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Morphological and cytochemical characterization of thrombocytes and leukocytes in hatchlings of three species of Amazonian freshwater turtles

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ABSTRACT

The aim of this paper was to characterize the morphological and cytochemical aspects of thrombocytes and leukocytes in hatchlings of *Podocnemis expansa, P. unifilis* and *P. sextuberculata* from the lower Purus River, Amazonas State, Brazil. Blood smears were submitted to staining by Periodic Acid-Schiff (PAS), Peroxidase (PER), Toluidine Blue (TB), Sudan Black B (SBB) and Bromophenol Blue (BB). Only the lymphocytes did not present a positive-PAS reaction, while heterophils of *P. unifilis* showed a weak positive reaction. Azurophils, heterophils and eosinophils had a weak positive reaction for PER, with the exception of heterophils in *P. expansa* which stained intensely. Positive staining with BB was observed in granules of heterophils, eosinophils and basophils of *P. expansa, P. unifilis* and *P. sextuberculata,* but this was weak in basophils of *P. sextuberculata*. Sudanophilia was observed in heterophils granules and in eosinophils of *P. expansa, P. unifilis* and *P. sextuberculata*. Metachromasia was demonstrated in basophils of these three species. In these turtle hatchlings, heterophils and eosinophils are the most frequent leukocytes and both have a similar role in defense, since the granules of these granulocytes present glycogen, sudanophilia, peroxidase and basic proteins.

Key words: blood cells, chelonians, cytochemistry, defense, *Podocnemis*

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Introduction

Chelonians of the genus *Podocnemis* (Reptilia: Podocnemididae) are widely distributed throughout the Amazon basin, from the Orinoco River to the Araguaia River (PRITCHARD and TREBBAU, 1984). Historically, these chelonians have been an important source of food for the traditional populations throughout the Amazon who consume their meat and eggs (PEZZUTI et al., 2008; OLIVEIRA-JÚNIOR et al., 2009). In addition, intense trade since pre-Columbian times has led to a drastic reduction in the natural population in some areas (PEZZUTI et al., 2008), while in other areas they have completely vanished. Nowadays, these freshwater turtles are protected by federal law and their reproduction in captivity for commercialization has been encouraged (OLIVEIRA-JÚNIOR et al., 2009).

The Amazon giant turtle (*Podocnemis expansa* Schweigger, 1812), the Yellow-headed turtle (*Podocnemis unifilis* Troschel, 1948), and the Six-tubercled turtle (*Podocnemis sextuberculata* Cornalia, 1849) are water chelonians of different sizes. The first species is the largest one, while *P. sextuberculata* is the smallest of the three. The *P. expansa* and the *P. unifilis* have a long life, with late sexual maturation, which leads to a low replacement rate of individuals. Their populations are characterized by low adult mortality, but high mortality of embryos and hatchlings (SALERA-JUNIOR et al., 2009). *P. expansa* deposits a large number of eggs (110-130) per nest, while *P. sextuberculata* (9-11) and *P. unifilis* (26-34) deposit a lower number of eggs (PEZZUTI et al., 2008), but the three species present different strategies in relationship to nest site selection behavior, and this has a direct influence on their survival (PANTOJA-LIMA et al., 2009).

Although the examination of peripheral blood cells is an important method for monitoring systemic changes in both experimental and clinically ill animals, little is known about the hematology base lines of the Amazonian species of turtles, mainly *P. unifilis* and *P. sextuberculata*. For both the cultivated *P. expansa* (MARCON et al., 2008; OLIVEIRA-JÚNIOR et al., 2009) and *P. expansa* from natural environments (MARCON et al., 2008), studies of biochemical and hematological parameters were recently concluded (OLIVEIRA-JÚNIOR et al., 2009). However, there is no study on the cytochemical properties of the blood cells of these three species of turtles.

Cytochemical staining helps to identify blood cells, when Romanowsky-type stains are not enough to allow for such identification (TAVARES-DIAS, 2006). Moreover, cytochemical staining may be used for understanding the immune function of thrombocytes and leukocytes as demonstrated for *Gopherus agassizii* (ALLEMAN et al., 1992), *Lepidochelys kempi* (CANNON, 1992), *Chelonia mydas* (WORK et al., 1998), *Chrysemys dorbignih* (AZEVEDO and LUNARDI, 2003), *Caretta caretta* (CASAL and ORÓS, 2006) and *Ocadia sinensis* (CHUNG et al., 2009). Peroxidase, Sudan black B, Bromophenol blue, Periodic Acid-Schiff (PAS), and toluidine blue have been used in these chelonians to differentiate blood cells, and different species can present differences in their cytochemical features.

Descriptions of the morphological and cytochemical characteristics of blood cells of Amazonian species of turtles are scarce. Therefore, these reports are very important, given that wild Amazonian turtles are intensively explored, and yet little cultured. Thus, the aim of this paper was to study blood cells of hatchling turtles (*P. expansa, P. unifilis* and *P. sextuberculata*) collected from Abufari Biological Reserve in central Amazon, Brazil, as a reference for future hematological studies of these species.

Materials and methods

Study area. The Abufari Biological Reserve (Fig. 1) is located in the lower Purus river, close to the municipal district of Tapauá, in the state of Amazonas, Brazil. It is 449 km from the capital Manaus. This biological reserve shelters a portion of the population of *P. expansa*, *P. unifilis* and *P. sextuberculata* turtles. The Purus River has white water, loaded with sediments that have been deposited through time, forming the soil that sustains the wetland ecosystem. The main beach at the Abufari Biological Reserve is the chelonians' area of reproduction. It consists of relatively fine grained sand and has a soft inclination (PEZZUTI et al., 2008).

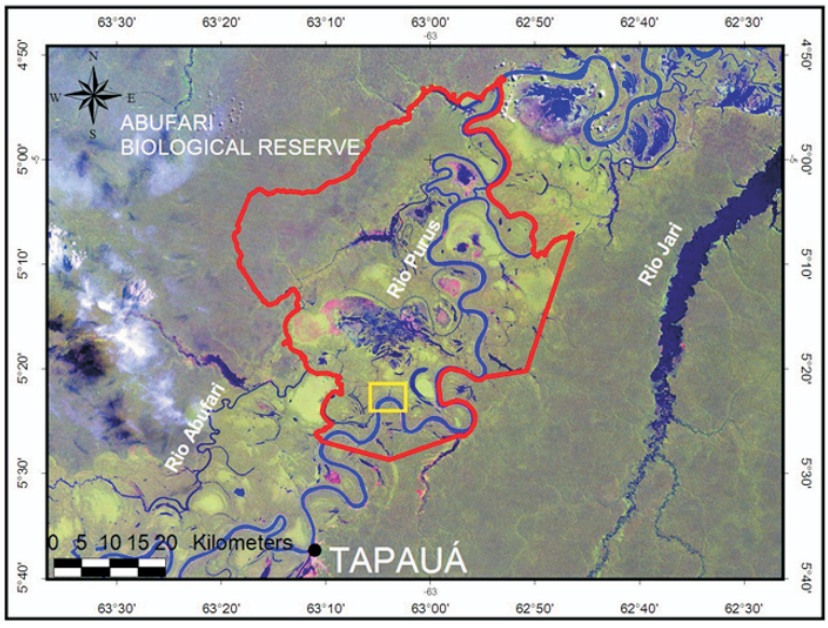

Fig. 1. The line in red shows the limits of the Abufari Biological Reserve located near the city of Tapauá, state ofAmazonas, Brazil.

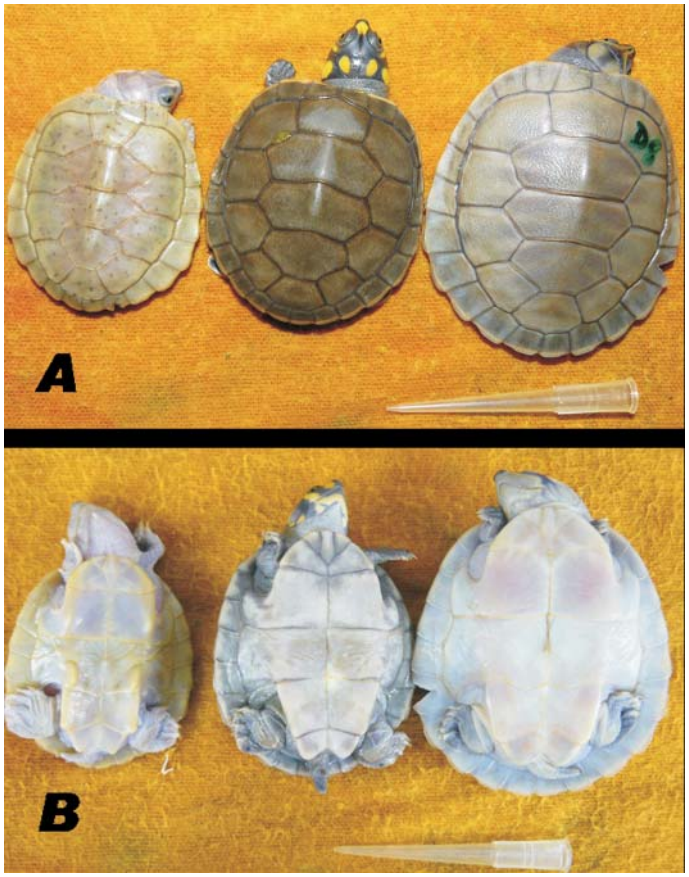

Fig. 2. From left to right: hatchling of Six-tubercled turtle *P. sextuberculata*, Yellow-headed turtle *P. unifilis* (tracajá), and Amazon giant turtle *P. expansa*. (A) Number plan and (B) ventral plan.

Twenty hatchlings of each species (*P. expansa, P. unifilis* and *P. sextuberculata*) (Fig. 2) were collected directly from nests on the main beach (5º22'12"S 63º01'06"W) of the Biological Reserve, during the hatching period (December of 2007). Soon after, they were transported to the Laboratory of Physiology of the Federal University do Amazonas, in Manaus (Brazil), acclimatized and maintained with periodic renewal (48-72 hours) of water, for blood studies. During this period, even with the presence of the vitelline sac, the hatchlings of *P. expansa* and *P. unifilis* were fed with cauliflower leaves (*Brassica oleracea*) and the hatchlings of *P. sextuberculata* with small pieces of raw fish.

Blood collection procedures and cytochemical methods. After the acclimatization period, ten hatchlings of each of the three species of turtles were randomly captured for blood collection, through puncturing the femoral vessel with syringes of insulin coated with sodium heparin (2.500 UI/mL) for making blood smears. After this procedure, the total body weight (g), the straight carapace length (SCL) and the length of the plastron (LP) were measured.

The blood smears were then stained with a combination of May Grünwald-Giemsa-Wright (TAVARES-DIAS and MORAES, 2003), for the morphological characterization of the cells. Other blood smears were used for demonstrating glycogen through the method of Periodic Acid-Schiff (PAS), with digestion by amylase; peroxidase staining using orto-toluidine in the presence of hydrogen peroxide; metachromasia staining with toluidine blue (TAVARES-DIAS, 2006); lipids staining with Sudan Black B 0.3% solution and basic proteins staining with a bromophenol blue solution (EGAMI and SASSO, 1988). The results were expressed according to the intensity of the cytochemical reactions: -: negative reaction; +: weakly positive reaction; ++: positive reaction.

Results

The biometric parameters for *P. expansa, P. unifilis* and *P. sextuberculata* hatchlings are shown in Table 1. In the blood smears of *P. expansa, P unifilis* and *P. sextuberculata* stained with May Grünwald-Giemsa-Wright (MGGW) the following were identified: erythrocytes, thrombocytes, lymphocytes, azurophils, heterophils, eosinophils and basophils. Some of the cytochemical stains used showed different results for these species (Tables 2, 3, 4, 5 and 6).

In *P. expansa*, *P. unifilis* and *P. sextuberculata*, thrombocytes are round shaped cells, but occasionally they can be oval. Their cytoplasm is hyaline and has no granules, with the nucleus accompanying the shape of the cell (Fig. 3A), and they had only positive-PAS staining (Fig. 3B and Tables 2, 3, 4, 5 and 6) after digestion by amylase. Azurophils are cells of different sizes and shapes, sometimes vacuolated, with the cytoplasm containing

Table 1. Mean, standard deviation, maximum and minimum length, weight values (g), straight carapace length (SCL) and length of the plastron (LP) of hatchlings of three species of Amazonian turtles

	Weight (g)	SCL (mm)	LP (mm)
P. expansa	29.4 ± 1.44 (25.93 - 30.38)	56.6 ± 1.56 (54.29 - 58.78)	49.8 ± 1.12 (48.24 - 51.21)
P. unifilis	18.94 ± 0.72 (17.79 - 20.24)	45.76 ± 0.67 (44.72 - 46.57)	41.45 ± 0.57 (40.72 - 42.35)
P. sextuberculata	14.48 ± 2.23 (10.64 - 16.37)	44.53 ± 2.81 (38.89 - 46.49)	40.44 ± 1.99 (36.89 - 42.43)

fine azurophilic granules (Fig. 3C). Azurophils presented positive-PAS staining (Fig. 3D) and weakly positive peroxidase staining. Lymphocytes are round and small, with basophilic cytoplasm and a round nucleus due to its high relationship with the cytoplasm (Fig. 3E), and no cell was positive for any of the cytochemical stains used (Tables 2, 3, 4, 5 and 6). Eosinophils are round and of assorted sizes, which can be small or large,

Table 2. PAS staining in thrombocytes and leukocytes of hatchlings of three species of Amazonian turtles

Blood cells	*P. expansa*	*P. unifilis*	*P. sextuberculata*
Thrombocytes	++	++	++
Lymphocytes	−	−	−
Azurophils	++	++	++
Heterophils	++	+	++
Eosinophils	++	++	++
Basophils	++	++	++

−: Negative; +: weakly positive; ++: positive

Table 3. Peroxidase staining in thrombocytes and leukocytes of hatchlings of three species of Amazonian turtles

Blood cells	*P. expansa*	*P. unifilis*	*P. sextuberculata*
Thrombocytes	−	−	−
Lymphocytes	−	−	−
Azurophils	+	+	+
Heterophils	+	+	+
Eosinophils	++	+	+
Basophils	−	−	−

−: Negative; +: weakly positive; ++: positive

Table 4. Bromophenol blue staining in thrombocytes and leukocytes of hatchlings of three species of Amazonian turtles

Blood cells	*P. expansa*	*P. unifilis*	*P. sextuberculata*
Thrombocytes	−	−	−
Lymphocytes	−	−	−
Azurophils	−	−	−
Heterophils	++	++	++
Eosinophils	++	++	++
Basophils	++	++	+

−: Negative; +: weakly positive; ++: positive

but they are relatively smaller than heterophils. Their cytoplasm is rich in eosinophilic granules, and the nucleus is generally eccentric and rarely segmented (Fig. 3F). Positive peroxidase staining was observed only in *P. expansa* eosinophils (Table 3 and Fig. 3G), while eosinophils of the three species of turtle presented positive staining for PAS (Fig. 3H), bromophenol blue (Fig. 4A) and Sudan Black B (Fig. 4B). Heterophils are predominantly round, with a cytoplasm rich in eosinophilic and basophilic granules. Their nucleus is small, eccentric, and occasionally segmented (Fig. 4C). Heterophils presented positive staining for PAS (Fig. 4D), Sudan Black B (Fig. 4E) and bromophenol blue (Fig. 4F). However, in *P. unifilis* the PAS staining was weak (Table 2), as was the peroxidase staining in the three species (Table 3). Basophils are round, with different sizes and a cytoplasm rich in basophilic granules, which obscure the rounded nucleus (Fig. 4G). Basophils had positive-PAS staining (Fig. 4H) and methacromatic staining with toluidine blue (Fig. 4I and Table 6).

Table 5. Sudan Black B staining in thrombocytes and leukocytes of hatchlings of three species of Amazonian turtles

Blood cells	*P. expansa*	*P. unifilis*	*P. sextuberculata*
Thrombocytes	−	−	−
Lymphocytes	−	−	−
Azurophils	−	−	−
Heterophils	++	++	++
Eosinophils	++	++	++
Basophils	−	−	−

−: Negative; +: weakly positive; ++: positive

Table 6. Toluidine blue staining in thrombocytes and leukocytes of three species of Amazonian turtles

Blood cells	*P. expansa*	*P. unifilis*	*P. sextuberculata*
Thrombocytes	−	−	−
Lymphocytes	−	−	−
Azurophils	−	−	−
Heterophils	−	−	−
Eosinophils	−	−	−
Basophils	++	++	++

−: Negative; +: weakly positive; ++: positive

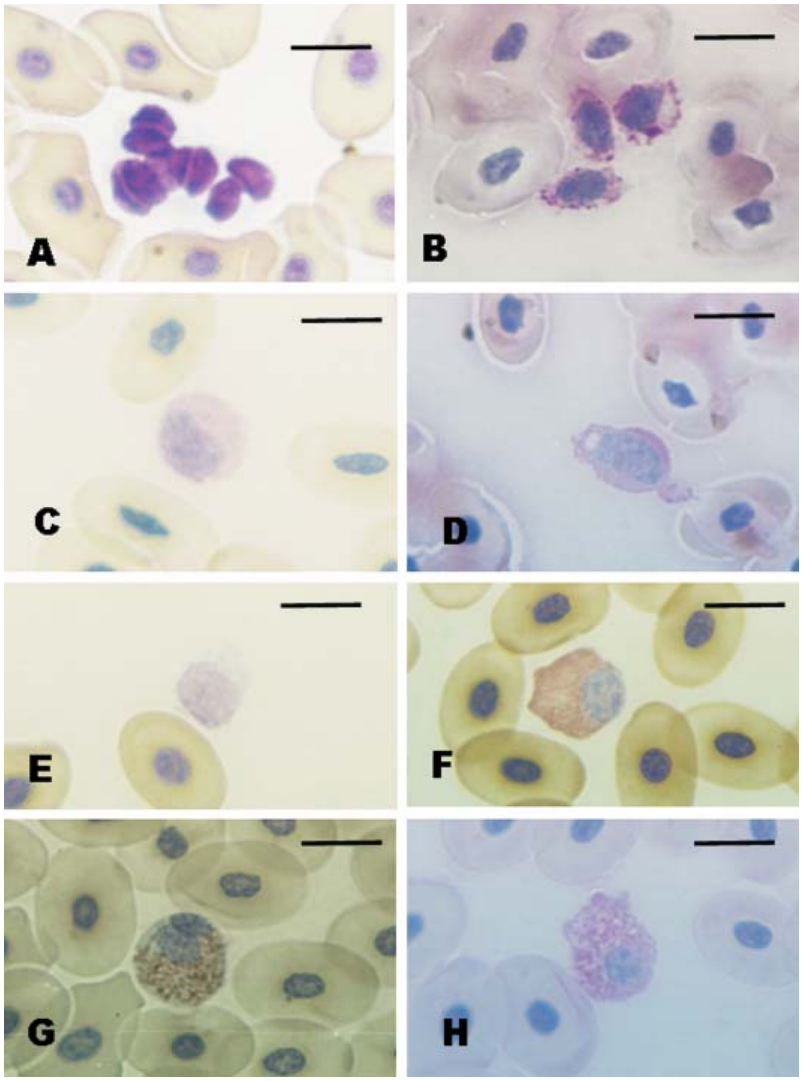

Fig. 3. Blood cells of hatchlings of three species of Amazonian turtles. Thrombocytes of *P. sextuberculata* stained with MGGW (**A**) and PAS (B). Azurophils of *P. unifilis* stained with MGGW (C) and PAS (D). Lymphocytes of *P. sextuberculata* stained with MGGW (E). Eosinophil of *P. expansa* stained with MGGW (F) and with positive reaction for peroxidase (G) and PAS (H). Scale bar = 10.0 µm.

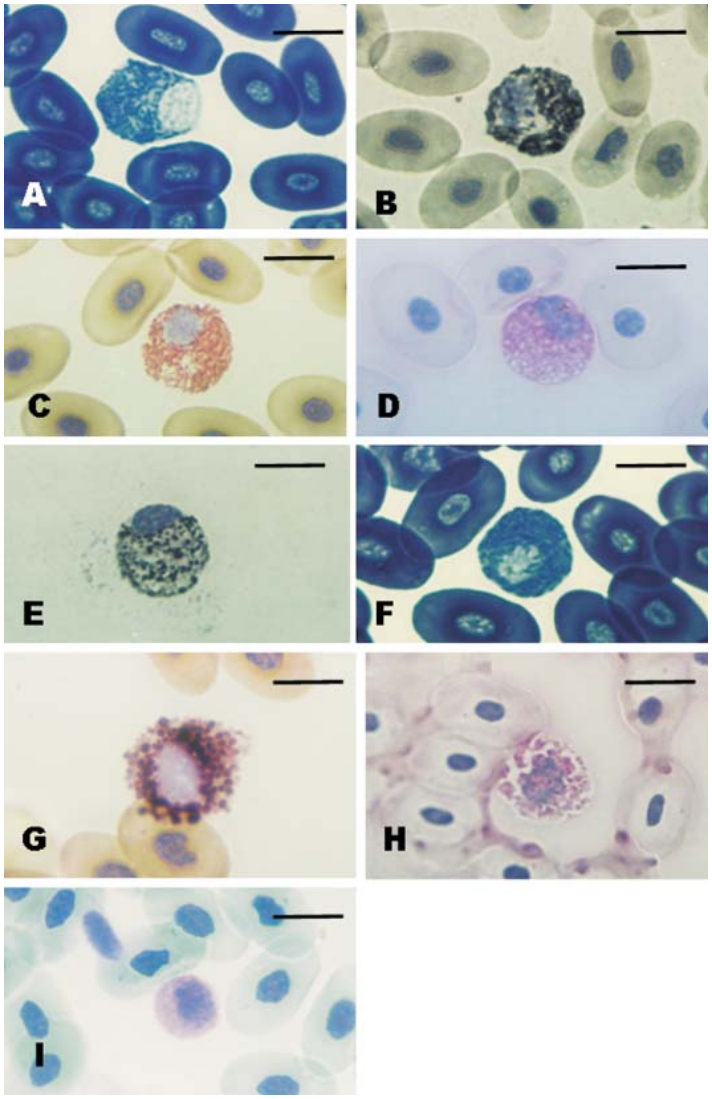

Fig. 4. Blood cells of hatchlings of three species of Amazonian turtles. Eosinophils of *P. unifilis* with positive reaction for bromophenol blue (A) and Sudan Black B (B). Heterophils of *P. sextuberculata* stained with MGGW (C) and with positive reaction for PAS (D), Sudan black (E) and bromophenol blue (F). Basophils of *P. expansa* stained with MGGW (**G**) and with positive reaction for PAS (H) and toluidine blue (I). Scale bar = 10.0 µm.

Discussion

In turtles, some studies using light microscopy have reported the existence of monocytes and azurophils (ALLEMAN et al., 1992; CASAL and ORÓS, 2006), whereas others reported only azurophils (OLIVEIRA-JÚNIOR et al., 2009) or monocytes (WORK et al., 1998; CHUNG et al., 2009). On the other hand, WOOD and EBANKS (1984) and CANNON (1992) did not report monocytes and azurophils. In addition, the existence of neutrophils and eosinophils (WOOD and EBANKS, 1984; CANNON, 1992; PITOL et al., 2007) has been demonstrated, whereas others reported the presence of eosinophils and heterophils (ALLEMAN et al., 1992; WORK et al., 1998; AZEVEDO and LUNARDI, 2003; CASAL and ORÓS, 2006; OLIVEIRA-JÚNIOR et al., 2009; CHUNG et al., 2009) for different turtles. However, classifying reptilian leukocytes as neutrophils is incorrect if these granulocytes contain heterophilic granulation. According to WORK et al. (1998) and CASAL and ORÓS (2006) neutrophils are rare in reptiles, and in the blood smears of turtles the presence of these leukocytes might be artifactual. Therefore, due to the wide morphological variation of granulocytes in different species it is difficult to characterize them solely on the basis of morphologic features (AZEVEDO and LUNARDI, 2003; PITOL et al., 2007).

Thrombocytes, lymphocytes, azurophils, heterophils, eosinophils and basophils were found in the peripheral blood of *P. expansa, P. unifilis* and *P. sextuberculata* hatchlings, collected from the natural environment. They presented morphologic characteristics similar to the ones described for cultivated youths and adults of *P. expansa* (OLIVEIRA-JÚNIOR et al., 2009), obtained by light microscopy. Heterophils are the most frequent granulocytes in the peripheral blood of these three species of Amazonian turtles, followed by eosinophils. Heterophils are the most common granulocyte in the blood of reptiles (SYKES and KLAPHAKE, 2008) and they are functionally analogous to neutrophils (WORK et al., 1998; CASAL and ORÓS, 2006; PITOL et al., 2007; SYKES and KLAPHAKE, 2008). However, morphological intra-specific variations can be found in this granulocyte due to different stages of maturation, since heterophils may mature while in circulation (SYKES and KLAPHAKE, 2008).

Cytochemical stains detect the contents of lipids, carbohydrates and enzymes in thrombocytes and leukocytes, and they are also useful for identifying undifferentiated (TAVARES-DIAS, 2006; CHUNG et al., 2009; ARAÚJO et al., 2009) and acute leukemia (CHUNG et al., 2009). These stains still have the advantage of being easy to use, besides having a low cost (CHUNG et al., 2009; ARAÚJO et al., 2009). The method of PAS identifies glycogen, an important source of energy for the phagocytosis in leukocytes (EGAMI and SASSO, 1988; CANNON, 1992; UEDA et al., 2001; TAVARES-DIAS, 2006; ARAÚJO et al., 2009). Positive-PAS staining was observed in thrombocytes, azurophils, heterophils, eosinophils and basophils of *P. expansa, P. unifilis* and *P. sextuberculata* hatchlings. However, in heterophils of *P. unifilis* this staining was weak. Glycogen granules have

also been reported in thrombocytes, eosinophils and heterophils of *C. caretta* (CASAL and ORÓS, 2006); in thrombocytes, monocytes, heterophils and eosinophils of *C. mydas* (WORK et al., 1998); in lymphocytes and eosinophils of *L. kempi* (CANNON, 1992) and in thrombocytes of *O. sinensis* (CHUNG et al., 2009). On the other hand, no blood cell of *G. agassizii* was stained with PAS (ALLEMAN et al., 1992). However, basophils do not contain glycogen, because the positive-PAS reaction is caused by the presence of acid glycosaminoglycans and phospholipids.

Peroxidase is an important lysosomal enzyme that participates in intracellular digestion, modulates the phagocytic activity, participating in the microbicide intracellular system involving oxidation reactions (EGAMI and SASSO, 1988; CANNON 1992; UEDA et al., 2001; AZEVEDO and LUNARDI, 2003; PITOL et al., 2007). The eosinophilic peroxidase is an enzyme characteristic of secondary granules or specific of eosinophils (ARAÚJO et al., 2009) and it influences two events during the inflammatory process - the margining of the neutrophils/heterophils and/or their accumulation in the place of the inflammation (ARAÚJO et al., 2009). Strong peroxidase staining only occurs in eosinophils of *P. expansa*, because in *P. unifilis* and *P. sextuberculata* the stain was weak. Weak peroxidase stains also happen in azurophils and heterophils of *P. expansa, P. unifilis* and *P. sextuberculata*. Positive peroxidase reaction was reported for eosinophils of *G. agassizii* (ALLEMAN et al., 1992) and of *L. kempi* (CANNON, 1992), as well as for heterophils of *C. caretta* (CASAL and ORÓS, 2006). In *O. sinensis*, eosinophils and basophils had a strong positive reaction while heterophils had a moderate positive reaction to peroxidase staining (CHUNG et al., 2009). On the other hand, this staining pattern was not reported in any leukocyte of *C. mydas* (WORK et al., 1998), possibly due to the insufficient amount of peroxidase to be identified through the techniques used or other factors. The absence of peroxidase in eosinophils can be accompanied by the compensatory development of other microbicide components, as for instance, cationic proteins (ARAÚJO et al., 2009).

The bromophenol blue method for basic proteins produced a variety of shades and intensities of stained blood leukocytes, according to the sequential stage of development of these in the peripheral circulation (EGAMI and SASSO, 1988). Staining with bromophenol blue made the identification possible of basic proteins in the eosinophils granules, heterophils and basophils of *P. expansa, P. unifilis* and *P. sextuberculata* hatchlings, but in that last species the staining was weak. These proteins which are present in the granules of leukocytes have a defense function against microorganisms, provoking their death when they are liberated, after the rupture of these cells (ARAÚJO et al., 2009).

The lipids correspond to part of the leukocytes' weight, but the ripe cells possess a larger amount of lipids, which can be stained with Sudan Black B. The leukocytes phagocytes can use lipids as a source of energy and they can degrade lipids through the action of cytoplasmatic enzymes. Sudan black B staining of granulocytes, which usually parallels that of peroxidase staining, indicated the presence of large quantities of lipids

(BEELEN et al., 2003). Intense Sudan black B staining was observed in the eosinophils and heterophils of *P. expansa, P. unifilis* and *P. sextuberculata* hatchlings. A similar discovery was reported in eosinophils and heterophils *C. caretta* (CASAL and ORÓS, 2006) and eosinophils of *L. kempi* (CANNON, 1992). Sudanophilia in eosinophils can be related to the presence of substances such as the more basic protein (MBP) or eosinophilic cationic protein (ECP) similar to what happens in humans (UEDA et al., 2001).

In our study with *P. expansa*, *P. unifilis* and *P. sextuberculata*, basophils were scarce, and their granules strongly stained with toluidine blue. OLIVEIRA-JUNIOR et al. (2009) reported that in *P. expansa* basophils are 7.8 ± 3.0% from leukocytes. This cytochemical pattern was similar to that described for basophils of *G. agassizii* (ALLEMAN et al., 1992), *C. mydas* (WORK et al., 1998) and *C. carreta* (CASAL and ORÓS, 2006).

In conclusion, in the blood of *P. expansa, P. unifilis* and *P. sextuberculata* there are heterophils and eosinophils whose granules present different morphological and dyeing characteristics. However, cytochemical staining indicated that heterophils and eosinophils possess similar functions in *P. expansa, P. unifilis* and *P. sextuberculata*, once the granules of both leukocytes contain glycogen, peroxidase, basic proteins and lipids. One of the most important mechanisms of the non-specific immune defense of vertebrates is phagocytosis. In these Amazonian turtles, phagocytic function may be carried out by heterophils and eosinophils. Therefore, the information on the cytochemical straining of the blood leukocytes from these turtles added to our understanding of leukocyte counts in healthy animals and may aid the knowledge of the potential role of these cells in the response against infectious and parasitic agents.

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SAŽETAK

Svrha ovog rada bila je odrediti morfološke i citokemijske značajke trombocita i leukocita mlađa kornjača *Podocnemis expansa, P. unifilis* i *P. sextuberculata* iz donjega toka rijeke Purus River u državi Amazona u Brazilu. Razmasci krvi bili su obojeni perjodnom kiselinom i Shiffovim reagensom (PAS), peroksidazom (PER), toluidinskim plavilom (TB), sudanskim crnilom B (SBB) i bromfenolskim plavilom (BB). Samo limfociti se nisu obojili metodom PAS, dok su heterofili vrste *P. unifilis* pokazivali slabo pozitivnu reakciju. Bazofili, heterofili i eozinofili pokazivali su slabu aktivnost peroksidaze s iznimkom heterofila u vrste *P. expansa* koji su pokazivali jaču aktivnost. Bromfenolskim plavilom obojila su se zrnca heterofila, eozinofila i bazofila u vrsta *P. expansa, P. unifilis* i *P. sextuberculata,* ali je bojenje bilo slabo u bazofilima vrste *P. sextuberculata*. Sudanskim crnilom B obojila su se zrnca heterofila vrsta *P. expansa, P. unifilis* i *P. sextuberculata*. Metakromatska zrnca bila su uočena u bazofilima svih triju vrsta. U njihova mlađa najčešće su dokazani heterofili i eozinofili, a i jedni i drugi imaju sličnu ulogu u obrani. Zrnca tih granulocita upućuju na sadržaj glikogena, peroksidaze, bazičnih proteina, a imaju afinitet prema sudanskom bojenju.

Ključne riječi: krvne stanice, Chelonia, citokemija, obrana, *Podocnemis*