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Standardizing a Method for Using Peripheral Blood Smears to Estimate Leukocyte Count in Sharks

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Introduction

Elasmobranchs have an immune system comparable to other vertebrates and they exhibit both innate and adaptive immune responses. Sharks have been used as model organisms for understanding how the immune system evolved across phyla and have been shown to have the same underlying molecular and cellular mechanisms observed in mammals. Peripheral blood cells are responsible for orchestrating immune responses. Sharks have peripheral blood leukocytes that have similar morphologies to their avian, reptilian, and mammalian counterparts. Peripheral blood smears (PBS) can be used to study the morphology and frequency of leukocyte types. Sharks have been shown to have multiple types of granulocytic cells in peripheral blood (neutrophils, fine eosinophilic granulocytes (FEG), coarse eosinophilic granulocyte (CEG), and basophils) that together are likely important as innate immune cells. They also contain mononuclear cells in the form of lymphocytes and monocytes. Cell counts can be conducted in whole blood and have been traditionally used in both human and veterinary medicine as a determination of health status. Studying these health markers in individuals in a wild population can be used to establish reference intervals (RI) for each species.

Currently, standardized nomenclature of the morphological characteristics of leukocyte types for each species and methods for using peripheral blood smears in shark studies are lacking. Conducting differential counts on peripheral blood smears can allow us to evaluate the proportion of cell types present and calculate the granulocyte-lymphocyte ratio (GLR) which can be used as a health marker. Complete blood counts (CBC) can also be used to assess health, but they require that counts be performed on fresh whole blood which can be difficult to do with wild caught sharks. As a result, peripheral blood smears have been used to estimate a complete blood count (CBC) of total leukocyte cells per milliliter of blood in wildlife medicine by calculating a cell count factor. The purpose of this study was to define the morphological characteristics of the leukocyte types in three species of shark and to use peripheral blood smears to formulate a calculated cell count factor (CCF) that can be applied to each study species to estimate CBC.

Methods

Study species: bull sharks (*Carcharhinus leucas*), blacknose sharks (*Carcharhinus acronotus*), and blacktip sharks (*Carcharhinus limbatus*)

- Sharks were caught using a circle-hook drumline system and blood samples were taken via venipuncture of the caudal vein
- Peripheral blood smears (3x) were prepared and stained (Giemsa stain) in the field for 10 individuals for each species
- Cell morphology for leukocyte types in each species were determined under light microscopy (100x oil immersion)
- Differential leukocyte counts (per 100 cells) were performed under light microscopy 100x oil immersion
- Mean number of each leukocyte type and 95% CV were determined as well as the mean and range of GLR values for each species
- Total leukocyte counts in fields at 40x magnification were performed to calculate the cell count factor necessary to obtain counts comparable to estimated CBC values found in the literature for shark species
- Minimum and maximum CCF values for each species were determined

Figure 1: Shark Blood Sampling performed by the UM Shark Research and Conservation Program.

Figure 2: A Bull Shark (https://www.aquariumofpacific.org/onlinelearningcenter/species/blacktip_reef_shark)

Figure 3: A Blacktip Shark (https://www.rbfishingcharters.com/bull-sharks/)

Figure 4: A Blacknose Shark (https://www.worldlifeexpectancy.com/fish-life-expectancy-blacknose-shark)

Granulocyte Morphology

Neutrophil

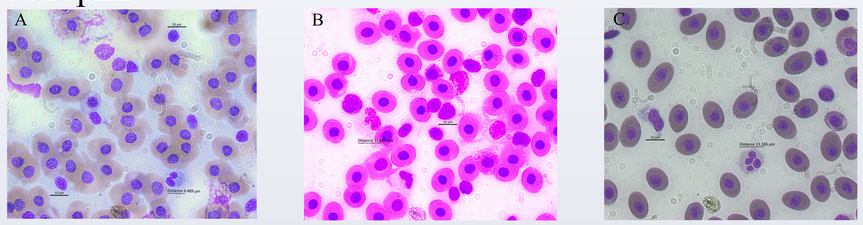

Figure 5: Neutrophils under light microscopy for **(A)** *Carcharhinus leucas* measured: 9.489 µm, **(B)** *Carcharhinus acronotus* measured: 12.585 µm, and **(C)** *Carcharhinus limbatus* measured: 13.165 µm. All images were at a magnification of 100x using oil immersion; scale bar:10 µm.

FEG

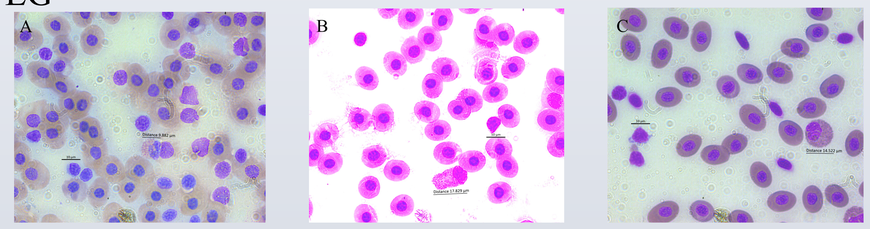

Figure 6: FEGs under light microscopy for **(A)** *Carcharhinus leucas* measured: 9.882 µm, **(B)** *Carcharhinus acronotus* measured: 17.829 µm, and **(C)** *Carcharhinus limbatus* measured: 14.522 µm; magnification 100x, scale bar:10 µm.

CEG

Type 1 Type 2

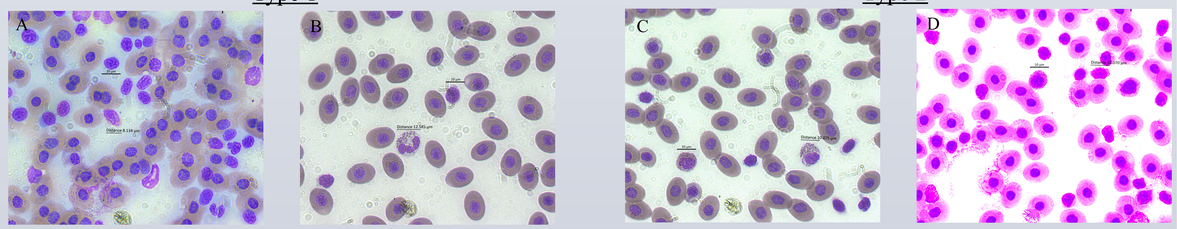

Figure 7: Type one CEGs under light microscopy for **(A)** *Carcharhinus leucas* measured: 8.134 µm and **(B)** *Carcharhinus limbatus* measured: 12.585 µm. Type two CEGs under light microscopy for **(C)** *Carcharhinus limbatus* measured: 10.075 µm and **(D)** *Carcharhinus acronotus* measured: 10.070 µm; magnification 100x, scale bar:10 µm.

Basophil

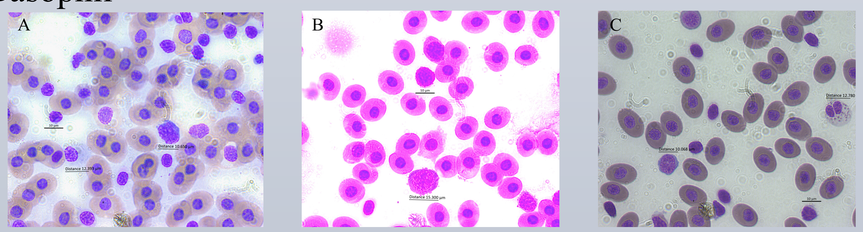

Figure 8: Basophils under light microscopy for **(A)** *Carcharhinus leucas* measured: 10.650 µm, **(B)** *Carcharhinus acronotus* measured: 15.300 µm, and **(C)** *Carcharhinus limbatus* measured: 10.068 µm; magnification 100x, scale bar:10 µm.

Differential Counts

Table 1: Leukocyte differential counts and the granulocyte-to-lymphocyte ratios (GLR) for **(A)** *Carcharhinus leucas*, **(B)** *Carcharhinus acronotus*, and **(C)** *Carcharhinus limbatus*. The GLR is the sum of granulocyte cells (FEG, CEG, Neutrophil, and Basophil) divided by the number of lymphocytes. Data was analyzed at 95% confidence.

A

Cell Type	Total Avg.	SD	95% CV	Min	Max
Lymphocyte	70.21	10.83	2.269	43	90
CEG	7.244	6.097	1.277	0	36
CEG2	N/A	N/A	N/A	N/A	N/A
FEG	5.611	3.762	0.7879	0	20
Neutrophil	6.4	3.534	0.7402	1	23
Monocyte	0.156	0.4952	0.1037	0	3
Basophil	10.4	6.699	1.403	0	24
Thrombocyte	15.98	14.53	3.043	1	55
Immature	0.8222	1.045	0.2188	0	4
GLR	**0.4376**	**0.1566**	**0.1120**	**0.1971**	**0.7156**

B

Cell Type	Total Avg.	SD	95% CV	Min	Max
Lymphocyte	70.28	11.17	2.34	34	93
CEG	4.822	3.012	0.6307	0	12
CEG2	N/A	N/A	N/A	N/A	N/A
FEG	8.722	5.321	1.115	2	25
Neutrophil	6.644	4.455	0.9331	0	22
Monocyte	0.3222	0.7469	0.1564	0	5
Basophil	9.278	6.278	1.315	0	26
Thrombocyte	11.68	7.865	1.647	0	38
Immature	2.667	2.652	0.5555	0	10
GLR	**0.4380**	**0.1799**	**0.1288**	**0.2265**	**0.8252**

C

Cell Type	Total Avg.	SD	95% CV	Min	Max
Lymphocyte	68.36	7.904	1.656	51	87
CEG	12.58	4.258	0.8919	3	23
CEG2	3.522	3.029	0.6344	0	12
FEG	12.46	4.394	0.9203	4	28
Neutrophil	0.6	1.099	0.2303	0	8
Monocyte	0.8333	1.256	0.2632	0	5
Basophil	1.711	1.351	0.283	0	6
Thrombocyte	59.02	29.31	6.139	5	121
Immature	1.478	1.756	0.3678	0	8
GLR	**0.4592**	**0.1037**	**0.0742**	**0.2376**	**0.5755**

Estimating Complete Blood Count (CBC)

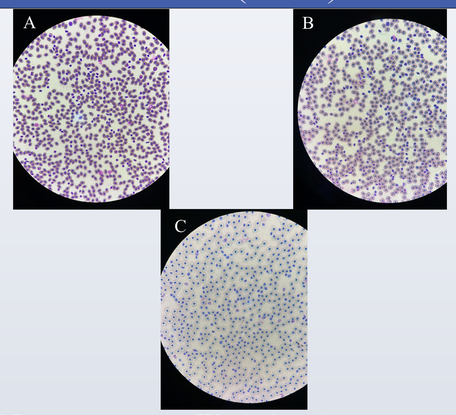

Figure 9: Fields on peripheral blood smear (PBS) under 40x magnification used to estimate CBC for **(A)** *Carcharhinus limbatus* , **(B)** *Carcharhinus acronotus*, and **(C)** *Carcharhinus leucas* . Total leukocytes were counted for 15 40x fields (5 fields per smear) for each shark. These values were used to calculate the cell count factor (CCF).

Calculating CCF

$$CCF = \frac{\text{expected CBC}}{\text{total avg. \# leukocytes at 40x}}$$

Figure 10: Equation used to calculate cell count factor (CCF) using CBC estimates. Total leukocyte count was averaged across 15 fields per individual. The estimated CBC obtained from literature was 5-10 x 10^6. The estimated CBC was divided by the mean leukocyte count per individual to get both a minimum and maximum CCF. The mean minimum and maximum CCF for each species was determined (Table 2).

Table 2: Mean of total leukocyte counts and minimum/maximum CCF values for *Carcharhinus leucas*, *Carcharhinus acronotus* , and *Carcharhinus limbatus*. Minimum CCF value was calculated by dividing 5 x 10^6 by the mean leukocytes per individual. The mean of those values was determined to be the min. CCF per species. Maximum CCF value was calculated by dividing 1 x 10^7 by the mean leukocytes per individual. The mean of those values was determined to be the max. CCF per species.

Species	Mean Leukocyte Count (40x)	CCF Min	CCF Max
Carcharhinus leucas	86.09	5.81 x 10^4	1.16 x 10^5
Carcharhinus acronotus	86.05	5.81 x 10^4	1.16 x 10^5
Carcharhinus limbatus	82.51	6.05 x 10^4	1.21 x 10^5

Conclusions

- Morphological characteristics of leukocyte types are variable across each species and within individuals
- Differential counts were estimated based on morphological characteristics for each species (95% CV)
- Mean GLR is relatively consistent within the genus Carcharhinus, which means GLR may be a useful health indicator for this species
- The minimum and maximum CCF values of each species are relatively consistent, which is promising in terms of determining a standard CCF value for sharks in the future
- Major limitation of this study was not having access to fresh CBC, however, consistent counts at 40x within and across species indicates that a CCF can be determined to estimate CBC across all three species from PBS

Acknowledgements

I want to especially thank Dr. Liza Merly for giving me the opportunity to perform this research project and for guiding me through every step of the way. I would also like to thank Dr. Josefina Olascoaga, Prof. Mohammed Moniruzzaman, and the UM Shark Research and Conservation Team.