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BLOOD PHYSIOLOGY OF FRESHWATER STINGRAYS FROM THE LOWER RIO NEGRO, AMAZONAS, BRAZIL

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ABSTRACT

Variations in the physiological characteristics of Potamotrigonines affect hematological parameters, revealing their health status. It is essential to obtain. This study aims to conduct a systematic review of the blood physiology of Potamotrigonines and determine the hematological parameters of freshwater stingrays from the lower Rio Negro, Amazonas. For the systematic review, searches were performed in 3 databases using combinations of keywords, resulting in 368 articles selected according to the inclusion and exclusion criteria. The results showed that only seven Potamotrigonines were studied regarding their blood physiology, with *Potamotrygon motoro* being the most investigated species. The articles identified analyses of erythrogram, leukogram, thrombogram, plasma biochemistry, hormone levels, enzymes and blood ions. For blood studies of stingrays from the lower Rio Negro, collections were carried out in the Tupé Development Reserve and on Açutuba beach, where 27 specimens were captured, 16 *Potamotrygon wallacei*, 3 *P. motoro* and 8 *Paratrygon* spp. After capture, the animals were anesthetized using an immersion bath, and then blood samples were collected by puncturing the gill vessel for hematological procedures. A multiparametric digital device was used to determine the physicochemical properties of the water. The results of the erythrogram demonstrated hematological differences between the species, as well as the results of plasma biochemistry, which showed high values in cholesterol levels in females, the leukogram and thrombogram demonstrated similar values between *P. motoro* and *Paratrygon* sp., while *P. wallacei* showed lower values for defense cells. The present study contributes with an overview of the physiological state of potamotrigonines and with hematological information on freshwater stingrays from the lower Rio Negro region for the first time. This knowledge is essential to improve care and management protocols for conserving this aquatic resource.

Keywords: Potamotrigoninae, Conservation, Hematology, Blood.

RESUMO

As variações nas características fisiológicas dos Potamotrigoníneos afetam os parâmetros hematológicos, revelando o estado de saúde. É fundamental obter. Este trabalho tem como objetivo conduzir uma revisão sistemática sobre a fisiologia sanguínea de potamotrigoníneos e determinar os parâmetros hematológicos de arraias de água doce do baixo rio Negro, Amazonas. Para a revisão sistemática foi realizado buscas em 3 bases de dados utilizando combinações de palavras chaves, resultando em 368 artigos que foram selecionados de acordo com os critérios de inclusão e exclusão. Os resultados mostraram que apenas 7 espécies de potamotrigoníneos foram estudadas quanto a sua fisiologia do sangue, sendo *Potamotrygon motoro* a espécie mais investigada. Nos artigos foram identificadas análises de eritrograma, leucograma, trombograma, bioquímica plasmática, níveis de hormônios, enzimas e íons sanguíneos. Para os estudos sanguíneos de arraias do baixo Rio Negro, foi realizado coletas na Reserva de desenvolvimento do Tupé e na praia de Açutuba, no qual foram capturados 27 espécimes sendo 16 *Potamotrygon wallacei,* 3 *P. motoro* e 8 *Paratrygon* spp., após a captura os animais foram anestesiados por meio de banho de imersão, em seguida foi realizado a coleta de amostras sanguíneas por punção do vaso branquial para a realização dos procedimentos hematológicos. Para propriedades físico-químicas da água foi utilizado aparelho digital multiparamétrico. Os resultados do eritrograma demonstraram diferenças hematológicas entre as espécies, bem como resultados de bioquímica plasmática, que mostrou valores elevados nos níveis de colesterol nas fêmeas, o leucograma e trombograma demonstrou valores semelhantes entre *P. motoro* e *Paratrygon* spp., enquanto *P.* wallacei mostrou valores inferiores para as células de defesas. O presente estudo contribui com visão geral do estado fisiológicos dos potamotrigoníneos e com informações hematológicas de arraias de água doce da região do baixo Rio Negro pela primeira, esses conhecimentos são essenciais para aprimorar os protocolos de cuidado e manejo, para conservação desse recurso aquático.

Palavras-chave: Potamotrigoninae, Conservação, Hematologia, Sangue.

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LIST OF ACRONYMS

Cl^-: Chloride

CONCEA: Council for the Control of Animal Experimentation

EDTA: Ethylenediaminetetraacetic Acid

Ht: Hematocrit

Hb: Hemoglobin concentration

K^+: Potassium

MCV: Mean Corpuscular Volume

MCH: Mean corpuscular hemoglobin

MCHC: Mean corpuscular hemoglobin concentration

Na^+: Sodium

RBC: Red Blood Cell Count

SUMÁRIO

ORGANIZATION

This master's degree was developed between 2023 and 2025, and it generated a dissertation structured in two chapters. The first chapter was submitted to the International Journal of Fish Physiology and Biochemistry (impact factor 2.5), and it addresses a systematic review of the blood physiology of freshwater stingrays.

The second chapter was submitted to the journal Scientific Reports (impact factor 3.8), with pioneering information on the physiological characteristics of freshwater stingrays from the lower Rio Negro region, Amazonas, Brazil.

I want to highlight the difficulties that occurred during the work's execution. Thus, capturing freshwater stingrays in their natural environment is an honorable, difficult and dangerous activity. Furthermore, in the years 2023 and 2024, the area had two historic droughts. There may be different formatting forms in the chapters, as this followed the formatting proposed by the journals.

1 INTRODUCTION

Freshwater stingrays belong to the subclass Elasmobranchii (Bonaparte, 1877), order Myliobatirformes (Compagno, 1973) and subfamily Potamotrygoninae (Carvalho, Loboda and Silva, 2016). They have a depressed body, with the presence of tails that usually have a stinger (Lameiras et al., 2013). Currently, this subfamily is composed of 4 genera, namely *Potamotrygon* (Garman, 1877), *Paratrygon* (Duméril, 1865), *Plesiotrygon* (Rosa, Castelo, Thorson, 1987) and *Heliotrygon* (De Carvalho & Lovejoy, 2011). The Amazon region has the most incredible diversity of freshwater stingrays, which present different color patterns, sizes, and shapes, contributing to ornamental activity and as a food source (Andrade et al., 2023; Carvalho, 2016; Duncan, 2010).

These elasmobranchs, which live restricted to freshwater, have been exploited in aquariums since 1970 and are currently being bred in captivity in Asian countries (Tribuzy-Neto et al., 2020). The importance of the freshwater stingray trade for consumption is also highlighted, especially for large species such as *Paratrygon aireba* and *Potamotrygon motoro* (Andrade et al., 2023).

Potamotrigonines, in addition to their importance in aquariums and consumption, are of utmost importance for maintaining the energy flow in the food chain since they are considered mesopredators, responsible for maintaining the trophic balance in the aquatic environment (Shibuya, 2022). Freshwater stingrays have characteristics similar to other elasmobranchs, such as slow growth and late sexual maturation, which consequently results in a low rate of population renewal in cases of impacts caused by overfishing. (Araújo et al., 2004)

Although freshwater stingrays' vulnerability has increased in recent decades, information on their blood physiology is still limited. Such information is essential for understanding their health conditions, especially given the increasing number of extreme climate events in the Amazon region.

2 LITERATURE REVIEW

2.1 Freshwater stingrays

Among elasmobranchs, the only group that lives exclusively in freshwater is the stingrays, which belong to the subfamily Potamotrygoninae (Loboda et al., 2016). Currently, this subfamily is composed of 4 genera: *Potamotrygon* (Garman, 1877) with 31 species, *Heliotrygon* (De Carvalho & Lovejoy, 2011) with two species, *Plesiotrygon* (Rosa, Castelo, Thorson, 1987) with two species and *Paratrygon* (Dumérill, 1865) being considered a complex with about 10 species (Loboda et al., 2021).

Freshwater stingrays are aquatic animals with a depressed, partially circular body and a tail that most often has spines (Lameiras et al., 2013). Potamotrigonines are endemic to the rivers of South America and are found in almost all countries in the region (Fontenelle; Carvalho, 2017). They are distributed in rivers that flow into the Atlantic Ocean and the Caribbean Sea, except the rivers in the São Francisco and Prata river basins and the rivers of Patagonia (Last et al., 2006).

A striking feature of potamotrigonids is the self-pattern of polychromatism observed in several species. This characteristic, combined with the round shape of the bodies of these animals, has attracted the use of these animals as aquarium animals for decades (Ferreira et al., 2021). In a study carried out by Gomes et al. (2024) on the diversity of freshwater stingrays carried out in the Uatumã River, the high degree of polychromatism found in the color pattern of potamotrigonids was observed, which reflects the tremendous adaptive capacity of the species in different environments. This characteristic is of utmost importance for the survival of stingrays since they use foraging for hunting and escape (Ortolani et al., 1996).

The Amazon region is the biome with the most incredible biodiversity and abundance of freshwater stingrays (Santos, 2022). They have reproductive complexity, including troponemata development, sexual dimorphism, and regulation based on the river flood pulse (Duncan et al. 2016). In addition, they have low fecundity, slow growth, great longevity, and late sexual maturation (Lameiras et al., 2013). These characteristics are similar to those of marine elasmobranchs, which hinder the species' population renewal capacity in the event of environmental impacts (Lucífora et al., 2017).

From the subfamily Potamotrygoninae, five species occur in the Rio Negro. However, *Potamorygon wallacei* (Carvalho, Rosa and Araújo, 2016) stands out, popularly called the “cururu” stingray. It is a species limited to the Rio Negro basin and prefers igapós, where the acidic water has low electrical conductivity with the leafy, sandy-clayey substrate (Oliveira et al., 2016). It is considered one of the smallest freshwater species and has pelvic fins and spiracles that are relatively large for its size (Duncan et al., 2016).

Potamotrygon motoro (Müller y Henley, 1841), also known as the fire stingray, occurs in almost the entire Amazon basin and can be found in various types of water (black, white, transparent) and adapts to muddy, sandy and flooded substrates (Oliveira et al., 2017). They have a circular disc with an ocellated pattern, a poorly developed labial groove and a single row of spines on the tail (Loboda et al., 2016; Rosa and Carvalho, 2016). This species has high dietary plasticity, showing a varied diet, which can be crustaceans, insects, fish and even mollusks, according to the availability of food in the environment (Vasconcelos and Oliveira, 2011; Shibuya, 2022).

Paratrygon is described as a species complex, as its characteristics have not yet been established (Loboda, 2016; Gomes et al., 2024). It is distributed throughout the Amazon basin and in clear, black-and-white waters (Duncan, 2016). It is one of the largest species, which makes it difficult to capture and handle. It has no labial groove, a slightly concave disc shape, a short tail in young individuals and a preference for sandy substrates (beaches) (Lasso et al., 2013; Loboda et al., 2021; Oliveira et al., 2017). Its diet is more specialized, with a preference for fish (Lasso et al., 2013; Shibuya, 2022).

Some factors, such as overfishing for commercial and artisanal purposes, damaging fishing, environmental pollution, extreme events and deep drought in the Amazon rivers, threaten the maintenance and conservation of freshwater stingray population stocks (Shibuya, 2022; Santos, 2022). Despite the increase in biological information about these three species of freshwater stingrays, studies on fishing and biological aspects are still essential to establish the health and well-being of a population in different environments.

2.2 Fishing and economic importance

Freshwater stingrays are a vital fishing resource in the Amazon basin and have been captured since the 1960s for the ornamental trade and since the 1990s for consumption (Santos, 2022). For exploitation for decorative purposes, there is a regulation by the Brazilian

Institute of the Environment and Renewable Natural Resources (IBAMA), which is based on Normative Instruction 204/2008. This regulation allows the commercialization of species of the genus Potamotrygon through a system of annual quotas in the states of Amazonas and Pará.

This IBAMA regulation (2008) includes 6 species with respective maximum sizes at which they can be captured and commercialized: *Potamotrygon motoro* with a maximum diameter of 30 cm, *Potamotrygon. wallacei* up to 14 cm, *Potamotrygon schroederi* 30 cm, *Potamotrygon orbignyi* 30 cm, *Potamotrygon henlei* 30 cm and *Potamotrygon leopoldi* also up to 30 cm. Amazonas has an annual quota of 12,200 stingrays, while the state of Pará has a yearly quota of 8,400 stingrays permitted for the ornamental trade.

Although control through the quota system has been in place by regulatory instruction for over 15 years, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) included freshwater stingray species in its Appendix 2, which lists species that, although not currently threatened with extinction, may face this risk if trade is not controlled (CITES, 2023). However, it is worth noting that even with the CITES warning, it does not prevent the trade of these endangered species. IBAMA uses information from CITES to evaluate and issue licenses, which suggests that there should be changes to Normative Instruction 204/2008, possibly by including species whose trade is legal in Brazil.

There is still no specific legislation regarding the fishing of freshwater stingrays for consumption purposes (Santos, 2022). Although the consumption of stingrays is not typical in the Amazon region, Batista (2008) observed that these animals began to be part of fishing landings in Manaus from 2004 onwards. Most stingrays captured are sold to Brazil's northeastern and southeastern states. This is due to the scarcity of marine stingray stocks, which are in conditions of overexploitation, and the trade in freshwater stingrays takes place to meet this demand in these regions, replacing marine stingrays (Batista, 2008).

In the states of Amazonas and Pará, there has been a significant increase in the exploitation and commercialization of freshwater stingray fillets, which were sold for R$1.00/kg in Colares, Pará, with an estimated consumption of 100 tons per year (Duncan, 2010). The species *P. aiereba* and *P. motoro* are the most frequently captured due to their large size and higher meat yield (Santos, 2022). Estimates of fishing pressure on freshwater stingrays indicated a population decline of species of Potamotrygon found in the Paraná Basin, with abundance decreasing by 15% annually between 2005 and 2016 (Lucifora et al., 2017). These estimates are poor due to freshwater stingrays' reproductive characteristics, making them more susceptible to extinction risks.

Furthermore, one of the threats is illegal trade a study by Andrade et al. (2023) used spectral and chemical reflectance to authenticate freshwater stingray meat samples from the natural environment and supermarkets. The results of the study showed that some samples from the supermarket sold as *P. motoro* and *P. orbignyi* meat were similar to *P. aiereba*. This indicates that *P. aiereba* stingray meat has been traded illegally since the species is critically endangered by the Ministry of the Environment (2022). These data show the difficulty that inspection agents from the competent agencies have in identifying the species (Oliveira et al., 2017).

In addition to these factors that threaten ray species, accidents and injuries involving the stingers of freshwater stingrays, with the period of most extraordinary occurrence during the low and dry seasons when beaches are exposed and consequently, there is a more significant presence of stingrays, have driven the practice of damaging fishing, which consists of capturing animals for mutilation, by removing their tails or even sacrificing the animal (Oliveira et al., 2015). This act harms the swimming movements and the reproduction of freshwater stingrays. Given this, actions that promote environmental education, management and conservation strategies are fundamental for the sustainability of freshwater stingrays.

2.3 Physiological characteristics

Physiology's principle is to understand how the animal body functions, so blood is one of the tissues that explains this function (Oliveira et al., 2021). This is composed of a liquid medium, plasma, which has water and chemical compounds such as glucose, proteins, triglycerides, cholesterol, urea, chlorides and others, which are also related to an organism's nutritional conditions (Brito et al., 2015; Oliveira et al., 2017).

Another blood component, the formed elements, are the cells: erythrocytes, leukocytes and thrombocytes (Maciel et al., 2016; Oliveira et al., 2018). The primary function of erythrocytes is to transport oxygen (Nikinmaa et al., 2019). Leukocytes have a defense function in the blood, acting in the immune system against diseases and infections (Zaminhan et al., 2017). Thrombocytes function in blood coagulation and defense of the organism (Maciel et al., 2016; Oliveira et al., 2018).

The study of each blood element is called erythrogram, leukogram and thrombogram, and all these analyses together form the hemogram (Lizama et al., 2020). The erythrogram consists of counting Red Blood Cells (RBC) and determining the Hematocrit (Ht) and the Hemoglobin level (Hb). With this data, it is possible to calculate hematimetric indices such as the Mean Corpuscular Volume (MCV) related to the volume of erythrocytes, the Mean Corpuscular Hemoglobin (MCH) related to the amount of hemoglobin in each erythrocyte, and the Mean Corpuscular Hemoglobin Concentration (MCHC) which is the index responsible for the concentration of pigment in the erythrocytes (Nikinmaa et al., 2019).

The leukogram and thrombogram consist of counting and identifying the morphology of leukocyte and thrombocytic cells (Oliveira, 2013). Thus, quantifying and identifying these blood compounds allows us to understand the physiological state of freshwater stingrays in their natural environment. In the literature, the physiology of potamotrigonines presents different characteristics compared to other marine elasmobranchs and teleost fish (Luer et al., 2004; Wood et al., 2002).

Potamotrigonines do not accumulate urea in their tissues and have a non-functional rectal gland (Duncan et al., 2016). In terms of osmoregulation, these rays function similarly to freshwater teleost fish, depending on the balance of sodium and chloride ions as osmoregulatory solutes. Thus, ionic and osmotic regulation patterns are directly related to environmental factors and the biological characteristics of the different river basins (Duncan et al., 2016). They have a smaller amount of erythrocytes in their blood, resulting in hemodiluted blood, but their cells are two to three times larger than those of teleost fish (Luer et al., 2004; Oliveira et al., 2017).

The physiological characteristics of freshwater stingrays can present changes between species, locations, ontogeny, sex, flood pulse and others. Therefore, it is crucial to generate hematological and biochemical reference information to establish the physiological state of freshwater populations (Pérez-Rojas et al., 2021), aiding in health diagnosis and improving care and management protocols, both in natural and artificial environments (Oliveira et al., 2021).

Previous studies carried out in the Middle Rio Negro have established physiological, biological and environmental aspects in freshwater stingrays, showing how water properties affect the physiology of these elasmobranchs. Thus, Oliveira et al. (2017) captured neonates and young individuals of 3 species of freshwater stingrays, *P. wallacei*, *P. aiereba* and *P. motoro*, and correlated them with the physicochemical parameters of the water and the hematological parameters, using Principal Component Analysis (PCA) and revealed a total variation of 72.92% in the hematological parameters. Concluding that *P. wallacei* has a lower red series index than the stingray *P. aiereba*, while *P. motoro* presented an intermediate red series pattern, varying between the two species. Regarding water properties, there was a differentiation of 68.57% in the PCA, indicating that the species *P. motoro* is widely distributed in the environment. In contrast, the species P. aiereba interacts more closely.

The study by Santos et al. (2024) was the first to provide data on the hematological and biochemical parameters of 2 species of freshwater stingrays in white water: neonates and juveniles of *P. motoro* and juveniles of *P. orbignyi* from a white water environment in the lower Solimões River. This study showed no significant difference in hematology and blood biochemistry between males and females. On the other hand, the erythrogram, glucose concentration and total proteins of neonates of *P. motoro* showed low values compared to juveniles. *P. orbignyi* revealed lower values of triglycerides and total cholesterol in both sexes compared to results from *P. motoro*.

The physical-chemical properties of the water showed results within the normal range for white water, with no significant differences between points, concluding that water was not a factor for hematological differences in this study but provided hematological references of the good health status of stingrays in white water. Therefore, physiological knowledge of stingrays and how they can be affected by environmental changes is fundamental to understanding their adaptive strategies since aquatic organisms have developed numerous methods to ensure survival (Santos et al., 2020). This study aims to contribute with hematological information on freshwater stingrays from the Lower Rio Negro, Amazonas, to generate relevant information for the development of management and conservation strategies for freshwater stingrays.

3 JUSTIFICATION

Freshwater stingrays play a fundamental role in aquatic fauna. They are mesopredators in the food chain (Shibuya, 2022), thus helping to maintain ecological balance in their natural habitats. In addition to their environmental importance, they are economically relevant as animals used in aquariums and as food sources.

Several factors threaten freshwater stingrays, including overfishing, damaging fishing, and environmental degradation. In addition, their biological aspects, such as late sexual maturation and slow growth, prevent a high rate of population renewal, making them more susceptible to the impacts suffered. In addition to being listed on the IUCN Red List of Threatened Species, such as *Paratrygon* spp., they present insufficient biological information, such as blood physiology, which is of utmost importance for establishing management and conservation strategies for these elasmobranchs that are restricted to freshwater.

Therefore, this work will provide data on the blood physiology of freshwater stingrays from the Lower Rio Negro. Notably, the number of studies related to physiology is still low in establishing reliable information about potamotrigonines. Therefore, establishing reference information regarding physiology will contribute significantly to managing and conserving these freshwater elasmobranchs that comprise the Amazonian biodiversity.

4 OBJECTIVES

4.1 General objective

- To determine the hematological parameters of freshwater stingrays from the Lower Rio Negro, Amazonas, Brazil.

4.2 Specific objectives

- Conduct a systematic review of the physiology of freshwater stingrays;
- Determine the biometric parameters of stingrays captured in the Lower Rio Negro, Amazonas;
- To establish the blood count and plasma biochemistry values of freshwater stingrays captured in the lower Rio Negro, Amazonas;
- Indicate the physical and chemical properties of the water in the locations where stingrays are captured.

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CHAPTER 1 - BLOOD PHYSIOLOGY OF FRESHWATER STINGRAYS: A SYSTEMATIC REVIEW

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Abstract

Freshwater stingrays from the *Potamotrygon*inae subfamily, are endemic species of South America. Blood physiology is crucial for assessing health and well-being and contributing significantly to supporting information for the management and conservation of these species threatened by overfishing and environmental degradation. Considering several factors that can influence blood properties and make it challenging to determine blood reference values, the present study systematically reviewed the blood physiology of freshwater stingrays. The articles showed that only 7 species have been studied regarding their blood physiology, with the most investigated stingray to date being *Potamotrygon motoro*, an endemic species to South America. In the analysis of the red blood series, the erythrogram, a total of four articles were

identified, of which collections were made from stingrays in the Paraná River, Rio Negro, Rio Solimões and one from captive animals. For plasma biochemistry, five studies were identified, with the species *P. motoro* and *Paratrygon aiereba* being the most investigated stingrays and the variables glucose, triglycerides, cholesterol, total proteins and urea being the most quantified. The levels of hormones, enzymes and blood ions, such as Cl^-, Na^+, K^+, progesterone, testosterone and estradiol, were also determined. Regarding the leukocyte count and thrombogram, several leukocyte types were recorded, such as lymphocytes, monocytes, heterophils and basophils. However, there was variation between species. Such information is essential for management and conservation, in addition to assisting in future animal production actions, thus contributing to the maintenance of natural stocks.

Keywords: Blood; Conservation; Freshwater stingrays; Hematology.

1 INTRODUCTION

Freshwater stingrays are elasmobranchs belonging to the Potamotrygonidae family and restricted to South America (Lasso et al. 2013). They have a depressed body, expanded pectoral fins attached to the head and trunk, an elongated tail that may have a stinger, and gill slits in the ventral region (Lameiras et al. 2013). Due to their evolutionary history, only the subfamily Potamotryfoninae have physiological characteristics that make them live exclusively in freshwater, such as the inability to retain urea due to the reduced rectal gland and modifications in the ampullae of Lorenzini (Duncan *et al.*, 2016).

Freshwater stingrays have complex polychromatic patterns (Gomes et al. 2024), making them valuable for the ornamental fish trade (Duncan et al. 2010). In addition to fishing for decorative purposes, stingrays are sold for food, mainly in Brazil's Northeast and Southeast regions, with an estimated 100 tons per year (Batista 2008). It is worth noting that in addition to their economic relevance, freshwater stingrays play extremely important roles in the trophic networks of aquatic ecosystems, mainly as an element of energy transfer between trophic levels, which is essential for maintaining the dynamics of marine populations (Navia et al. 2016).

Blood physiology studies are often used to assess the health status of individual or population freshwater stingrays (Oliveira et al. 2016). Thus, blood comprises a liquid medium called plasma containing water and various chemical compounds, such as glucose, proteins, triglycerides, cholesterol, urea, and chlorides. Furthermore, blood includes figurative elements, which are cells: erythrocytes, leukocytes and thrombocytes (Tavares-Dias and Moraes 2004; Oliveira et al. 2017), and it is from these elements and compounds that animal health and well-being are observed.

Studies on the blood physiology of freshwater stingrays were carried out on species of commercial importance. Oliveira et al. (2015) emphasize that blood physiology, leukogram, and thrombogram analyses make identifying diseases and animal well-being possible. From the biochemical profile of the blood, it is possible to evaluate stress, diet and ion regulation in freshwater stingrays (Brinn et al. 2012). However, many factors can interfere with the results of hematological and biochemical parameters (Oliveira et al. 2016), such as sex, size, developmental stage and habitat, consequently influencing blood properties and making it challenging to determine blood reference values for freshwater stingray species (Perez-Rojas et al. 2021).

Therefore, blood physiology can be attributed as an essential biological reference for freshwater stingray species, which currently have insufficient data when cited in the IUCN Red List of Threatened Species. This can contribute to generating support for managing and conserving freshwater stingray species. This work aims to conduct a systematic review of the blood physiology of freshwater stingrays to outline the physiological profile of species in the Potamotrigoninae subfamily.

2 MATERIALS AND METHODS

The systematic review was developed by the authors (Paixão, R. M. and Oliveira, A. T.) in the following steps: (i) formulation of the research focus question; (ii) search for studies related to the focus question; (iii) screening of selected studies (inclusion and eligibility criteria); and (iv) analysis and synthesis of results. The protocol proposed by the Preferred Reporting Items for Systematic Review (PRISMA) describes identifying and registering studies.

2.1 Focus question

This research has as its focus questions: “What is the physiological profile of freshwater stingrays?”; “Which are the main species of freshwater stingrays that had their blood parameters determined?”; “What is the geographic distribution pattern in physiological research with freshwater stingrays?”; “What is the most commonly used anatomical location for blood collection in freshwater stingrays?”; “What are the chemicals used for analgesia and as anticoagulants in freshwater stingrays?"; "What are the reference ranges found for the erythrogram, plasma biochemistry, metabolites, hormones, enzymes, leukogram and thrombogram of freshwater stingrays?".

The PICO strategy was used for this question, which stands for Population, Intervention, Comparison and "Outcomes," where P: Which species of freshwater stingrays? I: Which types of water and rivers do the species studied come from? C: Which blood parameters were found? and O: What is the physiological profile of freshwater stingrays?

2.2 Search for studies and screening

The studies were identified from the Scielo, Scopus and Web of Science databases from August to November 2024. The search was carried out using descriptors, and the studies were selected from full articles in the free period that were only in English. The Boolean operators AND (and) and OR (or) were used with the descriptors to construct the following search strategies in the databases:

Search 1: Elasmobranchs OR cartilaginous OR stingrays (SC1).

Search 2: Physiology OR Hematology OR Blood OR Biochemistry (SC2).

Search 3: Amazon OR freshwater stingrays OR potamotrigonines (SC3).

After selecting the search components, the Boolean operator "AND" combined searches between SC1, SC2 and SC3. Subsequently, the articles selected from an initial reading of titles and after reading the abstracts were imported and read in full, with all relevant data extracted and entered into a Microsoft Excel spreadsheet for evaluation. The extraction of articles from the databases was based on the Start application.

3 RESULTS

3.1 Literature search

368 studies were found in the searches, including 61 from the Web of Science database, 72 from Scopus ando 235 from the Scielo database. Only articles totaling 335 were selected, of which 45 were in English, were excluded. The Start program was used, which identified 31 duplicates, leaving 259 studies to be screened (Figure 1).

During the title reading, 181 articles were excluded, leaving 78. Of these, 56 studies were excluded based on the abstract reading, leaving 22 articles for full reading (Figure 1). After a thorough reading, twelve articles that presented the criteria for the physiology of freshwater stingrays were included. Three studies were included, totaling 15 articles used in the review (Figure 1).

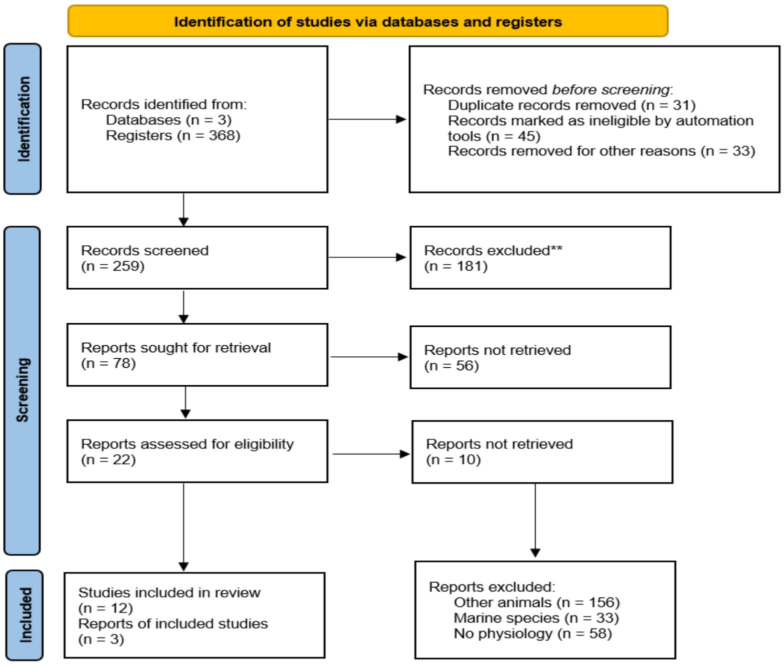

Figure 1: Flowchart with results of data searches in databases to PRISMA

Figure 2 identifies the freshater stingray species with blood physiology research. Thus, the stingray *Potamotrygon motoro* was the most investigated (Figure 2). Figure 3 shows the geographic location where the freshwater stingray species were captured. Occurrences were recorded in three countries: Brazil, Colombia and Singapore (Figure 3).

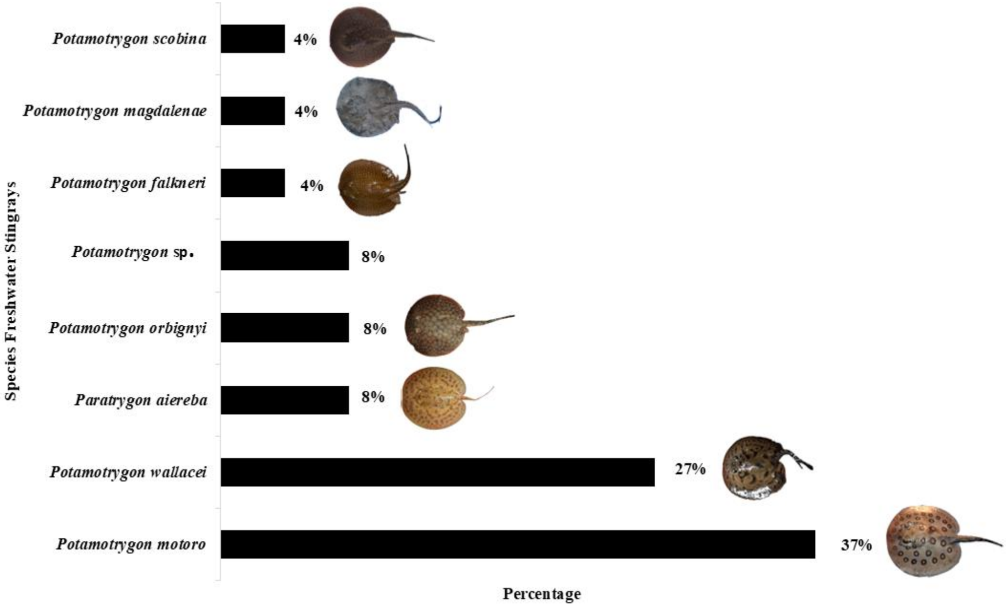

Figure 2: Freshwater stingray species with the determination of blood physiology.

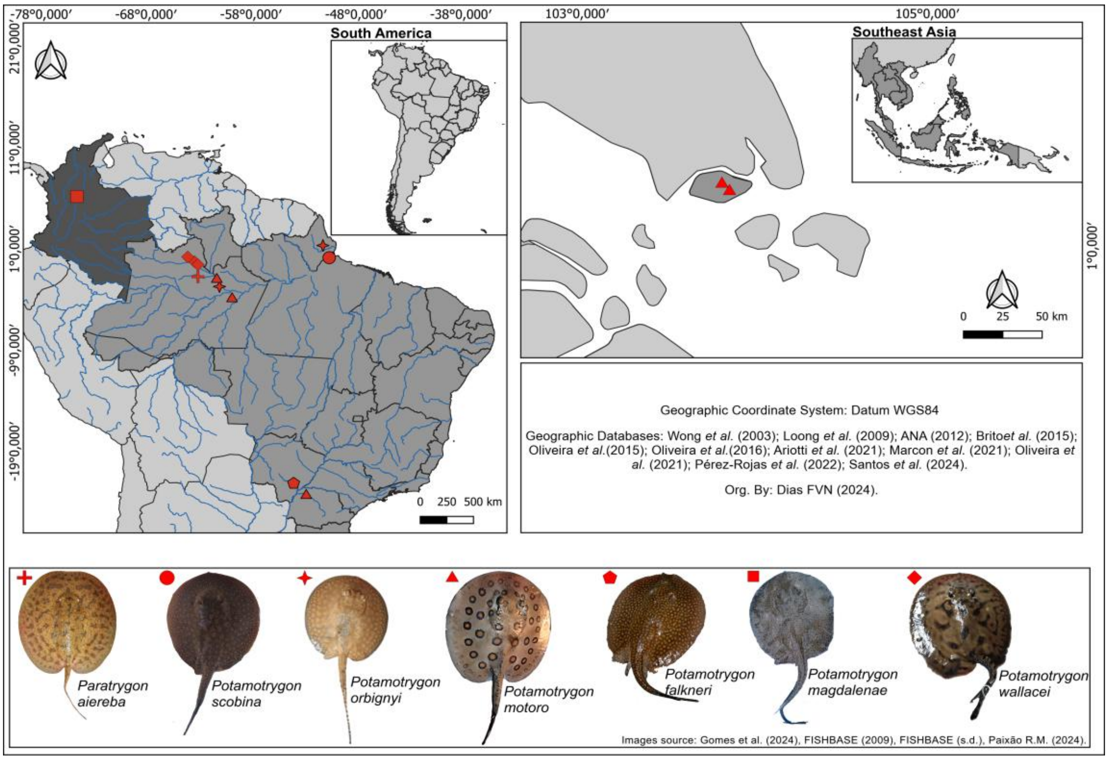

Figure 3: Geographical distribution and location of blood physiological research with freshwater stingrays

When evaluating which anatomical locations were used for blood collection, the anesthetics and anticoagulants used in freshwater stingrays, it was possible to observe that gill puncture, eugenol and 10% EDTA are usually used in studies with blood physiology (Table 1).

Table 1: Anatomical locations, anesthetics, anticoagulants, and analysis were used in blood collections from freshwater stingrays

Anatomical location	Anesthetic	Anticoagulant	Reference
Heart puncture	-	Ammonium heparina	Gerst and Thorson (1997)
Caudal vessel puncture / Heart puncture	MS-222 (tricaine methanesulfonate)	Heparin	Wood *et al.* (2002)
Caudal vessel puncture	-	Heparin	Wai *et al.* (2003)
Caudal vessel puncture	0.12% ethyl-3-aminobenzoate methanesulfonate	Heparin	Ip *et al.* (2003)
Caudal vessel puncture	-	Heparin	Ip *et al.* (2009)
Heart puncture	Benzocaine (100 mg L^{-1})	EDTA 10%	Brinn *et al.* (2012)
Heart puncture	0.05 g L^{-1} buffered 3–amino-benzoic acid ethyl ether	-	Kodra *et al.* (2014)
Caudal vessel puncture	Benzocaine (1:20,000) diluted in etanol (0.1 g mL^{-1})	EDTA 10%	Brito *et al.* (2015)
Branchial arterial vessel puncture	-	EDTA 5%, EDTA 10%, Heparin 2500 IU, Heparin 5000 IU and	Oliveira *et al.* (2015)

		sodium citrate 3.2%	
Branchial arterial vessel puncture	Eugenol (0.2 g L^{-1})	EDTA 10%	Oliveira *et al.* (2016)
Branchial arterial vessel puncture	Eugenol	EDTA 10%	Perez-Rojas *et al.* (2021)
Branchial arterial vessel puncture	Eugenol (0.2 g L^{-1})	EDTA 10%	Oliveira *et al.* (2021)
Branchial arterial vessel puncture	Eugenol (75 mg L^{-1})	EDTA 10%	Marcon *et al.* (2021)
Branchial arterial vessel puncture	Eugenol (0.2 g L^{-1})	EDTA 10%	Ariotti *et al.* (2021)
Branchial arterial vessel puncture	Benzocaine (1:20,000) in immersion	EDTA 10%	Santos *et al.* (2024)

In the analysis of the red blood series, the erythrogram, a total of seven articles were identified, of which collections were made from stingrays in the Paraná River, Piririm River, Negro River, Solimões River and three from captive animals (Table 2). Seven studies were identified for plasma biochemistry, with the species *P. motoro*, *P. wallacei* and *Paratrygon aiereba* being the most investigated stingrays, and the variables glucose, triglycerides, cholesterol, total proteins and urea being the most quantified (Table 3). The levels of hormones, enzymes and blood ions, such as Cl-, Na+, K+, progesterone, testosterone and estradiol, were also determined (Tables 4 and 5). Regarding the leukogram and thrombogram, several leukocyte types were recorded, such as lymphocytes, monocytes, heterophils and basophils, but there was species-specific variation (Table 6).

Table 2: Erythrogram values (average) of freshwater stingrays.

Species	River	Parameters						Reference
		RBC (million μL^{-1})	Hb (g dL^{-1})	Ht (%)	MCV (fL)	MCH (pg)	MCHC (g dL^{-1})	
Potamotrygon sp. (Freshwater)	Artificial environment	-	-	15.3	-	-	-	Gerst and Thorson (1997)
Potamotrygon sp. (Sea water)		-	-	15.5	-	-	-	
Potamotrygon sp. (Acclimatized in an artificial environment)	Negro	-	-	15.5	-	-	-	Wood *et al.* (2002)
Potamotrygon wallacei (Basal)	Artificial environment	0.3	-	22.6	588.1	-	-	Brinn *et al.* (2012)
Potamotrygon wallacei (Before transport)		0.3	-	22.5	713.0	-	-	
Potamotrygon wallacei (Control 24h)		0.3	-	19.6	650.1	-	-	

Potamotrygon wallacei (Antiobiotic 24 h)		0.3	-	21.6	722.7	-	-	
Potamotrygon wallacei (Probiotic 24h)		0.3	-	21.6	724.9	-	-	
Potamotrygon motoro	Paraná	0.7	4.19	19.6	325	59.4	22.2	Brito *et al.* (2015)
Potamotrygon falkneri		0.8	4.31	21.3	288	57.1	20.8	
Potamotrygon orbignyi	Piririm	0.9	4.96	23.1	267	54.5	21.9	
Potamotrygon scobina		1.3	5.22	22	171	39.5	23.7	
Potamotrygon wallacei (whithout anticoagulant)	Middle River Negro	0.5	3.9	19	367.1	72.1	22.3	Oliveira *et al.* (2015)
Potamotrygon wallacei (Edta 5%)		0.5	3.5	17.8	364.7	71.9	23.9	
Potamotrygon wallacei (Edta 10%)		0.4	4.7	17.8	403.1	114.9	30.3	
Potamotrygon wallacei (Heparin 2.500 IU)		0.5	4.8	17.8	376.2	97.3	28.4	
Potamotrygon wallacei (Heparin 5.000 IU)		0.4	3.9	18.7	376.7	89.2	19.9	
Potamotrygon wallacei (Sodium citrate 3.2%)		0.4	3.6	16.2	346.2	67.4	19.9	
Potamotrygon motoro (newborns)	Negro	0.3	2.6	18.0	709.1	101.5	14.5	Oliveira *et al.* 2016

Potamotrygon motoro (Young)	0.4	3.9	21.3	484.9	88.6	18.6
Potamotrygon motoro (Subadults)	0.3	3.7	20.0	569.7	104.3	17.9
Potamotrygon motoro (Adults)	0.3	3.8	20.6	615.8	106.6	18.2
Paratrygon aiereba (newborns)	0.4	4.5	23.0	642.8	110.3	19.4
Paratrygon aiereba (Young)	0.4	3.8	27.4	753.0	110.9	14.3
Paratrygon aiereba (Subadults)	0.4	3.8	25.7	734.5	107.6	14.0
Paratrygon aiereba (Adults)	25	3.8	0.3	629.5	96.6	15.3
Potamotrygon wallacei (Newborns)	0.3	2.2	14.6	420.4	62.8	15.4

Potamotrygon wallacei (Young)		0.4	4.0	29.0	501.0	73.0	21.1	
Potamotrygon wallacei (Subadults)		0.4	4.3	23.7	552.1	97.8	18.4	
Potamotrygon wallacei (Adults)		0.4	4.4	23.2	578.7	105.4	19.3	
Potamotrygon magdalenae (Adults)	Artificial environment	0.2	4.7	19.25	756.0	192.5	25.2	Perez-Rojas *et al.* (2021)
Potamotrygon motoro (Neonate)	Solimões	0.2	2.5	16.1	638.4	99.5	15.7	Santos *et al.* (2024)
Potamotrygon motoro (Juvenile)		0.3	3.8	20.3	573.8	109.1	18.9	
Potamotrygon orbignyi (Juvenile)		0.3	3.9	20.4	591.8	112.6	19.1	

Table 3: Plasma biochemistry values (average) of freshwater stingrays.

Species	**River**	**Parameters** **Glucose** (g dL^{-1})	**Triglyceride** (mM L^{-1})	**Cholesterol** (mM L^{-1})	**Total Protein** (g dL^{-1})	**Albumin** (g dL^{-1})	**Urea** (mM L^{-1})	**Reference**
Potamotrygon sp. (Acclimatized in artificial environment)	Negro	41.4	-	-	1.6		1.2	Wood *et al.* (2002)
Potamotrygon wallacei (Basal)	Negro	29.0	-	-	-	-	1.5	Brinn *et al.* (2012)
Potamotrygon wallacei (Before transport)		27.5	-	-	-	-	4.2	
Potamotrygon wallacei (Control 24h)		22.5	-	-	-	-	1.1	

Potamotrygon wallacei (Antiobiotic 24 h)		29.7	-	-	-	-	1.1	
Potamotrygon wallacei (Probiotic 24h)		35.4	-	-	-	-	1.3	
Potamotrygon sp.	Negro	-	-	-	-	-	2.0	Kodra *et al.* (2014)
Potamotrygon sp.	Branco	-	-	-	-	-	2.5	
Potamotrygon motoro	Paraná	61.8	52.4	74.2	3	0.4	5	Brito *et al.* (2015)
Potamotrygon falkneri		66.9	66.7	88.7	3	0.4	4.8	
Potamotrygon orbignyi	Piririm	44 .0	38.8	34.6	2	0.2	6.3	
Potamotrygon scobina		39.0	37.5	21.8	2	0.1	4	
Potamotrygon wallacei (whithout anticoagulant)	Middle River Negro	14.0	-	-	1			Oliveira *et al.* (2015)

Potamotrygon wallacei (Edta 5%)	16.0	-	-	0.3			
Potamotrygon wallacei (Edta 10%)	18	-	-	0.4			
Potamotrygon wallacei (Heparin 2.500 IU)	14.0	-	-	0.6			
Potamotrygon wallacei (Heparin 5.000 IU)	16.0	-	-	0.6			
Potamotrygon wallacei (Sodium citrate 3.2%)	16.0	-	-	0.7			
Potamotrygon motoro (newborns)	31.9	58.7	69.8	0.8	-	6.7	Oliveira *et al.* (2016)
Potamotrygon motoro (Young)	34.6	60.8	51.6	1.0	-	1.6	

Potamotrygon motoro (Subadults)		32.0	82.0	37.8	0.7	-	1.0
Potamotrygon motoro (Adults)	Middle river Negro	24.8	78.6	76.9	1.4	-	1.5
Paratrygon aiereba (newborns)		13.0	87.5	72.6	1.4	-	4.8
Paratrygon. aiereba (Young)		17.2	105.4	63.6	1.3	-	4.3
P. aiereba (Subadults)		17.6	104.9	56.7	1.5	-	4.3
Paratrygon. aiereba (Adults)		20.5	86.5	48.9	1.6	-	1.5
P. wallacei (Newborns)		41.2	62.0	76.6	0.8	-	1.6
P. wallacei (Young)		20.4	30.8	32.6	1.0	-	3.1

P. wallacei (Subadults)		32.4	56.8	50.9	1.0	-	1.4	
P. wallacei (Adults)		30.0	62.1	53.3	1.1	-	1.3	
P. magdalenae (Adults)	Artificial environment	23.5	50.7	47.7	2.3	-	4.0	Perez-Rojas *et al.* (2021)
P. motoro (Neonate)	Lower river Solimões	32.4	44.2	61	0.7	0.1	6.4	Santos *et al.* (2024)
P. motoro (Juvenile)		48.6	44.2	46.4	1.1	0.2	4.5	
P. orbignyi (Juvenile)		45.0	35.4	42.5	0.7	0.2	4.5	

Table 4: Plasma ions values (average) of freshwater stingray.

Species	River	Cl^- (mM L^{-1})	Na^+ (mEq L^{-1})	K^+ (mEq L^{-1})	Reference
Potamotrygon sp. (Freshwater)	Artificial environment	135.0	146.0	-	Gerst and Thorson (1997)
Potamotrygon sp. (Sea water)		175.0	185.0	-	
Potamotrygon sp. (Acclimatized in artificial environment)	Negro	146.2	178.2	-	Wood *et al.* (2002)
Potamotrygon motoro	Artificial environment	163.0	157.0	-	Wai *et al.* (2003)
Potamotrygon motoro (In 13% salinity)		180.0	166.0	-	
Potamotrygon motoro	Artificial environment	163.0	157.0	-	Ip *et al.* (2003)
Potamotrygon motoro (Exposure to ammonia)		172.0	147.0	-	
Potamotrygon motoro (Control Freshwater)	Artificial environment	159.0	174.0	-	Ip *et al.* (2009)
Potamotrygon motoro		203.0	236.0	-	

(15% water)					
Potamotrygon sp.	Negro	156.4	152.7	4.7	Kodra *et al.* (2014)
Potamotrygon sp.	Branco	135.3	124.8	4.9	
Potamotrygon motoro	Paraná	159.0	173.5	10.1	Brito *et al.* (2015)
Potamotrygon falkneri		157.7	174.7	14.9	
Potamotrygon orbignyi	Piririm	154.9	164.7	6.04	
Potamotrygon scobina		150.9	156.1	8.02	
Potamotrygon motoro (newborns)	Middle River Negro	122.2	126.1	11.6	Oliveira *et al.* (2016)
Potamotrygon motoro (Young)		119.1	137.3	9.6	
Potamotrygon motoro (Subadults)		124.3	134	9.9	
Potamotrygon motoro (Adults)		121.2	275.4	8.2	
Paratrygon aiereba (newborns)		124.2	135.1	8.7	
Paratrygon aiereba (Young)		122.4	144.3	9.2	
Paratrygon aiereba (Subadults)		114.5	136.4	9.6	
Paratrygon aiereba (Adults)		133.2	125.9	8.4	
Potamotrygon wallacei (Newborns)		125.5	135.7	8.8	

Potamotrygon wallacei (Young)		139.6	137.6	7.8	
Potamotrygon wallacei (Subadults)		126.6	139.9	10.1	
Potamotrygon wallacei (Adults)		125.6	143.8.	9.2	
Potamotrygon motoro (Neonate)		122.3	216.1	8.4	
Potamotrygon motoro (Juvenile)		120.9	240.5	7.7	
Potamotrygon orbignyi (Juvenile)	Lower river Solimões	122.7	232.3	1.8	Santos *et al.* (2024)

Table 5: Hormones and enzymes values (average) to freshwater stingray.

Species	River	Progesterone ($ng\ mL^{-1}$)	Testosterone ($ng\ mL^{-1}$)	Estradiol ($ng\ mL^{-1}$)	Alanine aminotransferase ($U\ L^{-1}$)	Aspartate aminotransferase ($U\ L^{-1}$)	Reference
Potamotrygon motoro	Paraná	-	-	-	25	111	Brito *et al.* (2015)
Potamotrygon falkneri	Paraná	-	-	-	18	119	Brito *et al.* (2015)
Potamotrygon orbignyi	Piririm	-	-	-	13	68	Brito *et al.* (2015)
Potamotrygon scobina	Piririm	-	-	-	19	79	Brito *et al.* (2015)
Potamotrygon wallacei (Regressing)	Middle river Negro	9.3	0.5	0.1	-	-	Marcon *et al.* (2021)
Potamotrygon wallacei (Capable to reproduce)	Middle river Negro	7.5	1.0	0.1	-	-	Marcon *et al.* (2021)
Potamotrygon wallacei (Active)	Artificial environment	16.2	1.1	0.2	-	-	Marcon *et al.* (2021)
Potamotrygon wallacei (Control)	Artificial environment	-	-	-	20.3	55.5	Ariotti *et al.* (2021)
Potamotrygon wallacei (With *Lippia alba* essential oil)	Artificial environment	-	-	-	10.4	37.3	Ariotti *et al.* (2021)

Potamotrygon motoro (Neonate)	Lower river Solimões	-	-	-	3.4	38.2	Santos *et al.* (2024)
Potamotrygon motoro (Juvenile)		-	-	-	3.9	38.9	
Potamotrygon orbignyi (Juvenile)		-	-	-	3.8	41.0	

Table 6 White blood cell count and thrombogram values for freshwater stingrays.

Species	River	Leukocytes (µL)	Lymphocytes (%)	Lymphocytes (µL)	Monocytes (%)	Monocytes (µL)	Heterofilos (%)	Heterophiles (µL)	Basophils (%)	Basophils (µL)	Thrombocytes (µL)	Reference
Potamotrygon motoro	Paraná	2520	-	718	-	234	-	147	-	8	1077	Brito *et al.* (2015)
Potamotrygon falkneri		3618	-	1123	-	337	-	232	-	9	1255	
Potamotrygon orbignyi	Piririm	2555	-	403	-	150	-	782	-	43	1232	
Potamotrygon scobina		2299	-	594	-	73	-	851	-	43	1736	
Potamotrygon motoro (newborns)	Middle river Negro	3055.0	50.0	1527.5	28.0	855.4	20.0	611.0	2.0	61.1	940.0	Oliveira *et al.* (2016)
Potamotrygon motoro (Young)		2908.2	42.5	1700.2	27.8	1367.8	23.7	860.9	4.2	181.0	816.0	
Potamotrygon motoro (Subadults)		8140.0	43.2	457.5	26.5	2251.7	28.0	891.0	2.2	325.1	850.0	
Potamotrygon motoro (Adults)		2510.0	52.3	1276.7	28.1	699.8	17.3	471.05	2.85	86.7	1000.0	

Paratrygon aiereba (newborns)	2970.0	48.1	1447.3	29.0	878.3	20.0	652.4	2.8	159.9	636
Paratrygon aiereba (Young)	2696.7	47.0	1228.8	28.3	733.3	21.7	899.4	3.4 8	190.9	610.0
P. aiereba (Subadults)	2859.5	44.5	1220.9	26.8	789.9	23.2	760.1	3.5	177.7	700.0
Paratrygon. aiereba (Adults)	3757.5	48.5	1795.8	28.2	1078.5	18.2	672.5	5.0	210.6	1595.0
Potamotrygon wallacei (Newborns)	2763.0	45.8	1240.8	27.1	748.3	23.8	761.1	3.0	136.0	564.0
Potamotrygon wallacei (Young)	3112.7	44.5	1386.7	22.4	894.7	28.5	656.6	4.6	114.2	1406.5
Potamotrygon wallacei (Subadults)	5925.0	43.4	2342.9	30.3	1882.8	21.0	1028.5	4.2	244.1	791.3
Potamotrygon wallacei (Adults)	3286.7	42.5	1904.0	30.0	951.9	19.5	652	2.5	95.2	-

Potamotrygon magdalenae (Adults)	Artificial environment	6643.7	55.0	4006.0	2.7	142.7	37.2	2065.5	-	-	6532.5	Perez-Rojas *et al.* (2021)
Potamotrygon motoro	Middle River Negro	2998	45.6	1367	26.2	785	25.1	752	2.7	81	826	Oliveira *et al.* (2021)
Potamotrygon wallacei		3629	46.1	1673	30.7	1114	20.2	733	3.0	109	890	
Paratrygon aiereba		3297	43.6	1437	28.5	939	24.9	820	3.0	99	690	
Potamotrygon motoro (Neonate)	Lower River Solimões	2821.8	58.5	1704.6	21.9	584.5	18.5	507.1	0.7	17.7	927.8	Santos *et al.* (2024)
Potamotrygon motoro (Juvenile)		3784.9	51.1	1939.6	26.6	1004	20.3	773.3	1.2	46.9	1370.5	
Potamotrygon orbignyi (Juvenile)		4184.2	52.4	2162.0	22.8	967.6	23.1	990.0	1.1	45.7	19.1	

4 DISCUSSION

4.1 Species of freshwater stingrays and location

The first studies involving blood physiology in freshwater stingrays were carried out with the species *P. motoro* (Gerst and Thorson 1997). Over the years, 7 species of freshwater stingrays that had studies on evaluating blood properties were counted (Figure 2). Of the species investigated, 6 were from the genus *Potamotrygon* and one from the genus *Paratrygon*, in addition to one undescribed species, with the species *P. motoro* (37%) and *P. wallacei* (27%) being the species with the highest percentage in the determination of blood physiology (Figure 2). *P. motoro*, the species with the highest record, is justified by its wide geographic distribution throughout South America (Rosa et al. 2010), with studies concentrated in Brazil, Colombia and Singapura (Figure 3). Although freshwater stingrays are only found naturally in South American countries, the stingrays in Singapore, Asia (Ip et al. 2003; Wai et al. 2003; Ip et al. 2009), was due to the commercialization of stingrays for ornamental purposes. Thus, the stingrays present in Singapore are exotic species in the region and are, therefore, an invasive species in that country (Ng et al. 2010).

With the advancement of studies, *P. wallacei* is the second most studied species, present in 27% of the studies on blood physiology of freshwater stingrays (Figure 2). This stingray has a restricted distribution in the middle Negro River but has also been described in the lower Negro River (Duncan 2016). P. wallacei is a small stingray exploited for aquariums (Charvet-Almeida et al. 2005; Freire 2015). For this species, studies have been developed to understand the mechanisms of ionic regulation, demonstrating that it is adapted to acidic waters, justifying the geographic limitation of concentrating on a part of the Rio Negro (Silva et al. 2007). Figure 3 shows that studies with the species P. wallacei were carried out in the municipalities of Barcelos, Amazonas, Brazil, in the middle Rio Negro, where the species is endemic (Duncan 2016).

A percentage of 8% reported studies with the species *P. orbignyi* and *P. aiereba* (Figure 2), demonstrating that research on blood physiology is an excellent indicator of the health of stingrays in their natural environment (Ranzani-Paiva et al. 2013; Oliveira et al. 2021). Other species were also evaluated, such as *P. scobina* and *P. magdalena*, and an unidentified species classified as Potamotrygon sp. (Figure 2). Although the Amazon is where the most incredible diversity and abundance of freshwater stingrays (Lameiras et al. 2013) occur, the results of the present study demonstrate that few species are being studied. This low number is due to capture difficulties caused by difficult access, high costs of excursions, difficulty in capture and risk with the management of freshwater stingrays (Jézéquel et al. 2020).

4.2 Anatomical locations, anesthetic, anticoagulant and analysis used in blood collections from freshwater stingrays

Blood is drawn from freshwater stingrays through three anatomical locations: cardiac puncture, caudal puncture, and arterial branchial puncture (Table 1). Thus, the first study conducted by Gerst and Thorson (1997) used cardiac puncture. Over the years, arterial branchial puncture has proven to be the most reliable and safest method for both the animal and the researcher.

Cardiac puncture consists of tilting the syringe at a 60° angle between the first and second branchial slits of the stingrays. This method requires precision to avoid myocardial necrosis and is the most invasive for the animal (Ishikawa et al. 2010). Caudal branchial puncture is also used, which is problematic due to the rigidity of the tail and the small amount of blood (Oliveira et al. 2012). However, these collection methods persisted in most studies until 2012, when it was possible to notice a pattern of collection method through branchial vessel puncture (Oliveira et al. 2016; Marcon et al. 2021; Santos et al. 2024). Oliveira et al. (2012), in their studies on blood physiology in stingrays, found this method to be the most efficient, safe and least invasive since, in addition to reducing the risk of stinging the stingrays, it also reduces the chances of collecting other undesirable body fluids.

Regarding the use of anesthetic, eugenol was the most used due to its low toxicity and mortality properties, in addition to the fact that stingrays have a quick recovery (Oliveira et al. 2012; Santos et al. 2024). However, as long as the correct dosages are used, eugenol and benzocaine are effective anesthetics for blood collection in freshwater stingrays. Benzocaine (1:20,000) and eugenol (0.2 g L-1) are recommended in an immersion bath for rapid recovery and non-toxicity (Brito et al. 2015; Oliveira et al. 2021; Santos et al. 2024).

The leading anticoagulant used was 10% EDTA, as it interferes less with hematological parameters and prevents coagulation for longer in blood analyses of freshwater fish (Dantas et al. 2012; Bagé 2017]. Oliveira et al. (2015) conducted comparative studies to evaluate which anticoagulant is more efficient in studies involving the physiology of freshwater stingrays. Thus, a study was conducted using syringes without anticoagulant, EDTA 5%, EDTA 10%, Heparin 2500 IU, Heparin 5000 IU and Sodium Citrate 3.2%, in which it was concluded that the use of sodium citrate is not recommended for preserving blood samples, because this anticoagulant was not very effective (Oliveira et al. 2015).

However, anticoagulants did not affect the parameters evaluated in the determination of plasma glucose and erythrogram, although evidence of coagulation was observed in the erythrocyte count when EDTA 10%, heparin 2500 and 5000 IU were used (Oliveira et al. 2015). However, no RBC, MCV and MCHC changes were observed (Oliveira et al. 2015). For the determination of total protein in blood plasma, heparin 2500 and 5000 IU is the ideal anticoagulant (Oliveira et al. 2015). The results of studies conducted by Oliveira et al. (2015) indicate that blood samples should be collected with EDTA 5% or 10% and Heparin 2500 IU or 5000 IU directly with anticoagulant concentrations to prevent blood clotting.

4.3 Blood physiology of freshwater stingrays

In the blood physiology of freshwater stingrays, the red blood series (erythrogram) is undoubtedly where the values are most consolidated since only six variables are investigated: RBC, Hb, Ht, MCV, MCH and MCHC. Indeed, the most evaluated species were *P. motoro* and *P. wallacei*, but studies were conducted with captures in both natural environments and under experimental conditions. Oliveira et al. (2016) and Santos et al. (2024) reported in their studies that the properties of the water, the species, the ontogeny, the geographic locations and the type of river can influence the blood physiology of freshwater stingrays, but this observation does not seem to occur in the erythrogram. The study conducted by Gerst and Thorson (1997) and Wood et al. (2002) evaluated only the Ht value, which varied between 15.3 and 15.5%, values considered low compared to those found in several later studies that demonstrated a variation between 18.0 and 29.0%, but with most values ranging from 20.0 to 24.0%, which appears to be the reference range for freshwater stingrays. It is worth noting that several abiotic and biotic factors can interfere with the determination of blood in freshwater stingrays, including the seasonality of rivers as a fundamental factor (Oliveira 2008).

The RBC values ranged from 0.25 to 1.3 (million μL^{-1}), this range includes neonates, juveniles and adults of *P. motoro, P. wallacei*, *P. aiereba*, *P. magdalenae*, *P. falkneri* and *P. orbignyi*. Only the stingrays *P. falkneri* and *P. orbignyi* demonstrated high values of 0.8 to 1.3 (Brito et al. 2015) when compared to other studies (Oliveira et al. 2016; Brinn et al. 2012; Perez-Rojas et al. 2021; Santos et al. 2024), this is due to adaptations to the specific characteristics of the aquatic environment of the Paraná River and Piririm River. In addition, there are no details of the development stages of the species studied by Brito et al. (2015). Still, it was possible to observe through the weight that the species were significant, which demands more oxygen, justifying the high value of erythrocytes (RBC) (Brito et al. 2015). Standard RBC values for *P. motoro, P. wallacei* and *P. aiereba* are established as 0.3 to 0.4 for juveniles, subadults, and adults, and for neonates, a range of 0.2 to 0.3, with the lower range being due to the low metabolic demand of the stage in which it is found (Oliveira et al. 2016;Santos et al. 2024). Although three studies (Gerst and Thorson 1997; Brinn et al. 2012; Perez-Rojas et al. 2021) originated from an artificial environment, there was no difference in erythrogram values compared to natural ones.

For Hb, which determines the respiratory pigment that binds to oxygen, subsequently promoting gas exchange, the values ranged from 2.5 to 5.22 g dL^{-1}. Santos et al. (2024) report that Hb can vary according to ontogeny, flooding pulse and other biotic and abiotic factors. For the hematimetric indices MCV, MCH and MCHC, which are mathematical variables determined from RBC, Hb and Ht, it was observed that the MCV is high compared to marine elasmobranchs and freshwater and marine teleosts, such determination was demonstrated by Wilhelm Filho et al. (1992). Griffith et al. (1973) and Oliveira et al. (2016) determined that freshwater stingrays have low metabolism, high cell volume, and lower RBC, Ht and Hb values, indicating that from an evolutionary and physiological point of view, they are ancestral animals, with characteristics preserved over time, such as their blood physiology, the erythrogram, which shows a lower need for oxygen combined with the ancestral cardiovascular system.

The main plasma biochemical components analyzed were glucose, triglycerides, cholesterol, total proteins, albumin, and urea (Table 3). All results of the biochemical parameters showed no similarity between the studies, demonstrating that these parameters are influenced by the species of stingray, development stage, environment, and feeding habits (Oliveira et al. 2016; Oliveira et al. 2021; Santos et al. 2024). Oliveira et al. (2017) and Perez-Rojas et al. (2021) emphasize that feeding conditions can be observed through plasma biochemistry. The feeding of a few species is consolidated in the literature, *P. motoro*, for example, has high feeding plasticity, unlike P. orbignyi, which is described as an insectivorous species (Rincon-Filho 2006; Shibuya 2022). Therefore, it is not possible to establish a reference standard for all species since several abiotic and biotic factors must be considered.

It is also observed that the glucose variable of P. aiereba individuals presents the lowest level compared to species of Potamotrygon. Thus, more plasma biochemistry studies with other genera must be carried out to evaluate the possibility of this being a characteristic of the genus or species.

The plasma ions chloride, sodium and potassium were the most evaluated parameters in freshwater stingrays (Table 4). Thus, studies with adaptations in natural environments and experimental conditions were conducted (Table 4). These parameters presented 114.5 to 203 mM L^{-1} for chloride, 124 to 240.5 mEq L^{-1} for sodium and 4.7 to 14.9 mEq L^{-1} for potassium.

The highest values of these ions are due to saline or ammonia exposure that occurred in experimental studies regardless of species (Gerst and Thorson 1997; Ip et al. 2003; Wai et al. 2003). For the stingrays of the Paraná River, the authors emphasize that the river's characteristics determine their physiology (Brito et al. 2015). Given that the Paraná River is perennial and has a high impact from hydroelectric plants, plasma ions are therefore reflected according to the adaptations of freshwater stingrays in different environments.

For hormones, the quantification of progesterone and testosterone levels was identified only in the P. wallacei stingray (Table 5), demonstrating that this area still needs studies. Thus, the applicability in determining hormone levels can be applied to managing and conservating these elasmobranchs. Its applicability is also aimed at captive production, whether for conservation or animal production.

For enzymes, Aspartate aminotransferase (Ast) and Alanine aminotransferase (Alt) were quantified in the stingrays *P. motoro, P. falkneri, P. orbignyi, P. scobina* and *P. wallacei* (Table 5). AST and ALT are related to physiological stress in rays; AST ranged from 3.4 to 20.3 U L^{-1}, and ALT ranged from 37.3 to 119 U L^{-1}.

Regarding the blood cells of potamotrigonids, few studies have been carried out to identify and characterize blood cell types (Table 6). This lack of information is due to the difficulties encountered in the procedures used to stain blood extensions, mainly in correctly identifying the different cell types (Oliveira 2008; Oliveira 2013). The morphological characterization of the various cell types to date has only been proposed in studies carried out with the stingrays *P. motoro, P. falkneri, P. orbignyi, P. scobina* (Brito et al. 2015), *P. motoro, P. aiereba, P. wallacei* (Oliveira et al. 2016), *P. magdalenae* (Perez-Rojas et al. 2021), *P. motoro* and *P. orbignyi* (Santos et al. 2024), totaling 5 articles and 7 species of stingrays investigated (Table 6). Notably, all blood cell types are found in freshwater stingrays' blood, except the special granulocytic cell (LG-PAS), which occurs exclusively in teleost fish (Tavares-Dias and Moraes 2004). Erythrocytes are elliptical with abundant hyaline cytoplasm, generally with the nucleus in the central portion following the cell's shape. Many immature erythrocytes (erythroblasts) are also depicted in the blood of freshwater stingrays. These cells are generally rounded and have hyaline cytoplasm but have a more excellent nucleus-cytoplasm ratio when compared to mature erythrocytes.

Lymphocytes are irregularly shaped cells, which can be elliptical and rarely oval, with the nucleus occupying a large part of the basophilic cytoplasm and having cytoplasmic projections and the absence of visible granulations (Oliveira et al. 2021). Thrombocytes are generally fusiform and have hyaline cytoplasm, and the nucleus occupies almost the entire cell and follows its shape. Monocytes are the largest leukocytes, predominantly oval in shape, with a generally eccentric nucleus that occupies a large part of the cell and basophilic cytoplasm, which may present vacuoles (Oliveira et al. 2021). Heterophils are predominantly oval cells with many courses heterophilic granulations and a generally eccentric nucleus (Oliveira et al. 2021). Basophils are also predominantly oval cells, have basophilic granulations, and have eccentric nuclei, which are usually bilobed (Oliveira et al. 2021). Neutrophils are predominantly circular cells and may also have a pleomorphic shape, exhibiting cytoplasmic projections (Pádua et al. 2010). In the cytoplasm, there are fine neutrophilic granulations with many vacuoles and a nucleus with a purple coloration and, frequently, with lobes (Pádua et al. 2010). Eosinophils have a circular shape, a significant variation in the size and shape of the nucleus, and their cytoplasm contains a varied number of circular granules stained with eosinophilic staining (Pádua et al. 2010).

Quantifying leukocyte cells and thrombocytes is fundamental for determining immune defense (Tavares-Dias and Moraes 2007; Pavlidis et al. 2007). In many cases, thrombocytes are classified as leukocytes; however, they predominantly play a role in coagulation (Luer et al. 2004; Walsh and Luer 2004) and secondarily in immune defense (Oliveira et al. 2013).

Griffith et al. (1973) carried out the first studies, which quantified leukocytes (total leukocytes = 5.12 ± 1.49 103/mm3; lymphocytes = 73.8 ± 9.3%; monocytes = 13.8 ± 9.0%; neutrophils = 7.5 ± 4.8%; eosinophils = 5.0 ± 1.7%) and total thrombocytes = 33.8 ± 1.3 103/mm3. However, in that study, cell type counting was performed in a Neubauer Chamber, which is no longer used today (Tavares-Dias and Moraes 2004), due to the impossibility of differentiation between blood cell types in non-mammalian vertebrates. This approach was later developed in the research of Oliveira (2008), Brito et al. (2015) and again by Oliveira et al. (2016), who described more consistent results for leukocyte and thrombocyte counting in freshwater stingrays (Table 6).

5 CONCLUSIONS

Freshwater stingrays are a vital commercial resource, such as aquariums or edible animals, and in their natural environments, they help maintain ecological balance. The stingrays *P. motoro* and *P. wallacei* are the most studied in blood physiology. Different methods have been applied, such as restraint and the use of anticoagulants for blood collection in freshwater stingrays. However, blood collection by puncture of the gill vessel and EDTA and heparin are the safest and most widely used methods. Studies on blood physiology are still limited for these freshwater elasmobranchs, but research with this group has been consolidated, especially in the Brazilian Amazon. Such information is essential for management and conservation, in addition to assisting in future animal production actions, thus contributing to the maintenance of natural stocks.

6 REFERENCES

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CHAPTER 2 – HEMATOLOGICAL PARAMETERS OF FRESHWATER STINGRAYS FROM THE LOWER RIVER NEGRO, AMAZONAS, BRAZIL.

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Abstract

Stingrays belonging to the subfamily Potamotrygoninae are adapted to survive exclusively in freshwater environments. There are still many gaps in information about their biology and distribution, knowledge that is essential for the conservation of the species. Therefore, knowledge of blood physiology is fundamental to determining the health status, well-being, and adaptations of the wild species. This study aims to determine the blood parameters of erythrogram, leukogram, thrombogram and plasma biochemistry of freshwater stingrays from the lower Rio Negro, Amazonas. Three species were captured: *Potamotrygon motoro, Potamotrygon wallacei* and *Paratrygon* spp., totaling 27 specimens. They were then anesthetized for blood sample collection. The results of the blood analyses showed differences in the red series between the species, except for RBC. The results of plasma biochemistry also showed differences between species, except for albumin, and between sexes there was a difference only for cholesterol, with females having higher levels. The white blood cell series showed differences between species, with *P. wallacei* having low levels. In this sense, the hematological parameters of freshwater stingrays are altered according to species, sex and habitat**.,**

Keywords: Conservation. Blood. Potamotrigoninae, Amazonian.

1 INTRODUCTION

Stingrays of the subfamily Potamotrigoninae are endemic to South America and currently consist of four genera: *Potamotrygon* (Garman, 1877) being the most diverse genus, *Paratrygon* (Duméril, 1865), currently considered a species complex, *Plesiotrygon* (Rosa, Castelo, Thorson, 1987) and *Heliotrygon* (De Carvalho & Lovejoy, 2011) which have two species each (Carvalho, Loboda and Silva, 2016). These rays are important components of the Neotropical ichthyofauna and are threatened by anthropogenic actions, such as overfishing, habitat degradation, damaging fishing, dam construction (Marquez-Velasquez et al., 2019) and climate change (Gomes et al., 2025). In this scenario, conservation strategies and management protocols in natural environments and captivity are necessary.

Therefore, blood studies are essential to understand the physiology, health and adaptation mechanisms of these species to the aquatic environment (Seibel et al., 2021). Blood contains crucial information about nutritional status, the presence of diseases and parasites, in addition to providing data on resistance to environmental variations, such as water quality. Furthermore, analyzing hematological parameters makes it possible to detect changes in the immune system and assess the impact of changes in habitats (Seibel et al., 2021; Carvalho et al., 2009).

Studies carried out by Oliveira et al. (2016) with stingrays from the middle Rio Negro showed that the development stage is a determining factor in the blood parameters of stingrays of the species *Potamotrygon wallacei*, but the same was not observed for *Potamotrygon motoro* and *Paratrygon aiereba*. The study conducted by Santos et al. (2024) evaluated the hematological and plasma biochemical parameters of stingrays from the Solimões River. It was the first blood study with stingrays from a whitewater river. The results demonstrated good health conditions of the species *P. motoro* and *P. orbigniy*, concluding that the type of water in their study was not a factor influencing the blood physiology of these stingrays.

For this reason, it is essential to establish the physiological state of freshwater stingray populations to assess the animal's health and well-being, generate hematological and biochemical reference information, consider its biology, environmental conditions and species habits (Lizama et al., 2020), and thus provide references to improve care and management protocols for the species. Given this information, this study aims to i) Determine the blood count and plasma biochemistry values of freshwater stingrays captured in the lower Rio Negro, Amazonas; ii) Evaluate the physical-chemical properties of the water.

2 MATERIAL AND METHODS

2.1 Research ethics

This study was carried out with approval from the Biodiversity Authorization and Information System (SISBIO), by Normative Instruction No. ICMBio Ordinance No. 748/2022. Number: 91584-1 and by the Animal Use Ethics Committee of the Federal Institute of Education, Science and Technology of Amazonas (23443.006658/2024-05). Also following the regulations of ethical principles in animal experimentation considered by the National Council for the Control of Animal Experimentation (CONCEA).

2.2 Research area

The study area was designated as the outskirts of the city of Manaus, in the state of Amazonas, which is named after the location of the river as the lower Rio Negro, during the recovery period after severe drought. The sites were chosen due to their great importance as habitats for stingrays and being on the banks of the Amazon region's central city (Manaus). The collection points were: Açutuba Beach, approximately 40 kilometers from Manaus, is a place with a lot of human activity due to being a tourist region; the other point was the Tupé Sustainable Development Reserve (RDS), located 25 kilometers west of Manaus, a lake with characteristics of flooded forest (igapó) (Figure 4).

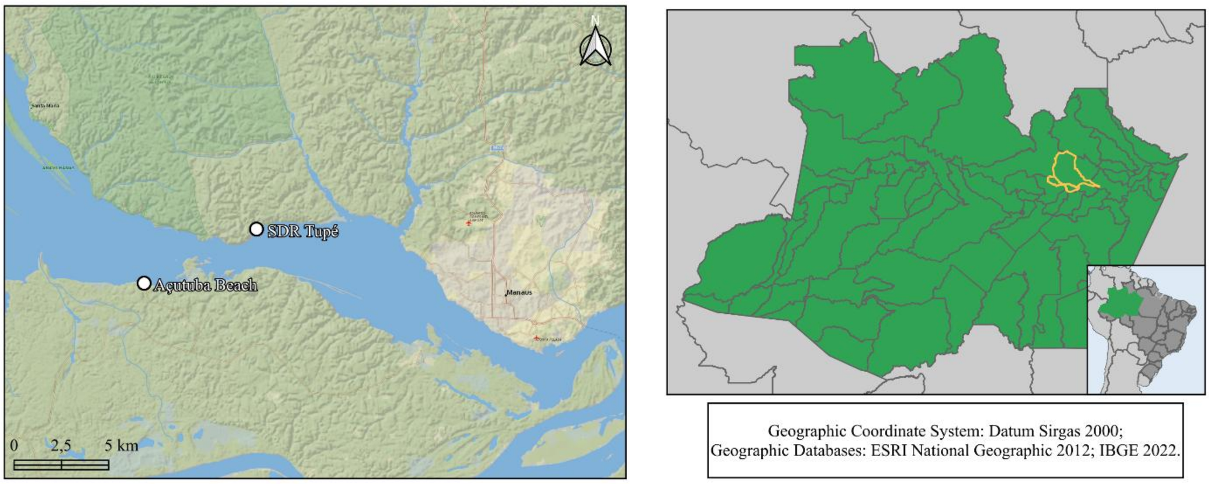

Figure 4: Map with collection points in the lower Rio Negro, Amazonas, Brazil**.**

2.3 Stingray capture

The capture was made through night fishing, using a hand net and headlamp. After capture, the rays were anesthetized with eugenol (200 µL L^{-1}) by immersion in plastic buckets. Then, the stinger was mechanically restrained with Foerster forceps following the recommendations of Oliveira et al. (2012).

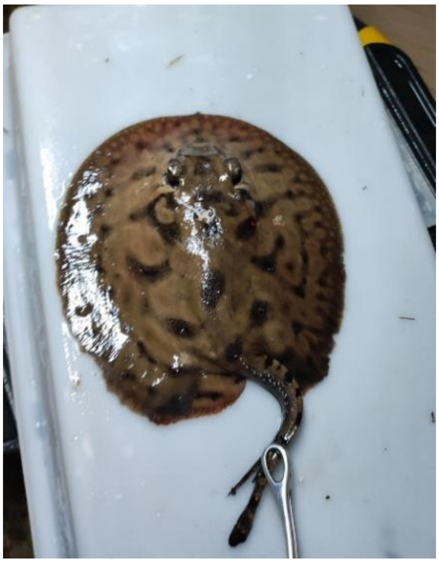

Figure 5: *Potamotrygon wallacei* contained with Forster fórcepsin to Sustainable Development Reserve Tupé, Amazonas, Brazil.

2.4 Blood collection

Blood collection was performed by puncture of the gill vessel, which is the least invasive method and is recommended for freshwater stingrays (Oliveira et al., 2012). 1.0 to 2.0 mL of blood was collected per captured stingray, using syringes containing approximately 15 µL of 10% EDTA anticoagulant. The samples were kept at low temperature (-4 °C) until inclusion in reagents for erythrocyte and hemoglobin analysis, preparation of slides for leukocyte and thrombocyte analysis, and centrifugation of plasma for plasma biochemistry analysis in the laboratory.

2.5 Identification

After blood collection, the following biometric parameters were measured: disc width (DL), total length (TL) and weight (W), using a tape measure and portable scale (Figure 6). Identification was made by observing the shape of the disc, the dorsal color pattern of the disc, the ventral color, the absence or presence of the labial groove and the number of rows of spines on the tail, using the identification key proposed by Rosa and Carvalho (2016). In addition, sexing was also performed by observing the presence or absence of claspers. The development stage followed the proposal of Araújo (1998) for *P. wallacei*, Araújo (1999) for *P. motoro* and Araújo (2011) for *Paratrygon* spp.

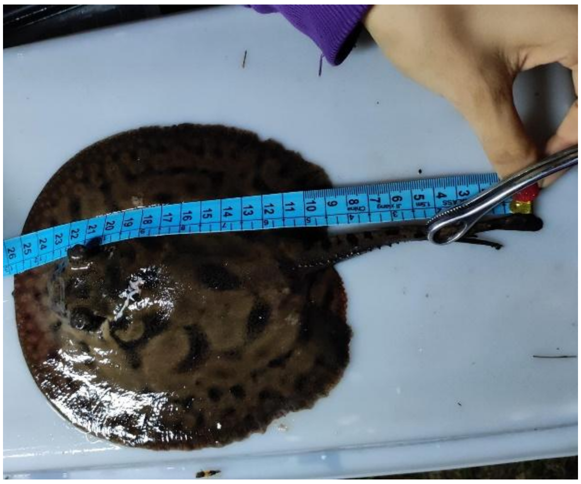

Figure 6: Measurement of the total length of *Potamotrygon wallacei*.

2.6 Blood tests: Erythrogram, leukogram and plasma biochemistry

In the erythrogram, the hematocrit (Ht, %) was determined by the micro hematocrit method and the erythrocyte count (RBC, 10^6 μL^{-1}) was determined by optical reading in a Neubauer chamber under an optical microscope (Leica®, DM 500, Wetzlar, Germany), after including the blood samples in a formalin-citrate solution (1:200) (Liebl et al., 2021).

The blood hemoglobin concentration (Hb, g dL^{-1}) is being determined by the cyanmethemoglobin method (Collier, 1944) after inclusion of homogenized blood (10 μL) in Drabkin's solution (2 mL), centrifugation of the material and reading at 540 nm, according to Ranzani-Paiva et al., (2013) and Machado et al. (2021). The hematological indices mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration (MCHC, g dL^{-1}) are being calculated according to Wintrobe (1934). For plasma biochemistry, the blood sample was centrifuged to separate the plasma. Commercial LabTest colorimetric assay kits were used for the analyses.

To prepare the leukogram, the total leukocyte and thrombocyte count will be performed on blood smears, where up to 100 cells will be counted, establishing the percentage of each cellular component of interest. The smears were stained with May-Grünwald, Giemsa, Wrigth (MGGW) (Tavares-Dias and Moraes, 2004). In addition, blood smears will also be used to differentiate total leukocytes into lymphocytes (%), monocytes (%), heterophils (%) and basophils (%), according to Oliveira et al. (2021).

2.7 Physical-chemical analysis of water

Amostras de água para a avaliação das propriedades físico-químicas, como temperatura (^{o}C), pH, condutividade (µS cm^{-1}) e oxigênio dissolvido (mg L^{-1}), foram determinados no local de captura de cada animal, utilizando-se aparelho digital multi-paramétrico (Hanna HI98194, Hanna Instruments Inc., Woonsocket, RI, USA).

2.8 Statistical analysis

The R® studio software was used for statistical analysis, in which the Shapiro-Wilk test was performed to assess normality. When the data presented normality, the Analysis of Variance (ANOVA) test was performed, followed by the Tukey test, to compare the species. The Student's t-test was applied to compare males and females. The statistical results were expressed as mean and standard deviation (SD), and all statistical tests applied were considered significant when $p<0.05$.

3 RESULTS

With the collections carried out in the RDS of Tupé and Praia do Açutuba, a total of 27 stingrays were captured, namely: *P. motoro*, *P. wallacei* and *Paratrygon* spp., with young, subadult and adult development stages (Table 7). The stingrays showed significant differences in biometric parameters between species and between sexes (Table 8).

Table 7: Sex and developmental stage of stingray species captured in the lower Rio Negro, Amazonas, Brazil.

Collection points	Species	Development Stage	Sex	N
Tupé Sustainable Development Reserve	*Potamotrygon wallacei*	Adult	F	3
			M	8
		Subadult	F	-
			M	1
AÇUTUBA	*Potamotrygon motoro*	Young	F	1
			M	1

		Adult	F	1
			M	-
	Potamotrygon wallacei	Adult	F	3
			M	1
	Paratrygon spp.	Young	F	4
			M	3
		Subadult	F	1
			M	-

Table 8. Mean values ± standard deviation of biometrics of three species of freshwater stingrays collected in the Lower Rio Negro, Amazonas, Brazil.

	Parâmetros biométricos			
	Disc Width (cm)	Total length (cm)	Disc Length (cm)	Weight (kg)
Species				
Potamotrygon wallacei	19.41 ± 2.77^{c}	30.11 ± 4.10^{b}	20.61 ± 3.39^{b}	0.74 ± 0.35^{c}
Potamotrygon motoro	30.63 ± 14.17^{a}	47.80 ± 13.38^{a}	29.60 ± 9.28^{a}	2.23 ± 2.19^{a}
Paratrygon spp.	26.92 ± 3.64^{b}	50.68 ± 7.23^{a}	29.92 ± 3.74^{a}	1.53 ± 0.50^{b}
Sex				
Male	20.37 ± 4.38	33.39 ± 8.92^{b}	21.66 ± 5.28^{b}	0.81 ± 0.50^{b}
Female	25.59 ± 7.52	43.32 ± 12.41^{a}	27.29 ± 5.92^{a}	1.51 ± 1.07^{a}

Lowercase letters in the column demonstrate a significant effect on the variables analyzed by Tukey's test at 0.05 significance level.

The values of Ht, Hb, RBC and hematimetric indices (MCV, MCHC and MCV) showed significant differences between the species for the erythrogram parameters, except for RBC. Furthermore, there was no statistical difference in the comparison between males and females (Table 9).

Table 9. Mean values ± standard deviation blood red series of three species of freshwater stingrays collected in the Lower Rio Negro, Amazonas, Brazil.

Species	Ht (%)	Hb (g dL^{-1})	RBC (millions μL^{-1})	MCH (pg)	MCV (fL)	MCHC (g dL^{-1})
Potamotrygon wallacei	20.22 ± 4.65[b]	9.59 ± 1.76[a]	0.30 ± 0.06	4.52 ± 1.85[a]	6.87 ± 2.27[b]	50.77 ± 16.98[a]
Potamotrygon motoro	23.00 ± 2.29[a]	8.99 ± 1.93[b]	0.24 ± 0.02	3.64 ± 0.48[b]	9.38 ± 0.17[b]	38.80 ± 4.88[b]
Paratrygon spp.	30.50 ± 4.69[a]	7.64 ± 0.88[c]	0.28 ± 0.03	2.72 ± 0.59[c]	10.61 ± 1.03[a]	25.87 ± 5.88[c]
Sex						
Male	22.43 ± 5.19	9.28 ± 2.05	0.31 ± 0.05	4.03 ± 1.76	7.42 ± 2.23	44.82 ± 17.84
Female	24.80 ± 7.44	8.58 ± 1.35	0.27 ± 0.05	3.73 ± 1.61	9.16 ± 2.56	39.10 ± 17.32

Lowercase letters in the column demonstrate a significant effect on the variables analyzed by Tukey's test at 0.05 significance level.

In the plasma biochemistry analysis, glucose, total cholesterol, total proteins, triglycerides and albumin levels were analyzed. Thus, the parameters showed significant differences between the species (Table 10). There was no statistical difference between males and females for the biochemistry parameters, except for cholesterol, in which females showed higher values (Table 10).

Table 10. Mean values ± standard deviation of blood plasma biochemistry of three species of freshwater stingrays collected in the Lower Rio Negro, Amazonas, Brazil.

Species	Triglycerides (mg dL^{-1})	Glucose (mg dL^{-1})	Cholesterol (mg dL^{-1})	Total Proteins (g L^{-1})	Albumin (g L^{-1})
Potamotrygon wallacei	47.16 ± 30.23[c]	27.75 ± 6.77[b]	32.46 ± 9.89[b]	0.99 ± 0.33[c]	0.45 ± 0.26

Potamotrygon motoro	66.96 ± 9.82[b]	58.34 ± 23.06[a]	57.50 ± 15.78[a]	1.50 ± 0.70[b]	0.38 ± 0.20
Paratrygon spp.	80.71 ± 31.05[a]	29.19 ± 21.05[b]	54.94 ± 10.12[a]	2.14 ± 0.73[a]	0.25 ± 0.13
Sex					
Male	58.79 ± 35.24	34.19 ± 21.01	41.59 ± 11.59[b]	1.29 ± 0.87	0.36 ± 0.22
Female	59.85 ± 29.86	28.76 ± 10.58	53.97 ± 14.06[a]	1.50 ± 0.53	0.41 ± 0.25

Lowercase letters in the column demonstrate a significant effect on the variables analyzed by Tukey's test at 0.05 significance level.

Four leukocyte types were found in the leukogram and thrombogram analysis: lymphocytes, monocytes, heterophils and basophils. *P. motoro* and *P. wallacei* presented the four types of leukocytes and thrombocytes. *Paratrygon* sp. showed the presence of thrombocytes and three types of leukocytes, but no basophils were observed (Table 11 and Figure 7).

Table 11. Mean values ± standard deviation of the differential leukocyte count (white series) of three species of freshwater stingrays collected in the Lower Rio Negro, Amazonas, Brazil.

Species	Parameters					
	Leucócitos (µL)	Trombócitos (µL)	Linfócitos (%)	Monócitos (%)	Heterófilos (%)	Basófilos (%)
Potamotrygon wallacei	6851.87 ± 1905.47[b]	1234.68 ± 1028.49[c]	82.93 ± 9.11[a]	8.25 ± 6.86[b]	7.93 ± 3.54	0.89 ± 0.12[a]
Potamotrygon motoro	5512.50 ± 724.72[c]	2858.33 ± 652.50[b]	71 ± 9.64[b]	18.66 ± 4.50[a]	9.33 ± 5.77	1.01 ± 0.10[a]
Paratrygon spp.	8000.62 ± 3002.98[a]	4835.31 ± 1395.8[a]	75.25 ± 6.43[b]	17 ± 3.27[a]	7.75 ± 4.71	0.00 ± 0.00[b]
Sex						
Male	6840.89 ± 1343.90[b]	2297.32 ± 1074.62[b]	79.92 ± 11.61	10.78 ± 8.58	8.78 ± 4.54	0.52 ± 0.14
Female	7261.53 ± 3012.67[a]	2680.76 ± 1767.68[a]	78.69 ± 6.49	13.38 ± 5.67	7.23 ± 3.32	0.70 ± 0.16

Lowercase letters in the column demonstrate a significant effect on the variables analyzed by Tukey's test at 0.05 significance level.

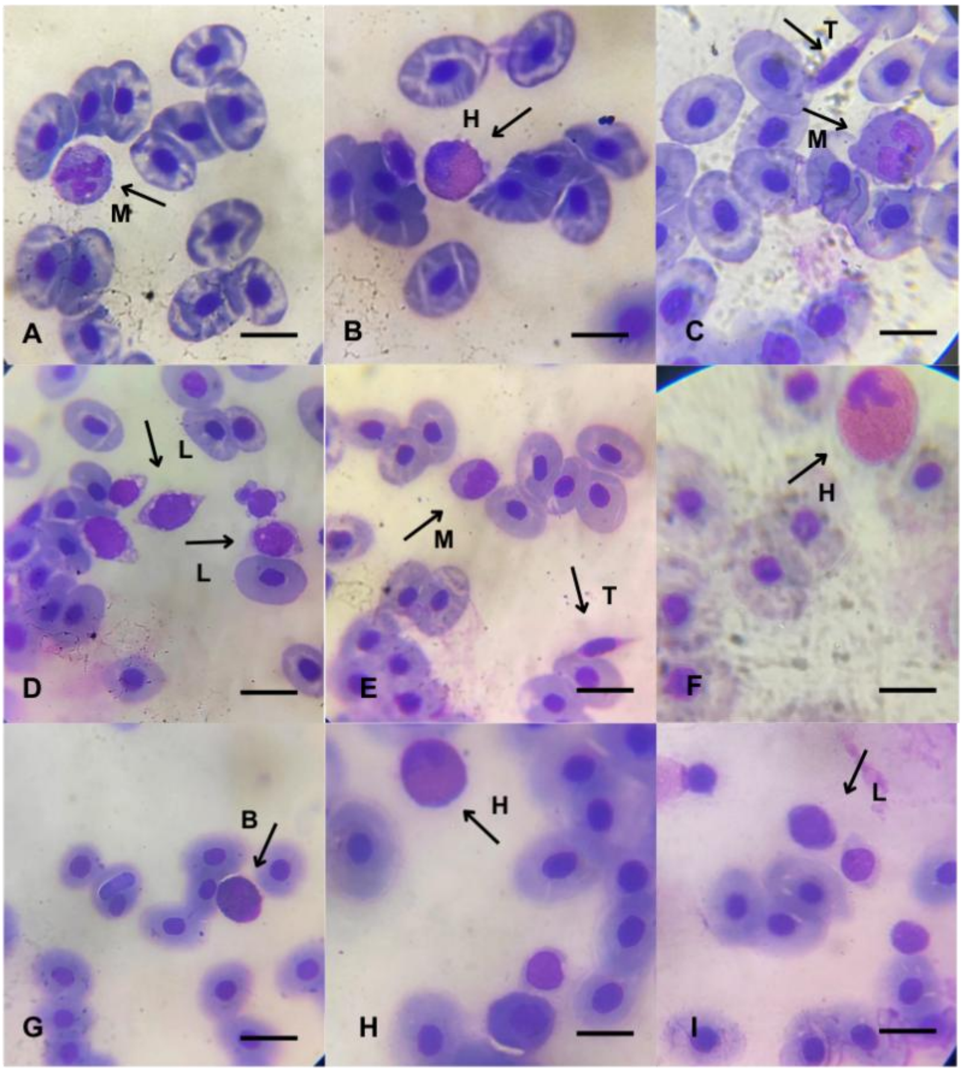

Figure 7: (A-I) Blood cells from three species of freshwater stingrays stained with May Grunwald-Giemsa-Wright. **(A)** M- Monocyte of *Potamotrygon motoro*; **(B)** H- Heterophile of *Potamotrygon motoro*; **(C)** M- bilobed monocyte *Potamotrygon. motoro*; **(D)** L- Leukocytes of *Paratrygon* spp.; **(E)** M-Monocyte of *Paratrygon* spp.; **(F)** H- Heterophile of *Paratrygon* spp.; **(G)** B- Basophil of *Potamotrygon wallacei*; **(H)** H- Heterophile of *Potamotrygon wallacei* e **(I)** L- Leukocytes of *Potamotrygon wallacei.*

The physical-chemical analyses of the water from the Tupé RDS and Praia do Açutuba presented values for the parameters of dissolved oxygen, temperature, pH, conductivity and total dissolved solids (Table 12).

Table 12: Mean values ± standard deviation of the physical and chemical parameters of the water in which each species of ray was captured in the lower Rio Negro, Amazonas, Brazil.

Species	Parameters				
	Dissolved Oxygen (mg L^{-1})	Temperature (ºC)	pH	Conductivity (mg L^{-1})	Total dissolved Solids (mg L^{-1})
Potamotrygon motoro	3.09 ± 0.37	31.34 ± 0	4.31 ± 0.05	11.33 ± 0.57	6 ± 0
Potamotrygon wallacei	3.47 ± 0.06	29.74 ± 1.05	4.27 ± 0.30	8.8 ± 1.95	4.53 ± 0.86
Paratrygon spp.	3.48 ± 0.05	31.4 ± 0.06	4.28 ± 0.03	12 ± 0	6 ± 0

4 DISCUSSION

In the biometric parameters of the stingrays captured in the lower Rio Negro (Table 8), it is observed that the three species are within the established pattern according to the development stages of freshwater stingrays, and when compared to the stingrays captured in the middle Rio Negro (Oliveira et al., 2016). Thus, the results of the present study presented similar results in biometry, with *P. wallacei* being one of the smallest species of freshwater stingray, limited to the Rio Negro basin (Duncan, 2016). While *P. motoro* and *Paratrygon* spp. are larger species and also distributed throughout the Amazon basin (Oliveira et al., 2017). The results demonstrated that females are larger than males, this larger size in female elasmobranchs is attributed to reproductive characteristics (Paesch and Oddone, 2020).

The blood physiology of these three species has already been established in other regions such as the middle Rio Negro (Oliveira et al., 2016, 2021) and the Solimões River (Santos et al., 2024). In this study of the lower Rio Negro region, the erythrogram (Table 9) showed that the hematocrit (Ht) of P. wallacei is lower than that of the other species. This is due to its small size, low metabolic demand, and adaptation to the flooded forest environment (igapó), in which they were captured (Oliveira, 2013). While P. motoro and Paratrygon sp., which are larger species, presented similar values. In addition, they were captured in beach environments, where there is more conductivity and less dissolved solids when compared to the igapó area. Hemoglobin (Hb) and hematometric parameters (MCH, MCHC) showed differences between species, with P. wallacei presenting higher values, demonstrating greater oxygen transport capacity, confirming that it is a physiological characteristic of adaptation to its habitat (Martin et al., 1978). However, the results of these indices are higher when compared to blood counts of *P. magdalenae, P. motoro, P. wallacei, Paratrygon aiereba* and *P. orbignyi* (Perez-Rojas et al., 2021; Brito et al., 2015; Oliveira et al., 2016, Santos et al., 2024). This result is possibly due to a physiological adaptation to the seasonal period, in which the river was still recovering from a severe drought.

No differences were observed in the RBC parameter between species, and the erythrogram did not show any difference between males and females. This result corroborates the studies by Oliveira et al., 2016, which emphasize that the sex of the species is not an influential factor in the red blood series.

Plasma biochemistry parameters (Table 10) are sources of metabolic energy in elasmobranchs and are strongly related to environmental conditions, such as prey abundance and quality, in addition to the biological cycles of reproduction and migration (Pethybridge et al., 2014). In this study, triglyceride and cholesterol values showed lower values in *P. wallacei* when compared to *P. motoro* and *Paratrygon* spp.. The size of the species is considered to be an influential factor; however, these values presented in the three species were lower when compared to other potamotrigonines (Brinn et al., 2012; Brito et al., 2015; Oliveira et al., 2016; Perez-Rojas et al., 2021), which may indicate a strong influence of feeding habits according to the availability of prey in that region (Shibuya, 2022). This is confirmed in the results of total proteins in which *Paratrygon* spp. demonstrated a higher level, due to its specific consumption of fish, unlike *P. motoro* and *P. wallacei*, which are species with more generalist eating habits (Shibuya, 2016; 2022). The glucose values of the three species, when compared to other studies, demonstrated consistent values; in this sense, in addition to the diet, it is possible to consider the seasonal period. Santos et al. (2020), in their study of blood physiology in different seasonal periods, demonstrated an increase in glucose levels in freshwater stingrays during periods of drought, reinforcing the idea of possible stress due to environmental stimulus.

There was no difference in albumin levels between species. However, this study showed that females of freshwater stingray species have higher cholesterol levels than males. This result may be mainly related to reproduction, in which after ovulation, females produce uterine fluids rich in lipids that are essential for the nutrition of their young (Hamlett et al., 1993). Due to the variation in plasma biochemistry values in response to environmental changes, developmental stage and, mainly, diet, more in-depth studies are needed that directly correlate the type of diet with the blood physiology of freshwater stingrays.

In the leukogram and thrombogram of stingrays, erythroblasts, mature erythrocytes, thrombocytes, lymphocytes, monocytes, heterophils, and basophils can be found, but there are no eosinophils, which suggests that heterophils are important in immune defense (Oliveira et al., 2021). In this study, there were significant differences in the leukocyte and thrombocyte values (Table 11) between species, and when compared to other leukocyte studies with freshwater stingrays, the values are very high (Brito, et al., 2015; Oliveira et al., 2016; 2021; Santos et al., 2024), however, similar to the results found in *P. magdalenae* from an artificial environment (Perez-Rojas et al., 2021). In this sense, it is suggested that total leukocytes and thrombocytes reflect immunological characteristics specific to the species and environmental variations (Oliveira et al., 2015).

In the differential leukocyte count of stingrays from the lower Rio Negro, similar lymphocyte values were observed for P. motoro and Paratrygon spp., when compared to P. wallacei, which demonstrated high values. In this sense, the natural environment (igapó) of P. wallacei should be considered as a determining factor for this result. In addition, the presence of hemoparasites in the cells of this species was observed during the leukocyte count. Oliveira et al., 2017) emphasize that hemoparasites can influence the number of leukocytes and attribute greater susceptibility to infection during periods of drought.

Regarding granulocytic leukocytes, the presence of heterophils and basophils was observed, but there was no difference between species, except for *Paratrygon* spp. in which there was no presence of basophils. Luer et al. (2004) emphasize that basophils are rarer in freshwater stingrays and heterophils are more common, both of which play an essential role in the immunological defense of stingrays. However, in the studies by Oliveira et al. (2016; 2021) carried out with *P. aiereba* from the middle Rio Negro, the presence of basophils in stingrays was reported, reinforcing the idea that different environments reflect on defense cells.

The physical and chemical parameters of the water from the lower Rio Negro were lower when compared to results from the middle Rio Negro (Oliveira, 2013), which suggests that some hematological parameters of the rays may be altered according to the spatial gradient of the rivers, adapting along the river (Vannote, et al., 1980). More studies should be done in the lower and upper Rio Negro to establish the physiological state of freshwater stingrays.

5 CONCLUSION

This study is the first to provide information on the physiological state of the blood of stingrays in the lower Rio Negro region, following a severe drought. In light of the climate changes that have been intensifying each year, this study provides a basis for better monitoring of the health conditions and conservation of freshwater stingray species in the wild, and even for management protocols in captivity.

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FINAL CONSIDERATIONS

Through blood physiology, it is possible to establish the health status of freshwater stingray populations from different habitats. However, studies related to hematology are still limited in terms of species diversity. Despite this, in recent years, these studies have been advancing and consolidating since consistent blood collection methods, anesthetics, and anticoagulants have been observed. This information is essential for carrying out further studies, thus filling the gaps that still exist regarding the biology and ecology of these species.

The stingrays from the lower Rio Negro region presented hematological differences between species, but not between sexes, showing that for the red series, sex was not an influential factor. However, for plasma biochemistry and white series, there were differences between species and sexes, suggesting that other factors can alter the physiological state of the species. In this study, it was considered that the severe drought may have affected the immune defense of these animals, and the characteristics of the environment in which they were captured.

Thus, this study provides an overview of blood physiology studies carried out with potamotrigonines through the systematic review and physiological information of stingrays from the lower Rio Negro, aiming to contribute to the conservation and management protocol of these species.