Morphological, Cytochemical and Ultrastructural Aspects of Blood Cells in Freshwater Stingray Species in the Middle Rio Negro Basin, Amazonian, Brazil

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Abstract

Examined the morphology, dimensions, cytochemical staining reactions and ultrastructure of blood cells from three freshwater stingray species, *Potamotrygon wallacei*, *Potamotrygon motoro* and *Paratrygon aieereba*, living in the waters of the middle Rio Negro basin (Barcelos, Amazonas, Brazil). We identified erythrocytes, erythroblasts, thrombocytes and four types of leukocyte (basophils, heterophils, lymphocytes and monocytes) in the blood of these stingray species. In all the freshwater stingrays studied, the shape and dimensions of these cells were similar to those of marine elasmobranchs. A positive PAS reaction occurred in heterophils and thrombocytes, and a weak reaction in lymphocytes and monocytes, while a metachromasia reaction only occurred in basophils. Sudan black staining was positive for thrombocytes and lymphocytes, and only a weak reaction occurred in heterophils. Basophils and heterophils were the only cells stained with bromophenol blue, while no peroxidase reaction was observed in any leukocyte type. This is the first study to establish the dimensions and cytochemical staining reactions of blood cells in Amazonian stingray species. Since these elasmobranch species are exported as ornamental fish to countries worldwide, this study can contribute towards establishing standards for blood constituents that may be helpful in assessing the health and welfare of these fish in artificial systems.

Introduction

The family Potamotrygonidae is a unique elasmobranch group composed of freshwater stingray species distributed along most of the great fluvial systems of South America ending at the Atlantic Ocean or Caribbean Sea (Compagno and Cook 1995; Lovejoy 1996). These Neotropical freshwater stingrays are currently classified into four valid genera: *Plesiotrygon*, *Paratrygon*, *Potamotrygon* (Carvalho et al. 2003) and, in a recent contribution (Carvalho and Lovejoy 2011), the new *Heliotrygon* genus, with two species, *H. gomesi* and *H. rosae*. However, great effort and research investment are needed in order to achieve better understanding of the diversity and taxonomic status of this family (Rosa et al. 2010).

Freshwater stingrays are an important component of Amazonian biodiversity. They have great socioeconomic importance, especially because of their use in the international ornamental fish trade, and because they represent an alternative source of income for riverine communities living along the tributaries of the middle Rio Negro basin (Chao et al. 2001). There is a relationship of freshwater stingrays with fishermen, especially by the presence of stingers that can cause accidents (Oliveira et al. 2015). Four valid species are found in this black water system: *Potamotrygon motoro* (Müller & Henle 1841), *Potamotrygon orbignyi* (Castelnau 1855), *Potamotrygon schroederi* (Fernández-Yépez 1958) and *Paratrygon aieereba* (Müller & Henle 1841). In addition, a new species known as *Potamotrygon wallacei* (cururu stingray) (Carvalho et al. 2016), is currently being identified and scientifically described. This species is probably endemic to this region, with a hotspot concentrated in the Mariuá archipelago, near the municipality of Barcelos (Amazonas, Brazil).
Investigations on the blood constituents of elasmobranch fish have been conducted on several marine species, especially sharks (Valenzuela et al. 2003; Luer et al. 2004; Walsh and Luer 2004; Cain et al. 2004; Arnold 2005; Dove et al. 2010). Nevertheless, only a few studies have been addressed towards freshwater elasmobranchs (Dove et al. 2010; Griffith et al. 1973; Oliveira et al. 2012; Magro et al. 2015; Oliveira et al. 2015a; Oliveira et al. 2015b; Oliveira et al. 2015c; Oliveira et al. 2016; Oliveira et al. 2017). It was reported the presence of erythrocytes, thrombocytes, lymphocytes, monocytes, heterophils and basophils in freshwater stingrays of the Potamotrygonidae family (Oliveira et al. 2016). However, these authors did not investigate the cytochemical features of these cell types.

Hematological evaluations are becoming routine practice for assessing the health of fish and other animals. Studies on blood leukocytes can indicate characteristics of the immune systems of different fish species (Tavares-Dias and Moraes 2007; Pavlidis et al. 2007), including free-living Amazonian stingrays. Hematological investigations have relied on classical Romanowsky staining with the Leishman, Wright, May, Grünwald and Giemsa used to identify leukocytes (Veiga et al. 2000; Tavares-Dias 2006; Tavares-Dias and Moraes 2006), but cell-based classifications of these stingray cells are not always reliable using classical staining methods. Thus, blood cytochemical staining of leukocytes may be particularly useful for identifying cell lineages and may suggest cell function.

This study aimed to investigate the morphology, dimensions, cytochemical staining reactions and ultrastructure of blood cells from three freshwater stingray species, *P. wallacei* (cururu stingray), *P. motoro* and *P. aiereba*, living in the black waters of the middle Rio Negro basin (Barcelos, Amazonas, Brazil). Since Brazil and other Amazonian countries export these species as ornamental fish to consumers around the world, these results will contribute towards establishing standards for blood constituents that may be helpful in assessing the health and welfare of these fish in artificial systems, especially in relation to the ornamental fish trade.

**Material And Methods**

**Study area and specimen collection**

Specimens of the Amazonian stingrays *Potamotrygon wallacei* (cururu stingray; n= 53), *Potamotrygon motoro* (n= 55) and *Paratrygon aiereba* (n= 32) were collected from the Mariuá archipelago (Collection Licence: 15116-1 IBAMA). This is the largest complex of islands that exists in continental waters (more than 700 islands; IBGE 2012), and it is located in the black waters of the middle Rio Negro basin, near the municipality of Barcelos (Amazonas, Brazil). These fish were caught at different sites within the archipelago, including beaches, lakes, small streams (igarapés), and areas of flooded forest (igapós), between January 2006 and October 2011. They were all caught by professional fisherman at night (19:00 to 03:00), through active searching with the aid of a head flashlight, a paddle and a typical hand net (rapiché). We immediately anesthetized the captured stingrays with eugenol (0.2 g/L), and withdrew a blood sample (1.0-1.5 mL) from the gill arterial vessel (Tavares-Dias 2006) used the anticoagulant EDTA 10%. After these procedures, we measured the total length (TL, cm), disc width (DW, cm) and body weight
(BW, kg) of each specimen. All the stingrays sampled recovered from the anesthetic and were safely returned to their respective capture site.

For cytochemical staining and ultrastructure examination of different blood cell types, ten individuals of *P. wallacei*, *P. motoro* and *P. aiereba* were caught near the Daracuá community, within the Mariuá archipelago, by professional fisherman. These stingrays were transported by boat (journey of 24 hours) to the Laboratory for Physiology Applied to Aquaculture (LAFAP), at the National Amazon Research Institute (Instituto Nacional de Pesquisas da Amazônia, INPA) in Manaus (Amazonas, Brazil). At the laboratory, they were acclimatized in 5000-liter tanks for 48 hours, with constant water changes and oxygenation so that the fish would recover from the stress that resulted from the capture and transportation procedures. After this period, blood sample (1.0 mL) was collected from the gill arterial vessel used the anticoagulant EDTA 10% (Oliveira et al. 2015c). Immediately after blood collection, blood smears were made. They were then determined the biometric parameters (TL, DW and BW).

**Morphological blood cells and morphometric measurements**

For this experimental procedure, we took fresh blood samples from *P. wallacei* (*n* = 43), *P. motoro* (*n* = 45) and *P. aiereba* (*n* = 32). We stained these blood smears with a combination of May-Grünwald-Giemsa-Wright in order to identify cells and make morphometric measurements (µm) 100 samples, with the aid of an optical microscope and a millimeter ruler.

**Cytochemical staining reactions**

For this experimental procedure, we took fresh blood samples from 10 specimens of each stingray species for smear preparation. The presence and intensity of glycogen deposits inside blood cells was confirmed by using the periodic acid-Schiff (PAS) method. Controls for this reaction were obtained through smears exposed to salivary amylase digestion for 60 minutes.

The peroxidase reaction was carried out by using the ortho-toluidine method in the presence or absence of hydrogen peroxide. The reactions products were subjected to nuclear staining using Harris hematoxylin (Tavares-Dias and Moraes 2006).

Reactions for metachromasia were tested in blood smears that were fixed in 1% lead subacetate for 10 minutes and subsequently stained with 0.2% toluidine blue for 50 minutes (Tavares-Dias and Moraes 2006). The presence of lipids in different blood cell types was confirmed in blood smears that had previously been fixed with 70% ethanol for 5 seconds and then were stained with 0.3% Sudan Black B solution.

In order to identify total protein, blood smears were fixed in formalin, stained with bromophenol blue for 15 minutes and then immersed in 0.5% acetic acid, washed in phosphate buffer and finally dehydrated in butyl alcohol. Reticulocytes were identified using a solution of brilliant cresyl blue and blood (1:1), which was homogenized, kept in a water bath for 20 minutes at 37 °C and stained with a combination of May-Grünwald-Giemsa-Wright (Tavares-Dias and Moraes 2006). The results from the cytochemical staining
were expressed qualitatively, according to the intensity of reactions observed for each blood cell type, i.e. negative reaction (−), weak positive reaction (+) and positive reaction (++).

**Ultrastructural analysis**

The blood cell types were characterized ultrastructurally in four out of the ten individuals of each stingray species that had been acclimatized for cytochemical studies. Blood samples were taken from the gill vessel (Oliveira et al. 2012), and were centrifuged at 750 g for 15 min to obtain pellets containing erythrocytes, thrombocytes and leukocytes. We immediately fixed these pellets in 0.1 M sodium cacodylate solution (pH 7.4) containing 2.5% glutaraldehyde and 2.0% paraformaldehyde, at 4 °C for 2.5 hours. We then immersed these samples in a 0.2 M sodium cacodylate solution (pH 7.4) containing 1% osmium tetroxide, at 4 °C for one hour. After these procedures, the samples were dehydrated and embedded in Araldite resin (Sigma-Aldrich, USA) and sections were cut using a Reichert OM-U3 ultratome, mounted on copper grids (200 mesh) and stained with 0.2% uranyl acetate solution and lead citrate solution for 15 minutes. The sections were analyzed using a transmission electron microscope at the Microscopy Center of the Institute of Biosciences, São Paulo State University (Universidade Estadual Paulista Julio de Mesquita Filho, UNESP), in Botucatu, São Paulo, Brazil.

**Results**

The mean values for total length, disc width and body mass of the specimens of *P. wallacei*, *P. aiereba* and *P. motoro* are shown in Table 1.

**Morphological blood cells and morphometric measurements**

The blood smears from *P. wallacei*, *P. motoro* and *P. aiereba* revealed erythroblasts, mature erythrocytes, thrombocytes, lymphocytes, monocytes, heterophils and basophils, of similar sizes among such species. Monocytes were the largest cells in these three elasmobranch species, in comparison with the other leukocyte cells (Table 2).

The mature erythrocytes were very similar in shape and size in these three Amazonian stingray species. Under an optical microscope, they presented as elliptical cells with abundant hyaline cytoplasm and a nucleus that was usually centered and condensed, and its shape followed that of the cell (Figure 1-I). The erythroblasts were rounded cells and easily differentiated from mature erythrocytes by their pale or hyaline cytoplasm and a higher proportion of nucleus in relation to cytoplasm (N:C ratio) (Figure 1-I).

The lymphocytes presented different sizes and irregular shapes, which were mostly elliptical and rarely oval, and with a nucleus occupying a large part of the basophilic cytoplasm. They presented cytoplasmic projections without visible granulations, and sometimes presented vacuoles (Figure 1-II). The thrombocytes were generally fusiform, with hyaline cytoplasm, their nucleus occupied almost the entire cell and its shape follows that of the cell (Figure 1-III). The monocytes were predominantly oval, with nucleus similar to thrombocytes (Figure 1-III). The heterophils were predominantly oval, with large amount
of heterophilic coarse granules and a nucleus that was generally eccentric (Figure 1-IV). The basophils were also predominantly oval, with basophilic granules and a nucleus that was eccentric and generally bilobulated (Figure 1-V).

**Cytochemical staining reactions**

The thrombocytes and leukocytes did not show any differences in cytochemical reactions between the three species of rays (Table 3). There was glycogen marking in thrombocytes (Figure 2-I) and heterophils (Figure 2-II), and there was a weak positive reaction in lymphocytes (Figure 2-III) and monocytes (Figure 2-IV).

Weak positive staining with Sudan black was observed in heterophils (Figure 3-I), lymphocytes (Figure 3-II) and thrombocytes (Figure 3-III). Positive identification of proteins using bromophenol blue occurred only in granules of heterophils (Figure 4-I) and basophils (Figure 4-II). Presence of reticulocytes was observed in erythrocytes, thus indicating the presence of crosslinking material fragments that did not stain with traditional dyes. There was no positive peroxidase reaction, although the metachromasia reaction was found (Figure 4-III). This was characterized by use of a blue reagent, which reacted with the red-colored blood of these freshwater rays.

**Ultrastructural analysis**

The thrombocytes were generally round and spindle-shaped. In their cytoplasm, a canalicular system with various sizes of vesicles of different sizes and canaliculi was occasionally found, along with glycogen pellets, granules and numerous mitochondria (Figure 5-I). Lymphocytes presented amorphous forms, with sparse cytoplasm. Presence of vacuoles and few mitochondria was observed, and the nucleus occupied almost the entire cell, with dense chromatin in the periphery and no evident nucleolus (Figure 5-II). The monocytes presented nuclei with peripheral heterochromatin and cytoplasm with mitochondria, secretion vesicles, secretion granules and endoplasmic reticulum. Because of scarcity of basophils in the blood, this type of granulocyte could not be found in these potamotrygonids in this study. Staining of heterophils revealed the presence of heterochromatin, and there were large numbers of granules that might have been glycogen, lipids and proteins, but could not be distinguished.

**Discussion**

Most vertebrates have seven blood cell types: erythrocytes, thrombocytes, lymphocytes, eosinophils, basophils, monocytes and neutrophils (Tavares-Dias and Moraes 2003; Canfield 1998). The morphology of each cell type appears to be similar, except for neutrophils. Which in some cases are replaced by heterophils, which present the same immunological function (Canfield 1998; Davis et al. 2008; Hawkey and Dennett 1989). It was reported that erythrocytes, thrombocytes, lymphocytes, monocytes, neutrophils and eosinophils were present in freshwater potamotrygonids (Griffith et al. 1973).
In contrast, no presence of eosinophils was observed in blood from Amazon stingrays, thus suggesting that heterophils have some importance in the immune defense of these potamotrygonids.

In the potamotrygonids of this study, reticulocytes were revealed through the presence of ribonucleoproteins inside some erythrocytes. High amounts of ribonucleoproteins indicate premature release of erythrocytes into the bloodstream (Tavares-Dias and Moraes 2006). Therefore, quantification of the number of circulating reticulocytes can provide information about erythropoietic activity, and therefore about animal health status.

Erythrocytes are generally larger in lower orders and variations in size may occur within species of the same order (Caneld 1998). The erythrocytes of freshwater stingrays were smaller than those of sharks *Centroscymnus coelolepis* (Barbosa do Bogo and de Brito Capello 1864) (Sherburne 1973), and about two times larger than in freshwater and marine teleosts (Vázquesz and Guerrero 2007) and *Dicentrarchus labrax* L.. (Esteban et al. 2000) The morphological features of erythrocytes of freshwater stingrays are similar to those of marine elasmobranchs such as the rays *Dasyatis sabina* (Lesueur 1824), *Raja eglanteria* (Bosc 1800) (Luer et al. 2004; Walsh and Luer 2004), *Raja microcellata* (Montagu 1818), *Raja brachyura* (Lafont 1871) and *Raja* sp. (Aragort et al. 2005), and the sharks *Squalus acanthias* (Linnaeus 1758) (Clewley et al. 2002), *Schroederichthyes chilensis* (Guichenot 1848) (Valenzuela et al. 2003), *Ginglymostoma cirratum* (Bonnaterre 1788) and *Carcharhinus limbatus* (Müller & Henle 1839).

In addition, in the shark *C. coelolepis*, immature erythrocytes (erythroblasts) may be smaller than mature erythrocytes (Sherburne 1973), and this characteristic was also found in these potamotrygonid stingrays. Therefore, these results do not show intraspecific differences relating to the environment.

Thrombocytes in elasmobranchs are blood cells with functions analogous to mammals' platelets, which play a role in homeostasis (Luer et al. 2004; Walsh and Luer 2004). In dogfish (S. *canícula*), it was demonstrated that blood thrombocytes remove antigenic substances, such as colloidal charcoal particles (Morrow and Pulsford 1980). The cell sizes and morphological characteristics of the thrombocytes of freshwater stingrays were similar to those reported in the sharks *S. chilensis* (Valenzuela et al. 2003) and *C. leucas* (Luer et al. 2004), and different from *C. plumbeus*, which presented cytoplasmic granules (Arnold 2005). Moreover, in the blood of the shark *C. coelolepis*, the form known as "drop" (with fingerlike cytoplasmic projection), was observed (Sherburne 1973), but this was not found in the Amazonian stingrays of this study.

In blood smears from marine elasmobranchs, leukocytes at different stages of maturation are frequently observed. This can cause incorrect identification (Luer et al. 2004), thereby contributing towards the confusing terminology of elasmobranch leukocytes (Luer et al. 2004), and also causing errors in identifying small monocytes and large lymphocytes (DaMatta et al. 2009). In the present study, lymphocytes presented shapes ranging from round to amorphous, and this has also been observed among lymphocytes in *C. coelolepis* (Sherburne 1973), *S. chilensis* (Valenzuela et al. 2003), *G. cirratum* (Valenzuela et al. 2003; Luer et al. 2004), *C. plumbeus* (Arnold 2005), *R. microcellata, R. brachyura*, R. sp. (Aragort et al. 2005), *O. maculatus, O. ornatus, O. sp.* (Old and Huveneers 2006) and *R. typus* (Dove et al. 2000).
The size of the lymphocytes of these Amazonian rays was slightly smaller than those of the shark *C. coelolepis* (Sherburne 1973).

The morphological characteristics of this cell type were similar to those observed in other elasmobranchs (Valenzuela et al. 2003; Luer et al. 2004; Arnold 2005; Dove et al. 2010; Aragort et al. 2005; Old and Huveneers 2006). Granulocytes have been reported in several elasmobranch species, but they are difficult to identify and classify because of the great variations in shape and size and the poor staining of the cells (Valenzuela et al. 2003). In the present study, in blood of freshwater stingrays, two types of granulocytes have been showed: heterophils and basophils. It was reported that the most common granulocytes in the blood of elasmobranchs were heterophils, while basophils were rare in blood (Valenzuela et al. 2003). It was shown that neutrophils and eosinophils were present in the blood of potamotrygonids (Griffith et al. 1973). Identification of neutrophils and eosinophils in these potamotrygonids can be correlated with the extreme difficulty of the methods for staining smears and/or with incorrect classification of the different types of leukocyte. Presence of heterophils and basophils with the same morphological features as in these Amazonian stingrays was observed in *C. coelolepis* (dogfish shark) (Sherburne 1973), *S. chilensis* (catshark) (Valenzuela et al. 2003), *C. limbatus* (blacktip shark) (Luer et al. 2004) and *R. typus* (whale shark) (Dove et al. 2010).

It was reported the existence of neutrophils and eosinophils in the blood of an individual of *P. motoro* and mentioned that difficulty in distinguishing neutrophils from heterophils had been found (Oliveira et al. 2015b). In the present study, no neutrophils were found. Instead, there were heterophilic granulocytes with morphological features distinct from neutrophils. However, these had heterophilic functions resembling phagocytosis, as also seen among neutrophils, as indicated by the presence of glycogen, lipids and proteins in *P. wallacei*, *P. motoro* and *P. aiereba*. Glycogen is an important source of cellular energy reserves for the innate defense mechanisms that occur, especially during the process of phagocytosis (Tavares-Dias and Moraes 2006; Ueda et al. 2001).

In the class Chondrichthyes, it was studied the cytochemical characteristics of leukocyte chimeras in the species *Callorhynchus milii* (Bory de Saint-Vincent 1823), *Chimaera phantasma* (Jordan & Snyder 1900), *Hydrolagus novaeezealandiae* (Fowler 1911), *Hydrolagus sp.*, *Harriotta raleighana* (Goode & Bean 1895) and *Rhinoclimaera pacifica* (Mitsukuri 1895) (Hine and Wain 1988). They reported that the enzyme esterase in the subclass Holocephali was very different from this enzyme found in elasmobranchs. However, the present study was the first aimed at determining the functions of blood cell types in potamotrygonid species. A positive PAS reaction was observed in thrombocytes of *P. wallacei*, *P. motoro* and *P. aiereba*, but the reaction in lymphocytes and monocytes was weak. Thrombocytes are cells that act on blood coagulation (Hayhoe et al. 1994), but they also play an important role in the immune activity of elasmobranchs (Luer et al. 2004).

There was no peroxidase reaction in any of the blood cells of *P. wallacei*, *P. motoro* and *P. aiereba*. Peroxidase is an important lysosomal enzyme involved in intracellular digestion, and one of its main features is that it marks absence of eosinophilic and neutrophilic granulocytes in the species investigated.
here. However, this lack of peroxidase may be accompanied by compensatory development of other antibacterial components, such as cationic proteins (Luer et al. 2004; Veiga et al. 2000).

Since basophils are rare leukocytes in the blood of *P. wallacei, P. motoro* and *P. aiereba*, their existence could be confirmed through the metachromasia reaction. In addition, these potamotrygonids demonstrated presence of lipids in thrombocytes and lymphocytes, but to a lesser degree than in heterophils. Similarly, in *Xiphophorus helleri* (Heckel 1848), a Sudan black reaction was also demonstrated in monocytes and lymphocytes (Schutt et al. 1997). However, in other teleosts, this reaction has been described in neutrophil granules (Tavares-Dias 2006). Phagocytic leukocytes can use lipids as an energy source, thereby degrading these constituents through the action of cytoplasmic enzymes.

The proteins in leukocyte granules are involved in host defense and microorganism death (Tavares-Dias 2006). The heterophils and basophils of *P. wallacei, P. motoro* and *P. aiereba* were positive for staining with bromophenol blue, similarly to what had previously been found in eosinophils from *S. brasiliensis* (Tavares-Dias 2006) in Amazonian turtles (Oliveira et al. 2011). It was observed a positive reaction in basophils, eosinophils and neutrophils from *P. motoro* (Oliveira et al. 2015b). Therefore, these results indicate that these proteins play an important role in the innate defense of animals, which is possibly performed by these granulocytes.

The ultrastructural analyses on leukocytes from *P. wallacei, P. motoro* and *P. aiereba* were similar to each other and comparable with the findings from the sharks *G. cirratum* (Hyder et al. 1983) and *S. canicular* (Morrow and Pulsford 1980). The morphology and sizes of the different cell types were similar to those of marine rays and sharks. It is very important to characterize the types of leukocytes in rays in order to provide basic knowledge of these cells and to make correlations with health conditions. In this manner, the cell types of these fish, which are extremely important for the aquarium market, can be quantified. The cytochemical characteristics of the heterophils indicated that these major granulocytes were important in the immune defense of Amazonian potamotrygonids. The blood cell features of wild native stingrays may be useful for making diagnoses and comparisons among these same species under farmed conditions.

**Declarations**

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Authors contributions

ATO and JLM conceived the study. ATO, JRGL, MQC, MLG and JLM designed the study. ATO, JRGL, MQC and MTD undertook laboratorial analyses. JPL, PHRA and MTD drafted the paper with contributions from all other authors. All authors read and approved the final manuscript.

Data availability

The datasets in this study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval and consent to participate

The experiment was developed in accordance with the rules of ethical principles for animal experimentation approved by the National Council for the Control of Animal Experimentation (CONCEA), subject to approval by the Ethics Commission on the Use of Animals (ECUA) of the Federal University of Amazonas under approval No. 005/2010. All experiments were conducted according to local and ARRIVE guidelines.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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**Tables**

**Table 1.** Mean (cm) ± standard deviation of the biometric variables *P. wallacei*, *P. motoro* and *P. aiereba* the Middle Rio Negro, Amazon.

| Species       | Total length (cm) | Disk Width (cm) | Body weight (g) |
|---------------|-------------------|-----------------|-----------------|
| *P. wallacei* (n= 53) | 19.1 ± 2.5        | 17.4 ± 1.1      | 226.0 ± 48.5    |
| *P. motoro* (n= 55)    | 25.1 ± 3.1        | 20.4 ± 1.8      | 351.0 ± 65.0    |
| *P. aiereba* (n= 42)    | 44.8 ± 13.9       | 29.3 ± 10.8     | 966.5 ± 856.9   |

**Table 2.** Mean diameter (µm ± SD) of largest and smallest axis of different blood cells (n= 50) from three freshwater stingray species living at the middle Rio Negro basin, Amazonas, Brazil.
| Cells                  | Potamotrygon wallacei | Potamotrygon motoro | Paratrygon aiereba |
|------------------------|-----------------------|---------------------|--------------------|
| Erythrocytes (µm)      | 20.1±0.7 x 14.1±0.6   | 20.2±0.8 x 14.1± 0.7| 20.0±0.8 x 14.0± 0.8|
| Erythroblasts (µm)     | 19.0±0.9 x 14.8±0.4   | 19.0±0.8 x 14.7±0.5 | 19.1±0.7 x 14.8±0.5|
| Thrombocytes (µm)      | 14.7±1.4 x 9.6±0.5    | 14.6±1.5 x 9.5±0.6  | 14.6±1.3 x 9.6±0.4 |
| Lymphocytes (µm)       | 14.4±1.8 x 12.4±2.7   | 14.7± 1.7 x 12.8±3.1| 14.8±2.1 x 12.7±2.9|
| Monocytes (µm)         | 21.4±1.1 x 21.4±1.1   | 21.3±1.2 x 21.3±1.2 | 21.5±1.0 x 21.5±1.0|
| Heterophils (µm)       | 14.5±0.5 x 14.5±0.5   | 14.4± 0.4 x 14.4±0.4| 14.4±0.5 x 14.4±0.5|
| Basophils (µm)         | 13.5±0.5 x 13.5±0.5   | 13.4±0.6 x 13.4±0.6 | 13.6±0.6 x 13.6±0.6|

Table 3. Cytochemical reactions of the blood cells of stingrays *P. wallacei*, *P. motoro* and *P. aiereba* the middle Rio Negro, Amazon.

| Cells       | PAS | Peroxidase | Toluidine blue | Sudan Black | Bromophenol blue |
|-------------|-----|------------|----------------|-------------|-----------------|
|             | 1   | 2          | 3              | 1           | 2               | 3              | 1   | 2   | 3 |
| Thrombocytes| ++  | ++         | ++             | -           | -               | -              | ++  | ++  | ++ |
| Lymphocytes | +   | +          | +              | -           | -               | -              | ++  | ++  | ++ |
| Monocytes   | +   | +          | +              | -           | -               | -              | -   | -   | - |
| Heterophils | ++  | ++         | ++             | -           | -               | -              | +   | +   | + |
| Basophils   | -   | -          | -              | -           | -               | -              | ++  | ++  | ++ |

(1) *Potamotrygon wallacei*; (2) *Potamotrygon motoro*; (3) *Paratrygon aiereba*

- Negative; + weak positive; ++ positive

**Figures**

**Figure 1**

(I – V). Morphology of blood cells of three freshwater stingray species stained with May Grunwald-Giemsa-Wright. (I) (E) erythrocyte and (Er) erythroblast of *P. wallacei*; (II) (L) lymphocyte of *P. wallacei*; (III) (T) thrombocyte and (M) monocyte of *P. wallacei*; (IV) (H) heterophil and (T) thrombocyte of *P. wallacei*; (V) (B) basophil of *P. wallacei*. Bar = 8µm.
Figure 2

(I-IV). PAS Reaction for demonstration of glycogen in stingrays of blood cells freshwater central Amazonia. (I) Thrombocyte of P. ariereba; (II) Heterophil of P. ariereba; (III) Lymphocyte of P. wallacei; (IV) Monocyte de P. motoro. Bar = 8 µm.

Figure 3

(I-III). Cytochemical markers of lipids in stingrays of blood cells freshwater central Amazonia, using staining with Sudan Black B. (I) Heterophil P. wallacei; (II) Lymphocyte of P. ariereba; (III) Thrombocyte of P. ariereba. Bar = 8 µm.

Figure 4

(I-III). Cytochemical markers of total protein and metachromasia in blood cells of freshwater stingrays in central Amazonia. (I) Heterophil of P. motoro in reaction total protein; (II) Basophil of P. wallacei in reaction total protein; (III) metachromasia in basophil of P. wallacei. Bar = 10 µm.
Figure 5

(I-II). Ultrastructural blood cell freshwater stingrays in central Amazonia. (I) Thrombocyte of *P. motoro*; (II) Lymphocyte of *P. wallacei*. Increase 4000 x.