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Hematology and blood pH reference intervals for wild Spiny Butterfly Rays (*Gymnura altavela*) in the Canary Islands

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Abstract

Background Despite the popularity of elasmobranchs in zoos and aquariums, data on hematological values for health monitoring remain limited, particularly for wild populations. In this study, reference intervals for key hematological parameters and blood pH were established from blood samples of 49 adult females of the Critically Endangered Spiny Butterfly Ray (*Gymnura altavela*) collected across three shallow beaches in the Canary Islands. Additional data were obtained from four adults maintained under human care. To our knowledge, this is the first report of hematological and blood pH reference intervals for free-ranging *G. altavela*.

Results Lymphocytes (56%) were the dominant leukocyte, followed by heterophils (23.6%), eosinophils (16.7%), monocytes (2%), neutrophils (1.1%), and basophils (0.2%). Median values included 356.6 × 10^3 cells/μL for red blood cell count (RBC), 31.7 × 10^3 cells/μL for white blood cell count (WBC), 28.2% for packed cell volume (PCV), and 8.1 g/dL for total plasma solids (TS). Blood pH averaged 7.25, rising to 7.38 with temperature correction. These values remained consistent across reproductive states and housing conditions, suggesting minimal physiological disruption.

Conclusion This study highlights the effectiveness of rapid, low-stress sampling and provides essential baseline data to support veterinary care and conservation strategies for *G. altavela*. Expanding future research with a larger sample size will enhance understanding of health variations by age, sex, and reproductive status.

Keywords Elasmobranch, Ray, *Gymnura altavela*, Hematology, Blood, pH, Reference intervals, Wildlife, Conservation

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Introduction

The Spiny Butterfly Ray (*Gymnura altavela*) is a species of ray of the family Gymnuridae distributed among coastal waters of the Atlantic Ocean, Mediterranean Sea and Black Sea. In 2021, it was officially listed as Critically Endangered in the Mediterranean Sea and Europe and Endangered worldwide in the International Union for Conservation of Nature's (IUCN) Red List as a result to declining populations primarily because of fishing pressure and habitat destruction [1]. This is a large ovoviviparous ray species, with a disc width reaching up to 260 cm, which has an annual reproductive cycle and a gestation period of approximately six months with 2-7 pups [2, 3]. Although this species inhabits depths up to 150 m deep [2], during the summer and autumn months, a large number of individuals of Spiny Butterfly Rays gather in shallow waters of some sandy beaches of the Canary Islands [4]. This archipelago, of eight islands, stands as a sanctuary for the conservation of many species of elasmobranchs, which have stable populations on their coasts [5–7]. However, the constant flow of mass sun and beach tourism throughout the year causes significant anthropogenic pressure on the shallow water local marine fauna, in addition to other pressures such as commercial and illegal fishing and the growing impacts of climate change, which collectively contribute to the global decline of elasmobranchs [8–10].

Veterinary medicine of aquatic species involves certain complexities since many diseases have an acute presentation with non-specific symptoms and the diagnostic tests routinely used in terrestrial vertebrates are limited. Thus, hematology and blood gases analysis represent valuable, rapid and cost-effective tools for assessing health status of elasmobranchs, both in the wild and under human care, facilitating early detection of diseases and monitoring their progression [11–14]. Reference intervals are defined as the range of values considered normal for a given parameter in a healthy population and are essential for the objective interpretation of blood tests in clinical evaluation [15, 16]. Although elasmobranchs are very popular species in zoos and aquariums worldwide, there is limited information available on hematology and blood gas values, particularly on wild populations, and reference intervals have not yet been established for many species. Consequently, clinical veterinarians must extrapolate from the values of related species, although in some cases, these species are not as taxonomically closely related which may reduce the reliability of the interpretation. In addition to reference intervals, the evaluation of blood smears provides crucial information for veterinary clinicians in decision-making, as conditions such as left shift, toxic changes, and intracellular parasites can be observed [17, 18]. Blood cells in elasmobranchs exhibit unique characteristics, which make their evaluation challenging if the species is unfamiliar. Elasmobranchs possess distinct types of granulocytes, and both their morphology and staining properties vary among species [11, 19]. These differences have led to confusion and the adoption of different nomenclature systems for granulocytes over time [19]. Lastly, blood gases and pH analysis has proven to be a valuable tool for assessing stress levels in elasmobranchs after capture and handling events [20–23].

In this study, the main objectives were to identify blood cells and to provide reference intervals for key hematological parameters and blood pH in free-ranging Spiny Butterfly Rays of the Canary Islands. Additionally, this study provides data from animals kept under human care and compares these values with those obtained from the wild populations.

Materials and methods

Animals

The Spiny Butterfly Ray is a species whose capture is prohibited in European Union waters (40/2013 ARM fin/2689/2009) and is listed in Spain under the LESPRE (List of Wild Species under Special Protection Regime). Therefore, handling these animals requires authorization from the Ministry of Ecological Transition and Demographic Challenge of the Government of Spain. Thus, the entire procedure described below has the authorization and direct supervision of the inspectors from the Ministry to ensure animal welfare. Blood samples were collected from 51 wild Spiny Butterfly Rays caught and released from three different shallow beaches from two islands of the Canarian archipelago in Spain: Los Cristianos located on the island of Tenerife, and Pasito Blanco and Amadores both located on the island of Gran Canaria (Fig. 1). Sampling was conducted from September to October over three consecutive years (2022-2024). Water temperature was estimated using publicly available data from Portus (Puertos del Estado, Ministry of Transport and Sustainable Mobility of Spain) [24].

Animals were captured by scientists and experienced aquarists using hexagonal-shaped nets at depths not exceeding 2 meters. After capture, specimens were transferred to an open stretcher specifically designed for the physical restraint of these rays in permanent contact with water. Once there, the sampling process was conducted in less than eight minutes, as stipulated in the permit granted by the Ministry of Ecological Transition and Demographic Challenge of the Government of Spain. The animals were implanted with acoustic (V16 Vemco-Innovasea) and visual (T-Bar) tags while different biometric measures were taken, and blood was immediately drawn to minimize the effect of stress on blood parameters. Additionally, ultrasound examination was performed to assess the organs of the coelomic cavity. All individuals

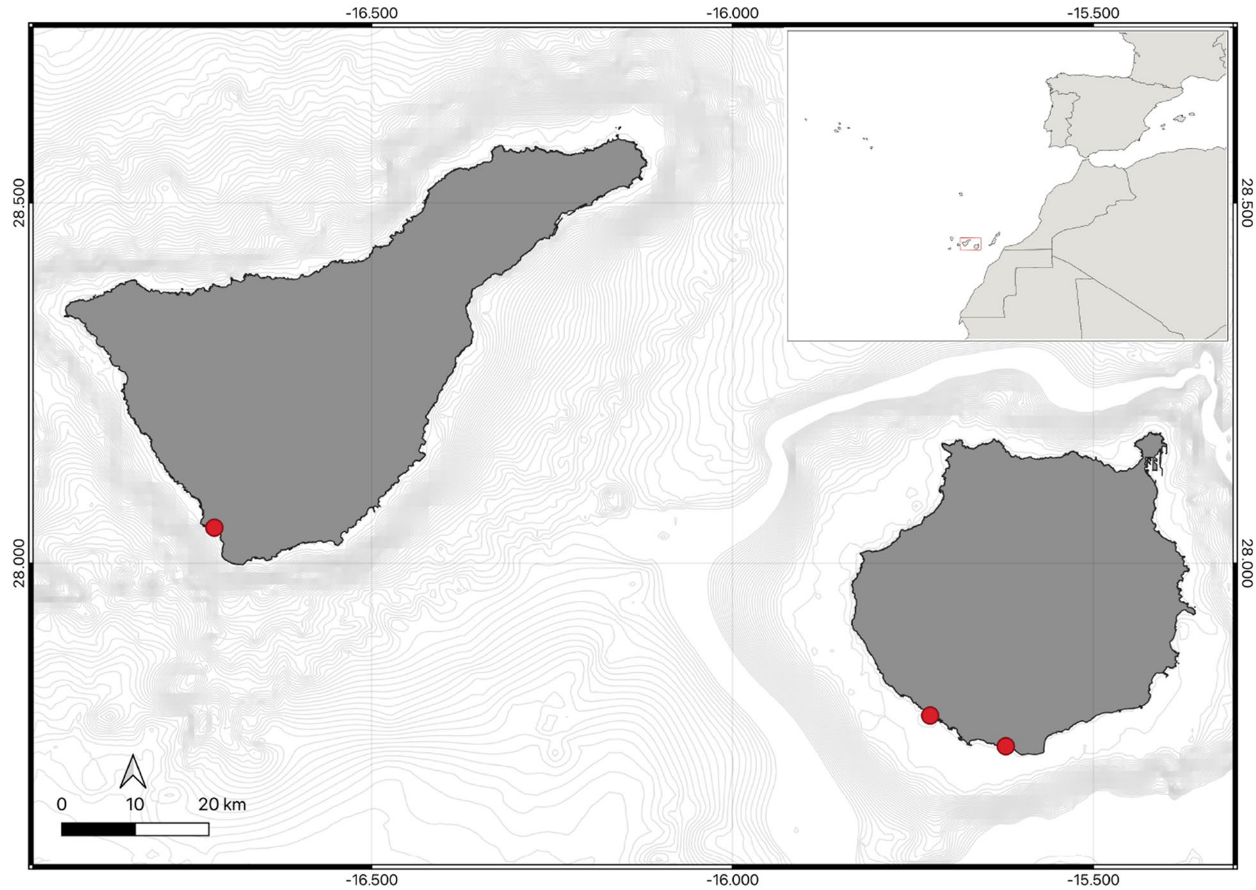

Fig. 1 Geographical situation of the Canary Islands and the sampling locations (from west to east: Los Cristianos, Amadores and Pasito Blanco)

sampled were considered clinically healthy on physical examination by the veterinary team. A total of 49 adult females (96.08%) and 2 juvenile males (3.92%) were sampled. This difference in sex distribution is because, during this time of the year, adult females tend to aggregate in shallow waters, presumably associated with seawater temperature and breeding behavior [4]. Due to this marked sex imbalance in the population sampled, the reference intervals analysis was limited to the adult females.

The adult females sampled exhibited disc widths ranging from 113 cm to 194 cm, with a mean of 158 cm (SD±13.6), while their total lengths ranged from 89 cm to 128 cm, with a mean of 107 cm (SD±7.8). The animals exhibited weights ranged between 20.3 kg and 68 kg, with a mean of 38.9 kg (SD±8.9). Ultrasonographic evaluation allowed for the diagnosis of gestation, based on the presence of developing embryos within the uterus. Thus, it was revealed that 30 females were pregnant, 4 were not, and in 15 cases, gestational status could not be determined due to time constraints or equipment unavailability.

Additional data is provided from blood samples from 4 adult Spiny Butterfly Rays (2 males and 2 females) maintained under human care in the Poema del Mar Aquarium (Las Palmas de Gran Canaria, Spain). These animals were housed in the 'Deep Sea', a 5500 m^3 tank, where water quality parameters are monitored daily. Mean temperature was 22°C, mean pH was 8.1 and mean salinity was 36.3‰. The animal density at the time of the study was approximately 0.001 kg/L. The mean weight of these animals was 23.7 kg, while the mean disc width was 136.3 cm and mean total length was 94.3 cm.

Sample collection and processing

The initial plan was to perform venipuncture in the caudal vein in the tail by ventral approach. However, due to the difficulty of this methodology in this species under field conditions, the dangerousness of the tail spine and the time limitation for sample collection, it was decided instead to use a ventral approach to the pectoral fin vasculature (Fig. 2). For this purpose, a methacrylate table (60x40 cm) was designed with a central opening (20x15 cm) that allowed access to the blood vessels of the pectoral fin vasculature while the pectoral wing is slightly elevated (Fig. 3). We collected blood samples using 21G and 23G needles attached to either 3 mL or 1 mL syringes, depending on the size of the animal. The volume of collected blood did not exceed 1% of the total weight of

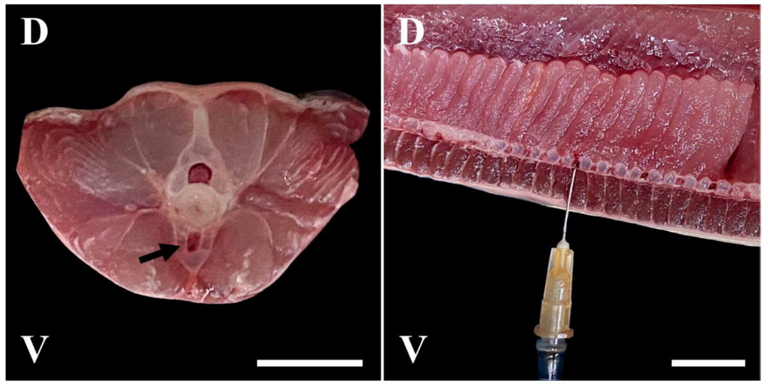

Fig. 2 Pictures of a Spiny Butterfly Ray necropsy (scale bar = 1 cm; (D) Dorsal side; (V) Ventral side). Left figure: Location of the caudal vein (arrow) in the tail of a Spiny Butterfly Ray, note that the calcified cartilage that surrounds this vessel may obstruct needle insertion for blood collection. Right figure: Sagittal section of the fin of a Spiny Butterfly Ray showing the pectoral fin vasculature between the ceratotrichia

Fig. 3 Venipuncture procedure. Elevation of the methacrylate table by the aquarists allows the veterinarian to access the pectoral fin vasculature of the ray for blood collection

the animal. After extraction blood was rapidly transferred into 1.3 mL lithium heparin anticoagulant tubes (Sarstedt™ Micro Sample Tube Li-Heparin LH). Within less than 5 minutes from sample collection, 100 µL were loaded into an CG4+ or CG8+ i-STAT® cartridge (Abbott Point of Care Inc.) for blood gas analysis, using a handheld blood analyzer (i-STAT® Alinity v). Blood smears were prepared for blood cell evaluation and differential leukocyte count, using the slide-to-slide technique, left to dry to ambient temperature and stored under cool and dry conditions. Fifty (50) µL of whole blood was preserved in Eppendorf tubes with 200 µL of 10% formalin for subsequent manual cell counts [25]. The methodology used to obtain blood samples from the animals maintained under human care was the same described for wild animals.

The blood samples were transferred within 6h of collection to the Poema del Mar Aquarium Laboratory, in Gran Canaria, or the Loro Parque Laboratory, in Tenerife. Samples that showed signs of hemolysis, clot formation or other alterations were not processed. Packed cell volume (PCV) was determined using microhematocrit tubes (BRAND Micro hematocrit capillary) centrifuged at 13000 rpm for 7 minutes in a centrifuge (Thermo Scientific, Heraeus Pico 17 microcentrifuge). Plasma total solids (TS) were determined using a clinical refractometer. Blood smears were stained using a Diff-Quick stain (T.R.H., Maim S.L.) and leukocyte differential counts were carried out by counting 100 consecutive leukocytes within the monolayer area of the smear for each sample, using high-magnification (100×oil immersion objective) in an optical microscope (Abaxis Global Diagnostics, Accu-Scope 3000-LED Series), and then determining cell percentages. Total estimation of red blood cell count (RBC) and total estimation of white blood cell count (WBC) were performed in an improved Neubauer chamber by using a 1:200 dilution of blood in Natt-Herricks solution without osmolarity adjustment. In addition, Wright, Giemsa and Brilliant Cresyl Blue stains were applied to blood smears from the animals maintained under human care at the Poema del Mar Aquarium, to assess whether these techniques improved the characterization of granules within granulocytes.

Statistical analysis

The determination of the reference intervals was performed following the guidelines established by the American Society for Veterinary Clinical Pathology (ASVCP) [16]. All statistical analyses were conducted using RStudio (version 5.4.1) and the package referenceIntervals [26]. Initially, a descriptive summary of the data generated, and histograms and boxplots were visually inspected to identify potential outliers. The Shapiro-Wilk test was applied to all variables in their original and Box-Cox transformed scales, aiming to normalize variables that did not originally follow a normal distribution. Subsequently, outlier detection was performed using Horn's method for normally distributed or normalized variables while van der Loo's method was applied to variables that could not be normalized [27]. In accordance with the guidelines [16], after identifying statistical outliers, a revised descriptive summary was generated excluding these values. For the construction of reference intervals, the parametric method with 90% confidence intervals for reference limits was used when the data were normally distributed or could be normalized. For non-normally distributed and non-normalizable data, the robust method with 90% Bootstrap confidence intervals was applied for the reference limits.

Results

Hematology

Eight blood cell types were observed: erythrocytes, lymphocytes, heterophils or fine eosinophilic granulocytes (FEG), eosinophils or coarse eosinophilic granulocytes (CEG), neutrophils or non-eosinophilic granulocytes (NEG), monocytes, basophils, and thrombocytes. Figure 4 illustrates all these different cell types in blood smears.

Erythrocytes presented the characteristic oval shape in fishes, with a central elongated nucleus. Lymphocytes exhibited the typical appearance, similar to that described in mammalians with a round shape, round nucleus and a small ratio between cytoplasm and nucleus and a basophil cytoplasm. Heterophils or FEG had a round shape with an eccentric nucleus that could be found segmented or not segmented, the cytoplasm contains small granules. Eosinophils or CEG were also round and easily recognizable because of their prominent pink rod-shaped granules, with an eccentric nucleus that could be observed segmented or not segmented. Neutrophils or NEG were round with a pale cytoplasm with no granules in it and an eccentric lobulated nucleus. Monocytes were round with a kidney shaped eccentric nucleus and blue-gray cytoplasm, which sometimes leads to difficulties to differentiate them from NEGs. Basophils were very rare to observe in blood smears, and they presented a round shape with an eccentric nucleus and a very basophilic cytoplasm and round basophilic granules. Thrombocytes were oval and elongated with a central and elongated nucleus and a light-gray cytoplasm. Presence of granulated thrombocytes was not observed in this species.

The data analyzed were divided into three groups: the total sample, the subgroup with confirmed pregnancy, and the subgroup with confirmed non-pregnancy. Descriptive statistics and reference intervals for total sample are presented in Table 1. The Supplementary Material includes descriptive statistics for both pregnant and non-pregnant subgroups, while reference intervals were derived solely for the pregnant subgroup (Table S1 and S2).

For both subgroups, the mean values lie within the reference intervals calculated for the total sample.

Blood pH

The i-STAT® measurements of pH are temperature-dependent and are measured at 37°C, but temperature can be corrected by introducing the patient's temperature on the chart page of the analyzer. As the Spiny Butterfly Ray is an ectothermic animal, blood sample temperature was estimated based on the seawater temperature at the time of collection (24°C). The mean pH was 7.25, while the temperature-corrected pH was 7.38. The variation in n between the two parameters is attributed to occasional device failures under field conditions. Furthermore, the limited number of i-STAT® cartridges available for this study precluded repeat measurements. Complete descriptive statistics and reference intervals for blood pH parameters are shown in Table 2 for the total sample. The Supplementary Material includes descriptive statistics for blood pH for both pregnant and non-pregnant subgroups, while reference intervals were derived solely for the pregnant subgroup (Table S3 and S4). Lactate reference intervals are provided in the Supplementary Material (Table S5); however, as the detection limit of the i-STAT® is 0.3 mmol/L, values below this threshold are reported as < 0.3, so an exact lower limit for the reference intervals cannot be determined.

The mean values of blood pH measurements of the subgroups lie within the reference intervals calculated for the total sample.

Hematological data from Spiny Butterfly Rays maintained under human care

Hematological values obtained from four adults Spiny Butterfly Rays, two females and two males, maintained under human care in the Poema del Mar Aquarium in Las Palmas de Gran Canaria (Spain), were comparable to those observed in wild Spiny Butterfly Rays. The mean values were: PCV 25.5%, TS 7.2 g/dL, RBC 312.5 $\times 10^3$ cells/μL and 44.5 $\times 10^3$ cells/μL. The differential leukocyte count showed mean percentages of 48.5% lymphocytes, 25.5% heterophils, 18.8% eosinophils, 2.3% monocytes and 0.8% basophils. Table 3 displays complete descriptive statistics for these hematological parameters.

Discussion

To the author's knowledge, this study provides the first-time reference intervals for hematological and blood pH in the Spiny Butterfly Ray. Although a minimum of 120 individuals is recommended to create reference intervals, in veterinary medicine with certain species this is not feasible, even more so when dealing with free-living animals, so smaller sample sizes are unavoidable [16, 28]. Thus, reference intervals can be determined with sample sizes of 20-40 animals using robust or parametric statistical methods and with sample sizes of 40-120 animals parametric, robust and non-parametric methods can be

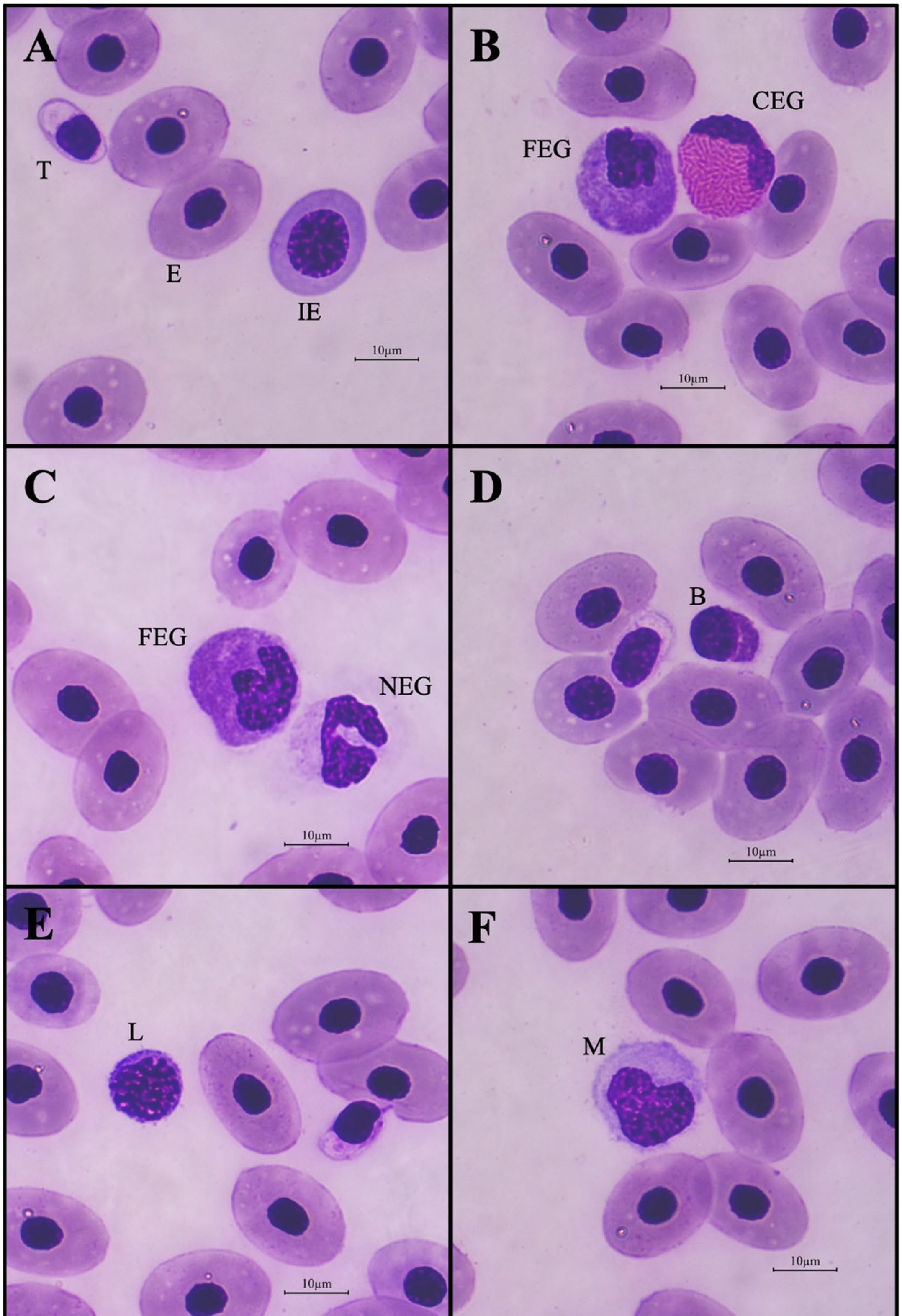

Fig. 4 Blood smears of Spiny Butterfly Rays (Diff-Quick, 100x). B = basophil, CEG = coarse eosinophilic granulocyte or eosinophil, E = erythrocyte, FEG = fine eosinophilic granulocyte or heterophil, IE = immature erythrocyte, L = lymphocyte, M = monocyte, NEG = non-eosinophilic granulocyte or neutrophil, T = thrombocyte

Table 1 Hematological reference intervals for free-ranging adult females Spiny Butterfly Rays

Analyte	n	Mean	SD	Median	Min	Max	RI	LRL	URL
PCV (%)	43	28.2	4.9	29	14	36.5	16.4-35.2	3.6-20.6	34.0-36.3
TS (*g/dL*)	38	8.1	0.9	8.1	6.2	9.8	6.4-9.8	6.0-6.8	9.4-10.1
RBC $(10^3/\mu L)$	41	356.6	113.4	350	110	640	134.4-578.8	84.6-184.2	529.0-628.6
WBC $(10^3/\mu L)$	39	31.7	10.7	29.5	14.5	56.5	13.8-55.9	11.0-17.0	49.3-63.1
L (%)	48	56.0	10.6	58	37	76	33.9-78.4	28.1-37.6	74.4-82.1
M (%)	47	2.0	1.6	2	0	6	0-6	0-0	4-5
H (%)	48	23.6	11.6	21	6	51	6.6-52.6	4.8-8.7	44.5-61.6
N (%)	46	1.1	1.22	1	0	4	0-3.5	0-0	3-4
E (%)	48	16.7	6.3	16	6	36	4.4-29.0	1.8-6.9	26.4-31.5
B (%)	47	0.2	0.4	0	0	1	0-1	0-0	1-1

[1]n, number of individuals; SD, standard deviation; Min, minimum; Max, maximum; RI, reference intervals; LRL, 90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit; PCV, packed cell volume; TS, total solids in plasma; RBC, red blood cell count; WBC, white blood cell count; L, lymphocyte; M, monocyte; H, heterophil; N, neutrophil; E, eosinophil; B, basophil

Table 2 Blood pH reference intervals for free-ranging adult females Spiny Butterfly Rays

Analyte	n	Mean	SD	Median	Min	Max	RI	LRL	URL
pH	38	7.25	0.11	7.22	7.1	7.4	7.03-7.46	6.98-7.08	7.41-7.51
pH*	27	7.38	0.08	7.38	7.3	7.6	7.25-7.57	7.22-7.28	7.5-7.65

[2]n, number of individuals; SD, standard deviation; Min, minimum; Max, maximum; RI, reference intervals; LRL, 90% confidence interval of the lower reference limit; URL, 90% confidence interval of the upper reference limit. pH* is the pH with temperature correction applied

Table 3 Hematological data from adult aquarium-housed Spiny Butterfly Rays in Poema del Mar

Ref. Animal	PCV (%)	TS (*g/dl*)	RBC $(10^3/\mu L)$	WBC $(10^3/\mu L)$	L %	M %	H %	N %	E %	B %
1(♀)	29	8.5	340	33.5	57	1	18	0	24	0
2(♀)	28	7.3	340	46	43	4	24	0	21	0
3(♂)	20	6.8	330	49	47	2	24	0	24	3
4(♂)	25	6.2	240	49.5	47	2	36	0	6	0
Mean	**25.5**	**7.2**	**312**	**44.5**	**48.5**	**2.3**	**25.5**	**0**	**18.8**	**0.8**

[3]PCV, packed cell volume; TS, total solids in plasma; RBC, red blood cell count; WBC, white blood cell count; L, lymphocyte; M, monocyte; H, heterophil; N, neutrophil; E, eosinophil; B, basophil

used [16]. The baseline health values provided in this work are from clinically healthy wild Spiny Butterfly Rays populations, these data add to the limited information available on hematological and blood pH reference values in elasmobranchs and may serve as guidance to veterinary professionals in interpreting blood tests for early disease detection and health status evaluation. As this species is Critically Endangered and has recently shown successful reproduction in individuals maintained under human care, this information is crucial for monitoring their health and welfare [3].

Ultrasound examinations performed during sampling revealed pregnancy or indications of recent parturition in some animals, which may have influenced the values obtained in this study. Nevertheless, no significant differences were detected in pregnant individuals, and all measured parameters remained within the reference intervals established for the overall population. Due to the limited number of confirmed non-pregnant animals, it was not possible to calculate separate reference intervals or conduct a robust comparative analysis. It would be advisable to extend the study by increasing the sample size and include plasma biochemistry parameters in the analysis. This would allow for the establishment of distinct animal subgroups with a n that allows to assess variations based on factors such as age, sex and reproductive status.

It is important to acknowledge that animals are inevitably exposed to stressors during the sampling process, primarily due to capture and handling. Although these procedures were completed in under eight minutes, such stress may still influence the parameters evaluated. Previous studies have shown that stress levels and capture duration can affect post-capture survival in sharks and rays [20–23]. Nonetheless, the swift and efficient performance of the trained team, along with the practice of keeping animals submerged throughout sampling, helped minimize hypoxic conditions.

To date, no carcasses of Spiny Butterfly Rays bearing acoustic or visual tags implanted during this study have been reported to the anatomopathological diagnostic service of the IUSA by the Canarian Wildlife Surveillance Network (Red Vigía Canarias, Government of the Canary Islands).

Hematology

The mean PCV obtained for wild Spiny Butterfly Ray was similar or slightly higher (28.2%, SD=±4.9) compared to those reported in other species of saltwater batoids (mean calculated from males and females) as the Bowmouth Guitarfish (*Rhina ancylostoma*) (22.2%, n=5, aquarium-housed), Southern Stingrays (*Dasyatis americana*) (25%, n=15, aquarium-housed), Cownose Rays (*Rhinoptera bonasus*) (26.6%, n=47, wild-caught), Spotted Eagle Rays (*Aetobatus narinari*) (28.1%, n=18, aquarium-housed) and juvenile Undulate Rays (*Raja undulata*) (15.3%, n=43, aquarium-housed) [29–33]. In addition to species-specific physiological traits, such variability may be influenced by the venipuncture site, as observed in elasmobranchs [31–34], and by the duration of centrifugation [12]. In the present study, a lower mean PCV was observed in aquarium-housed Spiny Butterfly Rays (25.5%, SD=±4), although this value remained within our reference intervals for wild specimens. Nonetheless, a larger sample size is required to enable more robust statistical comparisons and to better assess potential influences of housing conditions on hematological parameters. In this study, wild Spiny Butterfly Rays exhibited a mean TS concentration of 8.1 g/dL (SD=±0.9), while individuals housed at the Poema del Mar Aquarium showed a mean of 7.2 g/dL (SD=±1). These values are marginally higher than those reported for other aquarium-housed batoids, including Spotted Eagle Rays (*A. narinari*) (5.7 g/dL) [35], Southern Stingrays (*D. americana*) (7.0 g/dL) [31], and Cownose Rays (*R. bonasus*) (5.6 g/dL) [36]. These differences may be attributed to species-specific physiological traits, environmental conditions, or husbandry practices. However, a more robust comparison would require a larger sample size of animals maintained under human care to account for individual variability and potential confounding factors. Additionally, plasma total solids in this study were measured using a clinical refractometer. It is important to acknowledge that refractometer readings may vary slightly between devices, as minor inter-instrument discrepancies have been previously reported [37, 38].

The mean red blood cell (RBC) count observed in wild Spiny Butterfly Rays was 356.6 $\times$ 10^3 cells/µL (SD=±113.4), aligning with values previously reported for other batoid species [30]. Conversely, the mean white blood cell (WBC) count recorded in this study was 31.7 $\times$ 10^3 cells/µL (SD=±10.7), which is moderately elevated relative to findings from comparable research [31–33]. It is noteworthy that the specimens were wild-caught and, although they appeared clinically healthy upon physical examination, they may have harbored parasitic infections or other subclinical conditions potentially affecting hematological parameters [17].

Lymphocytes represented the predominant leukocyte type (56%), followed by heterophils or fine eosinophilic granulocytes (23.6%) and eosinophils or coarse eosinophilic granulocytes (16.7%), consistent with findings reported in previous studies on elasmobranchs [12, 17, 33, 35, 36, 39, 40]. Neutrophils (non-eosinophilic granulocytes; 1.1%) and basophils (0.2%) were present in very low proportions and were absent in the majority of samples—a pattern commonly observed in the blood of healthy batoids [17, 35, 36, 39, 40]. However, we acknowledge that the neutrophil-like cells observed could also represent degranulated or poorly stained FEGs. Although various staining techniques were employed for blood smear preparation, optimal results were achieved using rapid Romanowsky-type staining (Diff-Quik).

Blood pH

Blood pH analysis serves as a valuable tool for assessing acute stress levels in elasmobranchs following capture and handling [41]. Field measurements of this parameter can be easily conducted using portable devices such as the i-STAT® Alinity v handheld blood analyzer. The reliability of this system for measuring blood pH has been validated in both teleost fish and elasmobranchs, confirming its suitability for these species [42, 43]. In contrast, other parameters such as lactate have not been formally validated in elasmobranchs; while these measurements can still provide useful information, their interpretation should be made with caution. Moreover, i-STAT® enables the assessment of additional parameters. However, the blood gas parameters (PO_2 and PCO_2) exhibit high variability when measured with portable devices in these species [43], so this data requires cautious interpretation and was not included in the present study.

In this study, the mean blood pH recorded for wild Spiny Butterfly Rays was 7.25, and 7.38 after applying temperature correction. These values fall within the range previously reported for saltwater stingrays (7.3-7.6) [44] and closely match the mean value of 7.33 reported for Southern Stingrays (*Hypanus americanus*) [31]. Notably, our results are slightly higher than those documented for other wild elasmobranch species following capture, including Bull Shark (*Carcharhinus leucas*) (7.11), Bonnethead Shark (*Sphyrna tiburo*) (7.22), and Lemon Shark (*Negaprion brevirostris*) (7.19) [29]. For lactate, our values ranged from below 0.3 to 1.44 mmol/L, within the reported ranges of < 3 mmol/L in saltwater stingrays [44] and 0.3-2.7 mmol/L in *H. americanus* [31].

Conclusions

This study establishes, for the first time, reference intervals for hematological parameters and blood pH in wild Spiny Butterfly Rays, contributing valuable baseline data for veterinary assessment and conservation efforts. Despite the limitations posed by a modest sample size and the challenges of working with free-living, Critically Endangered animals, the results offer reliable indicators of health status. The findings underscore the importance of rapid, low-impact sampling techniques and highlight the need for expanded research incorporating plasma biochemistry and larger cohorts to refine subgroup analyses. These insights are especially relevant for monitoring the wellbeing of individuals maintained under human care and support broader efforts to safeguard this vulnerable species.

Abbreviations

ASVCP	American Society for Veterinary Clinical Pathology
CEG	Coarse eosinophilic granulocyte
FEG	Fine eosinophilic granulocyte
IUSA	Institute of Animal Health and Food Safety
IUCN	International Union for Conservation of Nature
NEG	Non-eosinophilic granulocyte
PCO^2	Partial pressure of carbon dioxide
PO^2	Partial pressure of oxygen
PCV	Packed cell volume
RBC	Red blood cell
TS	Total solids
WBC	White blood cell

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-05240-7.

Supplementary Material 1.

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Authors' contributions
Conceptualization, GMH and ACM; methodology, GMH; software, ASdP; validation GMH, MJC and ACA; investigation, GMH, MJC, ACA, LCH, DJA, AER, AGM, LCM, JJCH, FG, EPG, CFM, ACA; resources, DJA, AER and CFM; data curation, GMH and ASdP; writing-original draft preparation, GMH; writing-review and editing, all authors; funding acquisition, ACA and DJA. All authors have read and agreed to the published version of the manuscript.

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Data availability
All data used in the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The animal study protocol was approved by the Ethics Committee) of the University of Las Palmas de Gran Canaria (OEBA_ULPGC_05/2025).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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