose fish and chelonian health conditions (Fonseca et al. 2016; Marcon et al. 2008; Santos et al. 2005; Tavares-Dias et al. 2009), including those of *P. unifilis* (Duncan and Marcon 2009; Oliveira et al. 2011; Yoshioka et al. 2017, 2015). The environment and nutritional quality of food provided to animals in captivity can cause changes in plasma constituents, such as total cholesterol and triglycerides increased levels with increment of proteins levels in diets, which can be observed through blood biochemical analysis (Campbell 2015a; Marcon et al. 2008; Stacy, Alleman, and Sayler 2010; Yoshioka et al. 2017, 2015). The hematological and biochemical profile of turtles from the genus *Podocnemis* varies according to their nutritional condition (Marcon et al. 2008) and age (hatchlings, juvenile or adult turtles) (Tavares-Dias et al. 2012; Yoshioka et al. 2017, 2015). Thus, to understand how this happen overtime, the present study aimed to evaluate subadult *P. unifilis* hematological and biochemical responses to incremental levels of crude protein when feeding them with fish commercial ration over 180 days in captivity.

Materials and methods

The present study was authorized by the Embrapa Amapá Ethics Committee (CEUA) for the use of animals, protocol nr. 006/2016 and was registered in the Brazilian national system for the management of genetic heritage and associated traditional knowledge (SisGen), under number A237945.

Experimental protocol

Yellow-spotted Amazon river turtle *P. unifilis* (n = 120), aged approximately four years old, with body weights of 584.7 ± 49.6 g and carapace lengths of 162.3 ± 9.8 cm (mean ± SD), from the Embrapa Amapá vivarium were fed with different crude protein levels during 60, 120, and 180 days in captivity. They were randomly separated into 500-L experimental polyethylene tanks, containing ten animals each. The tanks were adapted to allow the animals access to both dry and wet areas. The wet areas received 300 L of water, thus maintaining a density of 33 individuals *per* cubic meter of water tank volume (Andrade et al. 2008). The tanks were cleaned, and the water was changed every 2 days. The animals were acclimatized for 10 days, before the experimental period, which began in February 2017.

Based on previous studies (Yoshioka et al. 2017, 2015) which concluded that 36% CP was the recommended level for *P. unifilis* hatchlings, a range of test levels of 28–40% CP was determined for the present study. Extruded commercial fish feed (Guabi Nutrição e Saúde Animal S.A., Campinas, São Paulo, Brazil) with 28%, 32%, 36%, and 40% crude protein (CP), as declared by the manufacturers, was provided. All the feed was submitted to bromatological analysis, they were ground up using a portable analytical mill (IKA, model A11, Staufen, Germany) and sent to the Food Analysis Laboratory of Embrapa Amapá. Crude protein was determined using the Kjeldahl method; ether extract was determined using a Soxhlet extractor (Tecnal, Piracicaba, SP, Brazil) with petroleum ether under reflux; dry matter was determined using the gravimetric method in an oven at 105°C until constant weight was reached; ash content was determined using the gravimetric method in a muffle furnace at 105°C for four hours; and phosphorus was determined using complexometry, with absorbance measured with a spectrophotometer (Femto, model CIRRUS 80MB, São Paulo, SP, Brazil). All the values were expressed as percentages and followed Silva and Queiroz (2002), Souza and Nogueira (2005), Instituto Adolfo Lutz (2008) and Gomes and Oliveira (2011). The bromatological analysis in the commercial feeds resulted in lower crude protein values than those described by the manufacturer, especially in samples of the diets containing 36–40% CP and the treatment levels considered in the present study were CP29, CP32, CP35, and CP39 (Table 1).

Table 1. Basal composition of four extruded commercial feeds (for fish) with 28%, 32%, 36%, and 40% CP (as informed by the manufacturer), and 29%, 32%, 35%, and 39% of CP, respectively, according to the bromatological analysis (values are expressed as mean ± standard deviation, n = 3 of each feed). CP = crude protein.

	28% CP	32% CP	36% CP	40% CP
	Manufacturer data			
Crude protein (%)	28	32	36	40
Ether extract (%)	5.0	6.5	6.5	8.0
Fiber (%)	7.0	7.0	6.0	6.0
Ash (%)	14.0	14.0	14.0	12.0
Phosphorus (%)	0.6	0.6	0.6	0.8
Calcium (%)	3.5	3.5	3.5	1.6
	Bromatological analysis			
Crude protein (%)	29.60 ± 0.44	32.04 ± 0.44	35.22 ± 0.48	39.31 ± 0.66
Ether extract (%)	4.37 ± 0.66	6.21 ± 0.28	4.10 ± 0.45	4.84 ± 0.10
Dry matter (%)	91.25 ± 0.19	91.99 ± 0.08	90.70 ± 0.26	91.31 ± 0.13
Ash (%)	13.35 ± 0.19	14.89 ± 0.17	10.81 ± 0.11	11.17 ± 0.04
Phosphorus (%)	0.63 ± 0.03	0.90 ± 0.02	0.62 ± 0.01	0.80 ± 0.02
Calcium (%)	0.69 ± 0.13	1.24 ± 0.05	2.07 ± 0.52	1.21 ± 0.16

Each treatment level had three replicates (each one containing 10 animals). Yellow-spotted Amazon river turtles were fed an equivalent of 3% of turtle biomass in each tank on a daily basis. Five animals from each experimental tank were used in physiological evaluations at 60, 120, and 180 days of the feeding trial (n = 15 animals per treatment level). Each animal was identified according to the methodology described by Yoshioka et al. (2015), to avoid the repetition of animals in sequential evaluations.

Hematological analysis

Blood samples were collected by caudal vessel puncture using disposable syringes and needles, with sodium heparin (5.000 UI/mL) used as an anticoagulant. Hematocrit (Ht), total hemoglobin concentration (Hb), and red blood cell count (RBC) were determined and the hematimetric indices calculated (Oliveira-Júnior, Tavares-Dias, and Marcon 2009; Ranzani-Paiva et al. 2013). Blood smears were stained with a combination of May Grünwald-Giemsa-Wright for the morphological characterization of the cells (Oliveira et al. 2011). Total leukocyte, thrombocyte, and differential leukocyte counts were obtained by an indirect method (Ishikawa et al. 2008).

The leukocyte respiratory activity (burst) assay was carried out following the Biller-Takahashi et al. (2013) protocol. This method consists of a colorimetric determination of the reactive oxygen species (ROS) produced by respiratory burst of the leukocytes, with the reduction of nitroblue tetrazolium (NBT, Sigma, St. Louis, MO, USA) into dark blue precipitate inside the phagocyte, known as formazan granules.

The supernatant optical density (OD) was determined on a spectrophotometer (Biospectro, model SP-220, Curitiba, PR, Brazil) at 540 nm. The remaining blood samples were centrifuged at 10,000 rpm (Centrifuge 5424, Eppendorf, Hamburg, Germany) for 5 min to obtain the plasma. Total protein, albumin, glucose, total cholesterol, and triglyceride plasma concentrations were determined with specific colorimetric kits (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil) and absorbance readings in a spectrophotometer (Biospectro, model SP-220, Curitiba, PR, Brazil).

Statistical analysis

For statistical evaluation of the effect of crude protein level and feeding time, yellow-spotted Amazon river turtle data were submitted to a normality test (Kolmogorov-Smirnov) and homogeneity of variance test (Bartlett). The parametric data were submitted to one-way ANOVA and Tukey's post-hoc and the non-parametric data (without any transformation) were subjected to Kruskal-Wallis analysis and Dunn's post-hoc. Differences were considered significant at 5% of probability (Zar 2010). The tests were performed with the GraphPad InStat (Version 3.00, 1997) statistical software.

Results

No significant differences were found in hematocrit, hemoglobin, and RBC values due to the variation of protein levels in the diet of *P. unifilis* when fed for 60 days (as shown in Table 2). In this feeding period, however, MCV and MCHC presented significant reductions with feed with 32% CP and MCV and MCHC were higher at CP39. Differences were not found in RBC and MCV values ($P > .05$) when *P. unifilis* were fed for 120 days, however Hb and CHCM were low at CP32 and Ht at CP35. After 180 days of feeding trial, Hb and MCHC presented higher values when fed with CP29; and Ht and RBC were higher at CP32, without significative difference for MCV.

Higher respiratory burst activity values ($P < .05$) were observed for *P. unifilis* at CP29 after 60 days, and at CP32 and CP35 after 120 days of feeding, and the lowest was observed at CP39 after 180 days (Table 3). Thrombocyte numbers were higher with elevated CP levels at all the feeding times. Total leukocyte numbers were higher in yellow-spotted Amazon river turtles fed diets with high protein levels (CP39 at 120 days and CP35 and CP39 at 180 days). Significant higher lymphocyte numbers were observed with CP32 at 60 days, and when fed with CP39 for 120 and 180 days. The highest azurophil number was observed with low CP diet after 180 days. Basophil numbers were significantly higher in *P. unifilis* fed diets with the highest CP levels at 120 and 180 days. Heterophil numbers showed great variation during

Table 2. Hematological parameters and hematimetric indices (mean ± standard deviation, n = 12) of *Podocnemis unifilis* subadults fed different crude protein diets (CP29, CP32, CP35, and CP39), at 60, 120, and 180 days.

Treatments	CP29	CP32	CP35	CP39
		60-day measurements		
Ht (%)	23.57 ± 2.11aA	24.07 ± 3.10aA	24.16 ± 3.01aA	24.00 ± 2.66aA
Hb (g/dL)	7.89 ± 1.93aA	6.43 ± 0.95aA	6.75 ± 1.68aA	7.70 ± 1.00aA
RBC (10^3/µL)	161.67 ± 36.58aA	195.00 ± 47.88aA	166.07 ± 36.44aA	157.50 ± 34.63aB
MCV (fL)	1544.37 ± 443.85abA	1221.07 ± 263.13 bA	1497.70 ± 213.92abA	1625.20 ± 382.23aA
MCHC (g/dL)	33.96 ± 9.48abA	26.84 ± 3.46bAB	28.56 ± 3.89abAB	32.19 ± 4.00aA
		120-day measurements		
Ht (%)	24.07 ± 2.75abA	27.83 ± 5.74aA	22.29 ± 4.18 bA	24.83 ± 4.45abA
Hb (g/dL)	7.40 ± 1.08abA	6.81 ± 1.07 bA	7.42 ± 1.79abA	8.77 ± 1.97aA
RBC (10^3/µL)	185.36 ± 35.49aA	192.14 ± 42.05aA	160.00 ± 35.63aA	201.33 ± 48.53aAB
MCV (fL)	1358.17 ± 216.90aA	1467.08 ± 268.19aA	1429.70 ± 251.77aA	1299.16 ± 193.17aB
MCHC (g/dL)	30.43 ± 1.93aA	24.80 ± 2.80bB	33.90 ± 6.74aA	35.64 ± 7.18aA
		180-day measurements		
Ht (%)	25.07 ± 2.85abA	26.50 ± 2.08aA	22.21 ± 1.72cA	23.64 ± 3.09bcA
Hb (g/dL)	8.51 ± 1.14aA	7.53 ± 1.45abA	6.13 ± 0.87cA	7.21 ± 0.91bcA
RBC (10^3/µL)	213.89 ± 51.77abA	228.93 ± 40.72aA	182.67 ± 44.19 bA	227.50 ± 43.88abA
MCV (fL)	1227.68 ± 300.16aA	1191.67 ± 283.92aA	1256.46 ± 280.81aA	1141.30 ± 208.49aB
MCHC (g/dL)	34.01 ± 3.25aA	28.88 ± 4.07 bA	28.05 ± 3.30bB	30.50 ± 2.22 bA

Hematocrit (Ht), hemoglobin concentration (Hb), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC). Different lower-case letters on the same line indicate a statistically significant difference in each measurement period ($P < 0.05$, Tukey-Kramer test or Dunn's test). Different upper-case letters in the same column for the same parameter indicate a statistically significant difference between periods of feeding within the same treatment ($p < 0.05$, Tukey-Kramer test).

feeding trial (see Table 3). Eosinophils numbers were higher when fed with CP29 and CP39 after120 day and 180 days; and the lowest number was observed at CP35.

Total plasma protein of *P. unifilis* was not affected ($P > .05$) with increments in dietary crude protein levels (Table 4), neither due to the feeding trial period. Albumin plasma levels presented lower value at CP29 and higher at CP39 fed for 60 days; and these levels were higher at CP35 after 120 days and at CP29 after 180 days of feeding. The feeding time promoted reduction of albumin levels at CP32, CP35, and CP39 and an increasing on CP29. The glucose plasma levels of *P. unifilis* were lower at CP29 fed for 60 and 180 days, and higher levels with CP32 and CP35 fed for 60 days, and with CP32 and CP39 at 180 days. However, glucose levels were lower after 180 days when compared to 60 days of feeding. There was no direct correlation of feeding time and CP levels over glycemia. The significant higher total cholesterol plasma levels occurred in animals fed with elevated levels of protein in their diets, although this relation (linear regression) was only about 37%. Triglyceride plasma levels were lower at CP29 after 60 days, and higher with CP39 at 60 days and when fed CP35 at 120 and 180 days of feeding, and there was no influence of feeding time, nor CP levels.

Table 3. Burst respiratory activity, total thrombocyte, total and differential leukocyte count (mean ± standard deviation, n = 12) of *Podocnemis unifilis* subadults fed different crude protein diets (CP29, CP32, CP35, and CP39), at 60, 120, and 180 days.

Treatments	CP29	CP32	CP35	CP39
60-day measurements				
Burst (OD)	0.62 ± 0.18aA	0.25 ± 0.04cB	0.30 ± 0.06bcB	0.35 ± 0.08abA
Thrombocytes (10^3/µL)	36.1 ± 9.24bB	47.99 ± 14.44 bA	33.59 ± 8.92bB	84.91 ± 16.23aA
Leukocytes (10^3/µL)	13.69 ± 3.88aA	13.33 ± 5.10aA	9.10 ± 3.71aAB	9.16 ± 2.67aB
Lymphocytes (10^3/µL)	0.42 ± 0.30 bA	1.49 ± 0.79aA	0.21 ± 0.12 bA	0.41 ± 0.24bB
Azurophils (10^3/µL)	0.12 ± 0.14aB	0.11 ± 0.14aA	0.09 ± 0.11aA	0.05 ± 0.06aA
Heterophils (10^3/µL)	11.25 ± 3.51aA	9.90 ± 3.88abA	7.62 ± 2.90abAB	6.95 ± 2.28bB
Eosinophils (10^3/µL)	0.65 ± 0.24aA	0.48 ± 0.27abA	0.34 ± 0.16 bA	0.51 ± 0.25abA
Basophils (10^3/µL)	1.24 ± 0.77aA	1.36 ± 0.93aA	0.84 ± 0.87aA	1.23 ± 0.83aAB
120-day measurements				
Burst (OD)	0.45 ± 0.07 bA	0.62 ± 0.10aA	0.61 ± 0.06aA	0.38 ± 0.06 bA
Thrombocytes (10^3/µL)	63.48 ± 25.28abA	57.57 ± 16.72abA	41.91 ± 13.88bB	78.29 ± 19.64aA
Leukocytes (10^3/µL)	10.22 ± 2.36bAB	10.25 ± 3.90 bA	6.36 ± 3.66bB	15.27 ± 3.83aAB
Lymphocytes (10^3/µL)	0.25 ± 0.14aA	0.29 ± 0.12aB	0.18 ± 0.30aA	0.84 ± 0.84aAB
Azurophils (10^3/µL)	0.05 ± 0.06aB	0.02 ± 0.04aA	0.02 ± 0.04aA	0.12 ± 0.14aA
Heterophils (10^3/µL)	8.67 ± 2.09aAB	8.63 ± 3.05aA	5.24 ± 2.83bB	10.85 ± 2.55aAB
Eosinophils (10^3/µL)	0.22 ± 0.09abA	0.13 ± 0.19 bA	0.24 ± 0.25abA	0.83 ± 0.63aA
Basophils (10^3/µL)	1.03 ± 0.43abA	1.18 ± 0.79abA	0.60 ± 0.35 bA	2.64 ± 2.47aA
180-day measurements				
Burst (OD)	0.29 ± 0.06aC	0.28 ± 0.07aB	0.27 ± 0.06aB	0.13 ± 0.03bB
Thrombocytes (10^3/µL)	82.49 ± 31.18abA	58.75 ± 34.74 bA	105.55 ± 45.87aA	117.25 ± 31.40aA
Leukocytes (10^3/µL)	7.29 ± 2.77bB	11.35 ± 5.04abA	12.85 ± 4.57aA	16.84 ± 6.44aA
Lymphocytes (10^3/µL)	0.30 ± 0.31 bA	0.44 ± 0.36abB	0.64 ± 0.36aA	1.73 ± 1.8aA
Azurophils (10^3/µL)	0.55 ± 0.55aA	0.02 ± 0.05 bA	0.01 ± 0.03 bA	0.07 ± 0.16 bA
Heterophils (10^3/µL)	5.75 ± 2.23bB	9.30 ± 4.37abA	11.01 ± 4.07aA	12.99 ± 4.60aA
Eosinophils (10^3/µL)	0.49 ± 0.35aA	0.15 ± 0.30cA	0.12 ± 0.15bcA	0.93 ± 1.23abA
Basophils (10^3/µL)	0.68 ± 0.39 bA	1.44 ± 0.59aA	1.07 ± 0.62abA	1.12 ± 0.69abB

Different lower-case letters on the same line indicate a statistically significant difference in each measurement period ($P < 0.05$, Tukey-Kramer test or Dunn's test). Different upper-case letters in the same column for the same parameter indicate a statistically significant difference between periods of feeding within the same treatment ($p < 0.05$, Tukey-Kramer test).

Discussion

Farming with inadequate nutritional conditions can result in the impairment of essential blood parameters, such as protein and iron, and cause health disorders (Paranzini, Teixeira, and Trapp 2008; Tavares-Dias et al. 2009). Malnourished *P. expansa* exhibited excessively low hemoglobin concentrations, impairing the health of these animals (Tavares-Dias et al. 2009). Moreover, diagnosis over long period of captivity, such as 180 days or more, shows the importance of adequate diets for animals kept in captivity (Morselli et al. 2016; Paranzini, Teixeira, and Trapp 2008; Stacy, Alleman, and Sayler 2010). When CP39 where fed for 120 and 180 days, a reduction in MCV can be observed in relation to 60 days and this could be associated with electrolyte imbalance of animals (Marcon et al. 2008; Paranzini, Teixeira, and Trapp 2008), probably due to the use of a fish ration to feed them, without a specific one to chelonians nutritional requirements. Under natural conditions, 90% of the food consists of vegetables (CENAQUA 2000), thereby

Table 4. Plasma metabolite profile (mean ± standard deviation, n = 12) of *Podocnemis unifilis* subadults fed different crude protein diets (CP29, CP32, CP35, and CP39), at 60, 120, and 180 days.

Treatments	CP29	CP32	CP35	CP39
60-day measurements				
Total Protein (g/dL)	3.50 ± 0.71aA	3.56 ± 0.48aA	3.45 ± 0.66aA	3.43 ± 0.91aA
Albumin (g/dL)	2.46 ± 0.45cB	3.46 ± 0.88abA	3.06 ± 0.54bcA	3.94 ± 0.88aA
Glucose (mg/dL)	44.49 ± 7.71 bA	65.85 ± 16.83aA	69.60 ± 17.16aA	60.19 ± 15.27abA
Cholesterol (mg/dL)	29.45 ± 7.60bB	75.44 ± 18.98aA	85.25 ± 21.49aA	88.86 ± 20.46aB
Triglyceride (mg/dL)	56.45 ± 17.31 bA	75.75 ± 24.26abA	68.76 ± 33.53abB	97.30 ± 40.18aA
120-day measurements				
Total Protein (g/dL)	2.96 ± 0.51aAB	3.18 ± 0.55aAB	2.82 ± 0.66aA	3.39 ± 0.76aAB
Albumin (g/dL)	2.87 ± 0.63abB	2.73 ± 0.46abAB	3.14 ± 0.65aA	2.35 ± 0.52bB
Glucose (mg/dL)	43.71 ± 8.96aA	38.87 ± 7.73aB	35.16 ± 6.85aB	40.52 ± 9.35aB
Cholesterol (mg/dL)	57.96 ± 12.89 bA	61.50 ± 10.89 bA	81.01 ± 18.62aA	81.37 ± 14.97aB
Triglyceride (mg/dL)	42.41 ± 11.58abA	35.50 ± 9.93bB	69.52 ± 30.77aB	51.17 ± 24.04abB
180-day measurements				
Total Protein (g/dL)	2.22 ± 0.76aB	2.38 ± 0.80aB	2.63 ± 0.37aA	2.49 ± 0.52aB
Albumin (g/dL)	3.60 ± 0.77aA	2.33 ± 0.60bB	2.03 ± 0.60bB	1.53 ± 0.61bB
Glucose (mg/dL)	26.67 ± 6.59bB	42.81 ± 10.09aB	33.29 ± 8.62abB	44.88 ± 14.22aB
Cholesterol (mg/dL)	49.60 ± 24.34cAB	80.17 ± 15.71 bA	62.35 ± 19.50bcA	139.52 ± 21.74aA
Triglyceride (mg/dL)	49.78 ± 34.64 bA	20.73 ± 13.42bB	147.15 ± 21.34aA	47.49 ± 25.92bB

Different lower-case letters on the same line indicate a statistically significant difference in each measurement period ($P < 0.05$, Tukey-Kramer test or Dunn's test). Different upper-case letters in the same column for the same parameter indicate a statistically significant difference between periods of feeding within the same treatment ($p < 0.05$, Tukey-Kramer test).

during captivity to feed subadult *P. unifilis* in addition to artificial fish feed, it could be provided some fresh food, as *Commelina longicaulis, Polyganum acuminatum, Aeschymene sensitiva, Macrolobium aeaciaefolium* and others, due to their protein, lipid, fibers, and mineral (calcium, phosphorus, potassium, and magnesium) composition (Portal et al. 2002).

The high respiratory burst activity observed in some *P. unifilis* from the present study could be a mechanism for restoring immunological balance, since respiratory burst increases frequently associated with incremental phagocytosis activity due to leukocyte action (Biller-Takahashi et al. 2013). At all the feeding times evaluated, *P. unifilis* which received elevated CP level diets showed higher thrombocyte number; these cells have phagocytic capabilities and are involved in hemostasis and wound healing (Stacy, Alleman, and Sayler 2010). Total leukocyte numbers were higher in yellow-spotted Amazon river turtles fed diets with high protein levels. The increased numbers of total leukocytes, lymphocytes, heterophils, and eosinophils indicate an improvement in animal immune defense response (Dias et al. 2019). The highest azurophil numbers were observed with low CP in the diet after 180 days and basophil numbers were significantly higher in *P. unifilis* that received diets with the highest CP levels at 120 and 180 days. All these different types of leukocytes act together to

maintain the integrity of the tissues against antigens and infectious agents and are responsible for the immunological balance of these animals (Marcon et al. 2008).

The environment and nutritional quality of food provided to animals in captivity may cause changes in plasma constituents (Marcon et al. 2008; Rangel-Mendoza et al. 2009). Interestingly, the total plasma protein of *P. unifilis* was not affected with increments in dietary crude protein levels. In healthy reptiles, plasma protein levels generally range between 3 and 7 g dL^{-1} (Campbell 2015b), as reported for captive bred subadult *P. unifilis* in the present study. Total plasma protein concentration can indicate the nutritional condition of the animal (Morselli et al. 2016), since the environment and nutritional quality of food provided to animals in captivity can be observed through blood biochemical analysis (Marcon et al. 2008; Rangel-Mendoza et al. 2009; Yoshioka et al. 2017). Albumin plasma levels were low with low diet CP and higher with high CP level fed after feeding for 60 days, and at CP35 fed for 120 days and C29 after 180 days of feeding, which coincides with increased feeding period, even when *P. unifilis* were fed with diets with low protein levels. Glucose plasma levels of *P. unifilis* were lower after 180 days when compared to 60 days analysis, without any relation of feeding time and CP levels over glycemia. Reduced blood glucose has been related to malnutrition in some reptiles (Campbell 2015b; Tavares-Dias et al. 2009). High glycemia can be a consequence of stressful condition in husbandry practices in captivity and due to the use of artificial diets (Oliveira-Júnior, Tavares-Dias, and Marcon 2009; Rangel-Mendoza et al. 2009); however, these were not observed in the present study (even Ht and Hb values did not show increased values), since animals from the present study was maintaining in lower stocking densities.

The higher total cholesterol plasma levels occurred in animals fed with elevated levels of protein in their diets, probably due to the diary feed, since food availability determines the amount of lipids that can be stored and used by the body (Derickson 1976). Oliveira-Júnior, Tavares-Dias, and Marcon (2009) reported that farmed turtles exhibited high plasma total cholesterol concentrations due to artificial diets and for being less physically active than wild animals. Elevated plasma total cholesterol and triglyceride levels were therefore exhibited by subadult *P. unifilis*, mainly when they received elevated protein levels (CP35 and CP39) diets. In contrast, according to Tavares-Dias et al. (2012), wild juvenile and adult *P. unifilis* triglyceride levels were higher than those bred in captivity. Nevertheless, the triglyceride levels of captive bred hatchlings were higher (Yoshioka et al. 2017) than the subadult animals from the present study, even fed with diets with elevated CP levels.

Conclusion

The elevated plasma total cholesterol and triglyceride levels presented by subadult *P. unifilis* fed artificial diets with incremented protein levels in captivity is a nutritional warning. However, an improvement in Yellow-spotted Amazon river turtle immune defense response was observed due to the increased total leukocyte, lymphocyte, heterophil, and eosinophil numbers. Therefore, commercial extruded fish feed was the main protein and nutrient source for *P. unifilis* when in captivity and to achieve adequate nutritional conditions and maintain animal health, it is particularly important to perform periodic physiological evaluations of these animals. In addition, alterations in husbandry practices are recommended and applied, such as maintaining lower animal stocking densities and, if possible, provide them with fresh food in addition to extruded artificial fish feed.

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