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Short Communication

Morphological characterization of blood cells in Amazon River dolphin *Inia geoffrensis*: a case study

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ABSTRACT. *Inia geoffrensis* is an endangered species of the Amazon River basin, but there has been limited research regarding its health, particularly in describing normal cell morphology by traditional techniques. This study aimed to identify the peripheral blood cells of *I. geoffrensis* through microscopic evaluation. Blood smears were collected from wild adults and stained with Wright's stain. We differentiated leukocyte cells (neutrophils, eosinophils, lymphocytes, and monocytes) and platelets. Additionally, we observed signs of inflammatory reactions in cell morphology by incrementing cell size and active cytoplasm in neutrophils, eosinophils, lymphocytes, and platelets. These findings provide important considerations for hemogram interpretation in future research and individual clinical cases in Amazon River dolphins. Also, our study delivers baseline information for future characterization and understanding of hemogram and leukogram changes in response to disease and health assessment for dolphin species.

Keywords: *Inia geoffrensis*; boto; blood cell morphology; microscopic evaluation; Amazon cetacean; characterization

The Amazon River dolphin or boto (*Inia geoffrensis*) is a cetacean endemic to the Amazon-Orinoco river basin, and it has recently been categorized as Endangered under the IUCN Red List of Threatened Species (Martin et al. 2004, da Silva et al. 2018). Although the total population size of this species is unknown, the boto population is decreasing (da Silva et al. 2018, Martin & da Silva 2021). The main threats are habitat degradation and fragmentation due to dams, deforestation, and fisheries interactions (bycatch and direct take) (McGuire & Aliaga-Rossel et al. 2010, Campbell et al. 2022). Therefore, there are regional and national conservation efforts to protect this species, including in Peru with Decreto Supremo N°007-2018-PRODUCE (El Peruano 2018). As a top predator, *I. geoffrensis* can be considered a sentinel species of freshwater ecosystems since it can reflect environmental hazards' potential negative health impacts (Bossart 2011). However, limited information is available concerning the health status of individuals and their populations, which could help elucidate the impacts of disease, toxins, climate change, and habitat degradation (Campbell et al. 2017, Mello & da Silva 2019).

A hematological analysis is an important tool for monitoring the health and disease of wildlife populations (Mello & da Silva 2019). Hematological parameters like red and white cell counts (RBC and WBC, respectively) are particularly important in helping

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diagnose any infectious condition or inflammation that might affect wild animals (Stacy & Nollens 2022). Furthermore, RBC and WBC's morphological characteristics can help clinicians identify cell lineages better and give a more accurate hemogram interpretation because these features could differ between species and populations (Schwacke et al. 2009, Stacy & Nollens 2022). Studies addressing blood values in *I. geoffrensis* are scarce (Best & da Silva 1993, Mello & da Silva 2019). Mello & da Silva (2019) produced the first hematological baseline values for *I. geoffrensis* to monitor health and stress variation during captures of individual adults, juveniles, and calves; however, they did not include morphological features of blood cells for botos in their study. This information would improve hemogram interpretation in this species. Only one study includes RBC and WBC morphological features in a river dolphin species, the Irrawaddy river dolphin (*Orcaella brevirostris*), in Southeast Asia (Techangamsuwan et al. 2010). Therefore, this study aimed to characterize the morphology of peripheral blood cells of *I. geoffrensis* through microscopic evaluation to help health professionals discern normal from abnormal features at a cellular level which can help understand the impacts of possible diseases, environmental stress, and physiological conditions in wild animals.

During June 2018 (dry season), eight adults of *I. geoffrensis* were captured for a habitat ecology project at the Pacaya-Samiria National Reserve (04°39'S, 73°49'W) alongside the Marañón River in Loreto region, Peru (Mosquera-Guerra et al. 2021, Campbell et al. 2023). This procedure involved satellite tagging and tissue sampling lasting up to 25 min in total under University of Exeter Ethics Committee approval (eCORN001707) and Peruvian research permits (RD 515-2018 PRODUCE and RJ 003-2018) (Mosquera-Guerra et al. 2021, Campbell et al. 2023). Capture and release, handling, and sampling methods were carried out following reviewed protocols for dolphin and Amazon dolphin species (Fair et al. 2006, Mosquera-Guerra et al. 2021). Capture locations were based on accessibility, river width, and water depth. In water courses wider than 300 m, a dolphin group was encircled with a 300 m fishing net with a 5 cm mesh size forming a half-moon with a radius of 100 m upstream. Each individual was untangled and carefully herded into a stretcher to a shaded riverside bank for the procedure. Animals were clinically examined after capture and monitored continuously for signs of stress by a qualified veterinarian in marine mammals following the protocol established by Fair et al. (2006). Blood was drawn from the caudal vascular bundle in the peduncle (Stacy & Nollens 2022) with a 10 cc syringe with an 18G/21G needle and placed in EDTA and serum chemistries tubes. The samples were stored on ice at 4°C. After 3 to 8 h, two to four blood smears were obtained per individual, were air-dried, and immediately fixed in methanol until microscopic evaluation. Methanol permanent fixation allowed us that, in October 2019, blood smears were stained with Wright's stain for erythrocyte and leukocyte morphological characterization via light microscopy at the Universidad Peruana Cayetano Heredia, Faculty of Veterinary Medicine and Zootechnics, Histology and Pathology Laboratory, Lima. Identifying peripheral blood cells was based on the nuclear appearance and cytoplasmic granular distribution, size, and shape of blood cells. The peripheral blood cells were observed and identified at 40 and 100x magnification on a Leica DM LS2 microscope using a Multicam Plus camera. The measurement of the identified blood cells was performed using the ImageJ software.

Blood was collected from four individuals. During the blood smear analysis, three types of peripheral blood cells were identified: erythrocytes, leukocytes, and platelets. These were similar to other mammals, particularly Cetacea (Harvey 2001, Nouri-Shirazi et al. 2017, Mello & da Silva 2019). Leukocytes were identified as granulocytes (neutrophils and eosinophils), monocytes, and lymphocytes. Platelets were also identified in blood films. No basophils were observed.

Erythrocytes were round with a mean diameter of 7.7 ± 0.6 µm (6.7-8.8 µm), biconcave shape with central pallor observed, and enucleated (Figs. 1b-c). Some blood smears presented erythrocytes with Rouleaux formation (Fig. 1a), which could be attributed to increased concentrations of fibrinogen and globulin proteins with prominent formations in some domestic animals like horses, cats, and pigs, and it is considered a normal finding in healthy dugongs and manatees (Harvey 2001, Owen et al. 2018). In bottlenose dolphins (*Tursiops truncatus*), Rouleaux formation indicated low flow velocities in central veins in clinically normal individuals (Seitz et al. 2016). *I. geoffrensis* individuals in our study remained calm with no anesthesia in the dorsoventral position for up to 25 min; even though the animal release was performed as soon as possible, there is a possibility that central veins could experience low intravascular flow. However, ultrasonography could only confirm this (Seitz et al. 2016). Red blood cell morphology was similar to those described for other dolphin and mammal species (Kiehl & Schiller 1994, Techangamsuwan et al. 2010, Nouri-

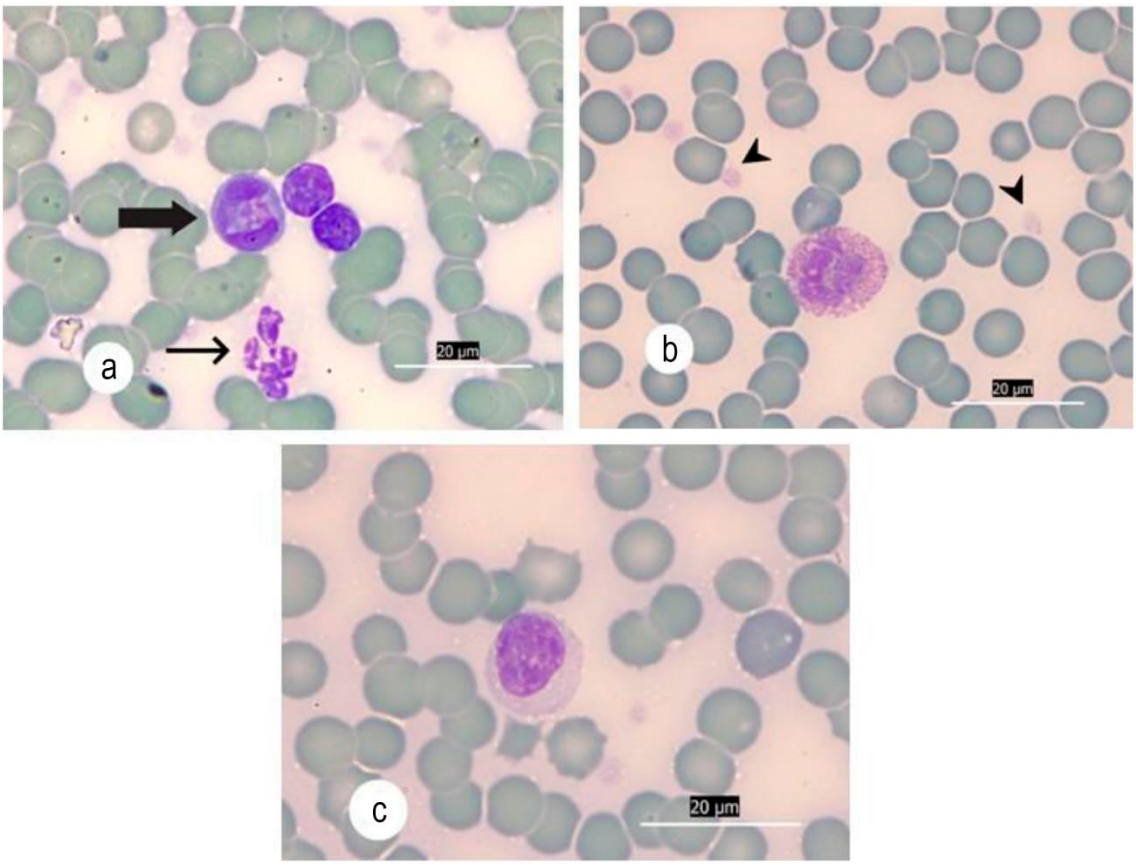

Figure 1. Photomicrographs of peripheral blood Wright-stained smears of Amazon River dolphin. a) Neutrophil with segmented nuclei (thin arrow), monocyte with a U-shaped nucleus (thick arrow), and lymphocytes with predominant nuclei, b) eosinophil with a segmented nucleus and acidophilic granules in its cytoplasm and platelets (arrowhead), and c) erythrocytes surrounding a large lymphocyte.

Shirazi et al. 2017, Nollens et al. 2018). Erythrocytes' mean diameters were slightly similar (5% bigger) to those reported in *T. truncatus* and 17% bigger than in the harbor porpoise (*Phocaena phocaena*) (Medway & Geraci 1964). Medway & Geraci (1964) reported that half of the six studied bottlenose dolphins were subjected to 36 h of stress due to transportation, but no changes related to erythrocytes were observed. These results do not imply any inflammatory changes, which, in contrast, could occur in WBC.

Leukocyte morphology identified in *I. geoffrensis* was similar to other dolphins and terrestrial mammals (Techangamsuwan et al. 2010, Nouri-Shirazi et al. 2017, Satyaningtijas et al. 2020). Neutrophils on Wright-stained blood films were 13.9 ± 2 µm (9.4-17.4 µm) in diameter and presented bi- to tri-lobed segmented nuclei with the presence of dust-like granules in their cytoplasm (Fig. 1a). Each segment of the nuclei was condensed with a mean diameter of 6.2 ± 1.7 µm (3.2-9.5 µm). Eosinophils have a similar size to neutrophils at 14.4 ± 1 µm (12.3-16.1 µm) in diameter, also with a condensed and segmented nucleus but with acidophilic granules in their cytoplasm (Fig. 1b). The segmented nuclei were slightly larger than the nuclei of neutrophils with a mean diameter of 10.6 ± 1.4 µm (8-13.3 µm). Granules' mean diameter ranged from 0.6 ± 0.2 µm (0.2-1.2 µm). Both neutrophils and eosinophils in *I. geoffrensis* were 26 and 60% bigger than in *O. brevirostris*, respectively (Techangamsuwan et al. 2010). When an inflammatory process occurs, neutrophilia and eosinophilia are present, generally related to bacterial or parasitic diseases, so their numbers increase in peripheral blood (Stacy & Nollens 2022). Variation in the size of these cells is not specifically reported in any dolphin or terrestrial mammal species except when toxicity is implied (Satyaningtijas et al. 2020, Stacy & Nollens 2022). Nevertheless, morphological structures also change in that case, which was not observed in our study. Monocytes presented a round shape with a mean

diameter of 11.3 ± 0.5 µm (10.3-11.9 µm) and a U-shape nucleus and granulocytes in their cytoplasm (Fig. 1a). The lobules of the U-shape nuclei presented a mean size of 7.6 ± 1.3 µm (5.2-8.8 µm).

We observed lymphocytes with a predominant nucleus (Fig. 1a), indicating a high nucleus-to-cytoplasmic (N:C) ratio with a mean diameter of 10.9 ± 2.9 µm (6.5-19.2 µm). Larger lymphocytes had a larger nucleus (13.1 µm diameter) as well as a paler and larger cytoplasm (Fig. 1c). The variable shape of their nucleus and basophilic cytoplasm distinguishes these large lymphocytes from monocytes and reactive lymphocytes, respectively (Harvey 2001, Stacy & Nollens 2022). Few normal size lymphocytes are found in healthy animals, with the highest N:C ratio in smaller cells (Harvey 2001, Nouri-Shirazi et al. 2017). Large lymphocytes with an N:C ratio larger than 1.0 are more common in ruminants than other domestic animals (Harvey 2001). Dolphins and ruminants are taxonomically related, sharing a common ancestor, so their physiology is very similar (Gatesy et al. 2013). The presence of large lymphocytes in our results could be explained due to this evolutionary feature. The typical lymphocyte morphology has been found and described in other dolphin species (Hall et al. 2007, Techangamsuwan et al. 2010, Nouri-Shirazi et al. 2017, Satyaningtijas et al. 2020). However, Nollens et al. (2018) explained that WBC in cetaceans could vary between blood samples taken over short intervals, affecting their interpretation. In our study, large lymphocytes were observed in more than one blood smear of the same individual. The smears were made from blood taken in short intervals for the same animal. Finally, platelets were 3.1 ± 0.7 µm (1.9-4.3 µm) in diameter, presenting an oval shape with small pseudopodia projecting from the membrane (Fig. 1b). These platelets are 50% larger than those thrombocytes reported in *O. brevirostris* (Techangamsuwan et al. 2010) which could be interpreted as the platelets in the Amazon River dolphin are more active or were activated due to their pale cytoplasm and hypogranular aspect (Harvey 2001).

In our study, RBC and WBC followed the regular morphology reported in marine and terrestrial mammals. However, the size difference in neutrophils and eosinophils, as well as the presence of large lymphocytes, could be considered morphological features to take into consideration when performing blood sampling for hematology analysis in *I. geoffrensis*. Our results complement baseline data in hematology and WBC differentiation for the health monitoring of Amazon River dolphin populations. Future similar studies could include larger sample sizes, cytochemical stains, and transmission electron microscopy for ultrastructural characterization and other geographic regions within the Amazon.

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