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Comparative hematological and biochemical analysis of giant turtles from the Amazon farmed in poor and normal nutritional conditions

Marcos Tavares-Dias[1]*, Antonio A. Oliveira-Junior[2], Michele G. Silva[2], Jaydione L. Marcon[2], and José F. M. Barcellos[3]

[1]*Empresa Brasileira de Pesquisa Agropecuária (Embrapa Amapá), Rodovia Juscelino Kubitschek, Macapá, AP, Brazil*

[2]*Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Amazonas (UFAM). Avenida Gal. Rodrigo Octávio Jordão Ramos, Manaus, AM, Brazil*

[3]*Departmento de Morfologia, Universidade Federal do Amazonas (UFAM). Avenida. Gal. Rodrigo Octávio Jordão Ramos, Manaus, AM, Brazil*

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ABSTRACT

Besides indicating the baseline values of the species, blood parameter assessments of chelonian may also be used as quick tools for diagnosing health status. An investigation was carried out to assess and compare red blood cells parameters, white blood cell (WBC) and total thrombocyte counts and certain blood biochemical parameters for freshwater turtles, *Podocnemis expansa* Schweigger, 1812 (Pelomedusidae) reared on normal (control group) and poor nutrition (malnourished group). For the malnourished turtles, a significant decrease ($P<0.05$) in red blood cell counts was found, including hematocrit, plasma glucose, plasma total protein, cholesterol and urea levels, as well as WBC counts, azurophils and heterophils. Malnutrition did not alter the hemostasis, but caused severe normocytic-hypocromic anemia and marked immune depression, which were diagnosed here. This study was the first to characterize the physiological and immune status of giant turtles from the Amazon under adverse nutritional conditions.

Key words: freshwater turtle, anemia, blood, leukocytes, malnutrition, *Podocnemis expansa*

Introduction

The exploitation of South American rivers as a food source has long been considered the main contributing factor to the decline of the natural populations of giant turtles from the Amazon. Due to the risk of extinction, the turtle was protected by Brazilian federal

*Corresponding author:

Dr. Marcos Tavares-Dias, Embrapa Amapá, Rodovia Juscelino Kubitschek, Km 5, N° 2600, Caixa Postal 10, 68903- 419, Macapá-AP, Brasil, Phone: +55 96 4009 9550; Phone/Fax: +55 96 4009 9501; marcostavares@cpafap.embrapa.br; mtavaresdias@pq.cnpq.br

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law and its farming was then introduced by environmental authorities in the State of Amazonas in 1999, in an attempt to decrease the capture of the natural populations. In 2004, there were estimated to be 92 turtle farms in Brazil, either for commercial purposes or conservation of the species, for a total of 880,000 animals (SÁ et al., 2004). However, in the Amazon, the implementation of conservation strategies involving sustainable use requires taking into account both biological and socio-economic factors.

Blood hematological and biochemical profiles are useful tools in measuring the physiological status of turtles because they may provide information for diagnosis and prognosis of diseases (CHRISTOPHER et al., 2003; WHITING et al., 2007; OLIVEIRA-JÚNIOR et al., 2009). Consequently, in some turtle species, such tools have been used as physiological disturbance indicators of diseases (SWIMMER, 2000; CHRISTOPHER et al., 2003; KNOTKOVÁ et al., 2005; WHITING et al., 2007), stress (KNOTKOVÁ et al., 2005) or exposition to contaminants (LUTCAVAGE et al., 1995; KELLER et al., 2004), as well as to assess degrees of dehydration (PETERSON, 2002; CHRISTOPHER et al., 2003). Sick turtles are often anemic and hypoproteinemic, thus serial hematocrit and plasma total protein determinations help to determine the status of the individual and the most appropriate therapeutic regimen (CAMPBELL, 1998; NORTON, 2005).

Blood parameters for diagnostic purposes of poor nutrition have been little used despite their potential, because of the difficulties associated with estimation of nutritional intake and the animals' living conditions in nature. FRAIR (1977) found low hematocrit values for malnourished turtles. For free-ranging desert tortoises (*Gopherus agassizii*), a decrease was also reported in hematocrit caused by chronic malnutrition in animals that were trapped for 11 months without food (CHRISTOPHER, 1999). For *Chelonia mydas* reared in laboratory conditions, starvation reduced glucose and plasma non-protein nitrogen levels, which was mainly constituted of urea (BONNET, 1979). For *Phrynops hilarii*, starvation did not alter plasma glucose levels; however, it induced a marked decrease in the synthesis of metabolic reserves (SILVA and MIGLIORINI, 1990). Despite all the research, knowledge of the giant turtle from the Amazon, as an important South American freshwater species, is still limited.

Ideally, a unique set of normal reference values should be obtained for the health of a group of turtles under a given set of environmental and nutritional parameters for comparison when these animals become sick (CAMPBELL, 1998) or malnourished. Therefore, the purpose of this study was to compare the red blood cell parameters, as well as thrombocyte and leukocyte counts and some blood biochemistry variables for *P. expansa* farmed in poor and normal nutritional conditions.

Materials and methods

Animals and rearing characteristics. The freshwater turtles from the Amazon (*P. expansa*) used in this study, were categorized into two major groups: a control group (n = 28) and a malnourished group (n = 25). The control group, obtained from one turtle farm, localized in the municipality of Manaus, was composed of turtles of both sexes that were kept in a pond (15 m × 31 m) together with fish (*Colossoma macropomum*, *Arapaima gigas* and *Oreochomis niloticus*). Control turtles were fed daily with aquatic macrophites (*Pistia stratiotes* e *Azolla* sp.) in addition to extruded fish meal, containing 34.0% crude protein. Wild *P. expansa* were fed basically on fruits, seeds, roots and stalks of aquatic macrophites from flood plains (TÉRAN et al., 1995). All these turtles were clinically healthy. The malnourished group of turtles was obtained from a pond (measuring 20 x 30 m) on a farm near the municipality of Iranduba, also in the State of Amazonas, Brazil. These animals were fed with cassava only, but once a week also with 27.0% extruded fish meal and vegetable remains.

Blood collection procedure and hematological assessment. Blood samples were drawn from the femoral vein using syringes containing heparin (2.500 UI) and divided into two aliquots. The first aliquot was stored on ice and used for determination of red blood cell counts (RBCC), hematocrit (Hct), hemoglobin concentration (Hb), mean cell volume (MCV) and cell hemoglobin concentration (MCHC) according to previous recommendations (OLIVEIRA-JÚNIOR et al., 2009). Blood smears were stained with a combination of May Grunwald-Giemsa-Wright (TAVARES-DIAS and MORAES, 2003) and used for differential white blood cell (WBC) counts. Counts of total WBC and total thrombocytes used methods described by TAVARES-DIAS et al. (2008). The identification and nomenclature of leukocytes was according to OLIVEIRA-JÚNIOR et al. (2009).

The second blood sample was immediately centrifuged at 750 g to separate the plasma, which was collected and frozen at -70 °C until required. Plasma glucose was determined by the glucose oxidase method, while plasma urea, triglycerides and cholesterol concentration was determined by enzymatic-colorimetric method using commercial kits (Doles®, GO, Brazil). The total plasma protein level was determined by biuret reaction. All analyses were performed using an automated chemical system regularly monitored for accuracy and precision.

After collecting their blood, the turtles were measured to obtain the total body mass, straight carapace length (SCL) and length of the plastron (LP), and then each individual's sex was determined using dimorphic characteristics, using the method described by (MOLINA and ROCHA, 1996). In addition, clinical examination using non-lethal macroscopic methods was performed (Norton, 2005), and the turtles reared in poor nutrition feeding conditions were apparently healthy.

Statistical analysis. The mean and standard deviation were calculated for each group of turtles. The control and malnourished group were compared using the Student's *t*-test and results were assumed to be significant at $P<0.05$.

Results

There was no difference in body size between the two groups ($P<0.05$), Fig. 1 and Table 1. There was no difference between sexes in the turtle groups.

A significant ($P<0.05$) decrease of the red blood cell count values, hematocrit, hemoglobin, MCHC, total protein, glucose, total cholesterol and urea values was observed in malnourished *P. expansa* when compared to controls on adequate feeding conditions. The malnourished turtles were severely anemic, presenting with normocytic-hipocromic anemia (Table 1).

Table 1. Comparison of biometric data, red blood cells and biochemical parameters for *P. expansa* control and malnourished groups. Values among parentheses are ranges. Different letters in the same line indicate significant differences ($P<0.05$).

Parameters	Control Turtles	Malnourished Turtles
Body mass (kg)	4.3 ± 3.3^a (0.5-12.8)	4.7 ± 3.3^a (1.0-12.5)
SCL (cm)	26.5 ± 8.0^a (15.0-41.0)	25.1 ± 5.0^a (17.6-36.0)
LP (cm)	28.0 ± 9.0^a (16.0-45.5)	28.1 ± 6.9^a (18.8-43.0)
Total protein (g/dL)	3.5 ± 1.3^a (1.2-5.9)	1.6 ± 0.8^b (0.8-3.5)
Glucose (mg/dL)	91.3 ± 17.7^a (63.3-134.6)	66.8 ± 25.1^b (37.1-154.4)
Triglycerides (mg/dL)	35.0 ± 24.3^a (5.5-104.4)	29.8 ± 15.1^a (11.6-75.5))
Total cholesterol (mg/dL)	62.7 ± 24.3^a (22.2-123.1)	35.7 ± 15.0^b (17.7-73.0)
Urea (mg/dL)	3.7 ± 2.7^a (0.7-9.9)	2.1 ± 1.0^b (0.8-4.7)
RBCC ($\times 10^6/\mu L$)	0.280 ± 0.070^a (0.160-0.500)	0.220 ± 0.060^b (0.160-0.360)
Hematocrit (%)	25.1 ± 7.0^a (14.5-41.0)	18.4 ± 5.2^b (14.0-32.0)
Hemoglobin (g/dL)	6.5 ± 1.9^a (4.1-11.8)	1.9 ± 1.3^b (0.5-5.4)
MCV (fL)	922.0 ± 150.0^a (625.0-1250.0))	852.0 ± 72.0^a (687.5-1.000.0)
MCHC (g/dL)	26.2 ± 5.4^a (13.4-42.9)	10.0 ± 5.1^b (3.9-27.5)

SCL = straight carapace length; LP= length of the plastron

Table 2. Comparison of thrombocyte and leukocyte counts for *P. expansa* control and malnourished groups. Values in parentheses are the range. Different letters in the same line indicate significant differences (P<0.05).

Parameters	Control Turtles	Malnourished Turtles
Thrombocytes (μL)	4058.0 ± 1915.0[a] (2080.0-9900.0)	3809.0 ± 2072.0[a] (800.0-8280.0)
WBC (μL)	6701.0 ± 4048.0[a] (2480.0-23,680.0)	4616.0 ± 1916.0[b] (2040.0-8960.0)
Lymphocytes (μL)	1090.0 ± 962.0[a] (342.0-5446.0)	846.0 ± 554.0[a] (268.8-1983.8)
Azurophils (μL)	441.0 ± 344.0[a] (99.0-1658.0)	216.0 ± 137.0[b] (0-448.0)
Heterophils (μL)	3085.0 ± 1492.0[a] (1017.0-8525.0)	1706.0 ± 704.0[b] (612.0-3491.4)
Eosinophils (μL)	1527.0 ± 1039.0[a] (504.0-5683.0)	1609.0 ± 795.0[a] (739.2-)
Basophils (μL)	558.0 ± 485.0[a] (106.0-2368.0)	246.0 ± 144.0[b] (3794.4)

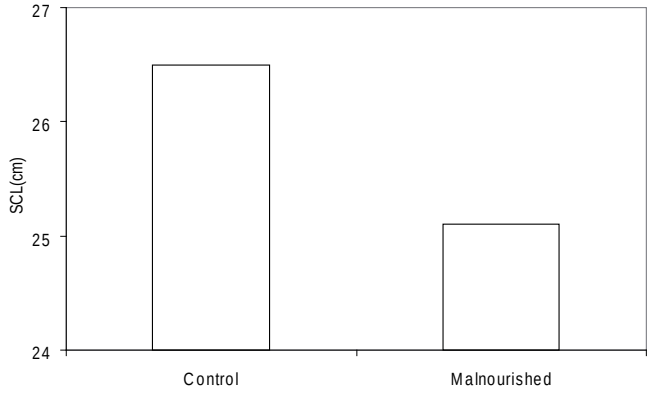

Fig. 1. Graphic representation of the SCL for *P. expansa* control and malnourished groups

For *P. expansa* from both groups, the thrombocyte counts were similar. However, the malnourished turtles had a significant (P<0.05) decrease in WBC, azurophils and heterophils counts when compared to the controls (Table 2), but in blood smears no toxic heterophil was seen.

Discussion

Blood parameter assessment and the ability to predict the subsequent health status are particularly important tools for monitoring the health and general condition of turtle individuals and populations (CAMPBELL, 1998; WHITING et al., 2007; OLIVEIRA-JÚNIOR et al., 2009). Blood glucose (LUTCAVAGE et al., 1995) and total protein levels (WHITING et al., 2007) have been related to the nutritional status and diet of turtles. In general, hypoglycemia is associated with starvation, malnutrition, severe liver disease, and, more commonly, septicemia (CAMPBELL, 1998). Hypoproteinemia occurs when there is an increase in the loss of proteins or when the body is unable to produce enough protein (SWIMMER, 2000) due to a lower crude protein intake. For *C. mydas*, starvation reduced glucose plasma levels (BONNET, 1979). For *P. expansa*, poor feeding conditions also caused a decrease in total protein and glucose levels. Total protein concentration was a good indicator of nutritional status because it only decreased due to a reduction in crude protein intake (WHITING et al., 2007). This low crude protein intake also led to a decrease in the plasma urea levels in malnourished *P. expansa*. Similarly, for *C. mydas*, starvation reduced plasma non-protein nitrogen levels, which was mainly constituted of urea (BONNET, 1979). Higher plasma urea levels have been attributed to an increased protein or increased protein turnover, either through a higher protein diet or from a breakdown of tissues (WHITING et al., 2007). However, the lower urea levels (2.1 mg/L) observed here were caused by a low protein diet in the feeding of *P. expansa*.

It has been stated that chelonians are extremely hardy animals and can be normally active even when they have severe anemia and hypoproteinemia (CHRISTOPHER et al., 2003; NORTON, 2005). In addition, this disturbance in lipid metabolism can be measured via changes in cholesterol, which is secreted from the liver in the form of bile acids (SWIMMER, 2000). Starvation did not alter plasma glucose levels for tortoises (*P. hilarii*); however, it caused a decrease in the synthesis of metabolic reserves (SILVA and MIGLIORINI, 1990). In the malnourished *P. expansa*, plasma triglyceride levels were not altered, yet decreased cholesterol levels were found. Lipids represent a biochemically efficient mechanism for storing energy to be used at a latter date for maintenance (CHRISTOPHER, 1999; DERICKSON, 1976). Food availability determines the quantity of lipids stored, when lipids are stored, and for what purposes these lipids are utilized (DERICKSON, 1976). In *P. expansa*, lower blood biochemistry values seem to indicate a reduced energy requirement because, despite decreased nutrient intake even for prolonged periods, this did not cause mortality in any animal. Therefore, it is reasonable to suppose that the turtles were conditioned to a reduced diet, which assured the minimum energy requirement necessary for their organic functions, and this condition is by far more ideal for good growth of animals in culture. Metabolic rate depression is an important survival strategy for many animal species under various conditions of prolonged food deprivation,

such as hibernation, torpor, and aestivation (STOREY and STOREY, 2004; MAKARIEVA et al., 2006). Nevertheless, animals kept under unsuitable feeding conditions must not be considered chelonian-culture, because improving the health protection of farmed turtles is one of the most important factors for higher production and economic progress in aquaculture.

In chelonian, red blood cell indices may be interpreted as a comparative index of condition, nutrition, or general health (CAMPBELL, 1998; PETERSON, 2002; WHITING et al., 2007; OLIVEIRA-JÚNIOR et al., 2009) because anemia is the common effect of chronically poor nutrition, particularly with respect to protein intake (CHRISTOPHER, 1999; PETERSON, 2002). Hematocrit levels less than 20.0% is suggestive of anemia (CAMPBELL, 1998). Therefore, it is clear that the poor feeding conditions of *P. expansa* caused severe anemia, with a decrease in values of red blood cell counts (0.220×10^6/μL), hematocrit (18.4%), hemoglobin (1.6 g/dL) and MCHC (10.0 g/dL). Similarly, in wild desert tortoises (*G. agassizii*), lower hematocrit levels caused by starvation when the animals were trapped for 11 months (CHRISTOPHER, 1999) was also reported. On the other hand, starvation for six weeks did not alter red blood cell counts, erythrocyte corpuscular constants, hematocrit, or hemoglobin concentration of *P. scripta elegans* (HIRSCHFELD and GORDON, 1965), probably due to short period used for the assay.

Nutritional support plays an important role in maintaining the optimum health condition of organisms by providing the building blocks of non-specific cellular and humoral immunity (SAURABH and SAHOO, 2008) and thus immune protection against diseases, in any organism, can be reduced by malnutrition (KELLER et al., 2004). Thereby, WBC assessments can be used as important indicators of the health status of turtles when suffering from any adverse condition and in many cases it may also be a helpful tool for evaluating the immunological system. *P. expansa* reared turtles showed lower WBC counts due to reduction of azurophils, heterophils and basophils numbers caused by poor nutritional conditions during culture. On the other hand, starvation did not alter leukocyte counts in *Pseudemys scripta elegans* (HIRSCHFELD and GORDON, 1965). *P. expansa* seems to be able to survive for prolonged periods on low feeding rates, under levels sufficient to support normal lymphocytes and eosinophils numbers. Nevertheless, these low feeding rates were not sufficient to support adequate circulating heterophils numbers, which are the major granulocytes of this Amazon turtle. Such immune disturbances can endanger the health of *P. expansa* by causing a decreased immunologic responsiveness that could result in opportunistic or uncontrolled infection. Lymphocytosis, basophilia, eosinophilia, heterophilia and azurophilia are immunologic responses regarded as typical of turtle patients with ongoing infection and suffering from malnutrition or chronic stress (CHRISTOPHER et al., 2003; KNOTKOVÁ et al., 2005). Immunity is an important physiological mechanism in animals for protection against infection and preservation of

internal homoeostasis (SAURABH and SAHOO, 2008). Thus, a healthy immune system directly relates to the survival of individuals and the stability or recovery of sensitive populations (KELLER et al., 2005). Therefore, further studies are required for knowledge of the interaction between the nutrition and defense mechanisms of the giant turtle from the Amazon, as this relationship is far more complex than has been discussed here.

In conclusion, the findings from this study contribute to the growing data-base on the blood profile of giant turtles from the Amazon, which are malnourished in captivity, and can be used to identify hematological and biochemical responses to low nutritional conditions. Furthermore, this confirms that the hematological and biochemical parameters investigated were good indicators of physiological state, except thrombocyte counts. Therefore, these blood parameters may be successfully used for monitoring the nutritional and health status of farmed *P. expansa*; hence they must be performed as part of the necessary veterinary inspection on turtle farms in Brazil. These results could also be extrapolated for use in projects monitoring this species in free-range, which is more vulnerable due to its use in meat as well as in egg production. This is the object of much concern as great efforts are currently being made for the conservation of this species in Brazil.

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SAŽETAK

Osim za utvrđivanje osnovnih vrijednosti osebujnih za vrstu, pretraživanje krvnih pokazatelja može u kornjača biti rabljeno za brzo određivanje njihova zdravstvenoga stanja. Istraživanje je poduzeto da se odrede i usporede pokazatelji crvenih krvnih stanica, bijelih krvnih stanica, ukupnog broja trombocita i neki biokemijski pokazatelji za slatkovodne kornjače *Podocnemis expansa* Schweigger, 1812 (Pelomedusidae) uzgajane u normalnim (kontrolna skupina) i lošim hranidbenim uvjetima (pokusna skupina). U kornjača uzgajanih u lošim uvjetima ustanovljen je značajno smanjen ($P<0,05$) broj crvenih krvnih stanica kao i smanjene vrijednosti hematokrita, razine glukoze u plazmi, razine ukupnih proteina plazme, kolesterola i mokraćevine te broj bijelih krvnih stanica, azurofila i heterofila. Loša hranidba nije utjecala na hemostazu, ali je prouzročila tešku normocitnu hipokromnu anemiju i znatnu imunodepresiju. Ovo je prvo istraživanje u kojem je prikazan fiziološki i imunološki status divovske kornjače iz Amazone, držane u nepovoljnim hranidbenim uvjetima.

Ključne riječi: slatkovodna kornjača, anemija, krv, leukociti, loša htranidba, *Podocnemis expansa*