UNIVERSITY OF MIAMI
ROSENSTIEL SCHOOL of MARINE & ATMOSPHERIC SCIENCE

STANDARDIZING PERIPHERAL BLOOD LEUKOCYTE COUNTS IN SEVERAL SHARK SPECIES

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Introduction

- Blood samples from wild sharks can provide an efficient way to gain insight into an animal's health status
- Peripheral blood leukocyte (PBL) counts are one way to assess the immune status or health condition of an animal
- Measures of shark health are scarce in the literature and have mainly involved their care in an aquarium setting; information on hematological assessments in wild sharks is rare and is related to the difficulty of in-field testing
- Standardized methods for assessing peripheral blood counts in wild sharks are needed to provide a comprehensive picture of normal health and physiology in a variety of species that have not been studied extensively
- Due to the nucleation of erythrocytes in sharks (and most non-mammalian vertebrates) the automated cell count method does not work
- Obtaining accurate total leukocyte counts from peripheral blood smears that can be obtained in the field, fixed, stained, and evaluated later is a possible solution to some of these challenges
- To obtain estimation of complete blood counts (CBC) from peripheral blood smears, it is necessary to determine the calculated count factor or CCF that yields the total count based on the number of leukocyte counted in 40x field under light microscopy
- The first specific aim was to determine whether a peripheral blood smear could be used to conduct a total white blood cell count (CBC) and establish the CCF appropriate for use with shark blood
- The second specific aim was to evaluate PBL in the Lemon shark (*N. brevirotsris)* as a sample species to determine what leukocyte types are present and conduct differential counts
- Establishing methodologies to conduct effective complete blood counts and differential counts can provide critical information for studies assessing the health condition of wild sharks

Methods

- Sharks whole blood was collected by caudal venipuncture by the Shark Research and Conservation team at the University of Miami during regular shark tagging trips between September 2019 and February 2020

For the CBC Estimation project:

- 5mL of anticoagulated blood was mixed with 995μl of Natt & Herrick solution
- WBCs stained by solution were counted on a hemocytometer immediately following collection to determine CBC count
- For the same shark sample, multiple counts of stained leukocytes were counted on the blood smear at 40x
- The calculated count factor or CCF was determined using the equations developed in Figure 5

For Lemon shark differential counts:

- Leukocytes were observed under light microscopy under oil immersion at 100x and the cell types were compared to other known leukocytes to identify cells present
- For each blood smear, replicate counts of 100 leukocytes were made for the different leukocytes observed; the number of thrombocytes and immature cells seen for every 100 leukocytes was recorded
- Cell diameters of each cell type (min. 10 cells) were determined using Zen software program
- Granulocyte to Lymphocyte ratio was recorded for each shark
- Descriptive statistics for cell counts were determined using Excel

Results

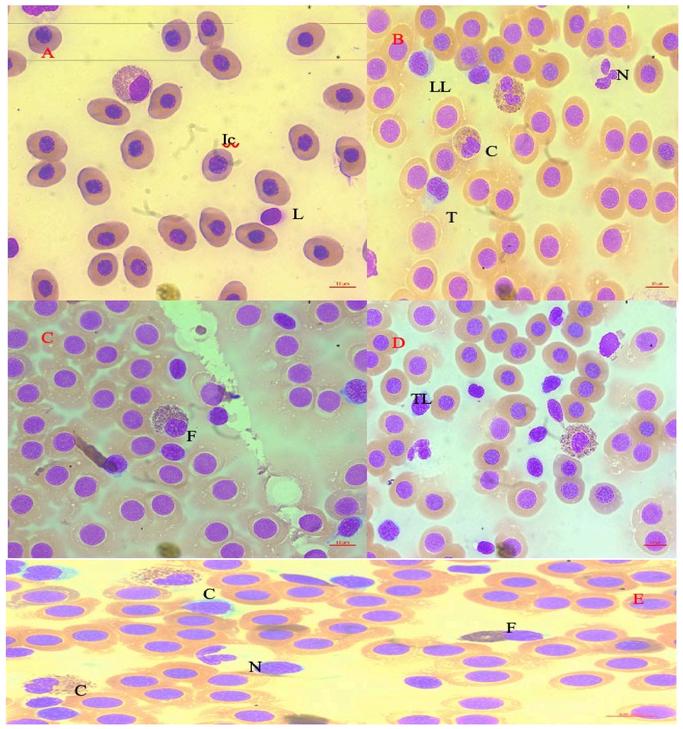


Figure 1:Photos of Giemsa Stained leukocytes of Lemon Shark (N. *breviotris*): Ic (immature Cell, L (lymphocyte), LL (large lymphocyte), TL (twinning lymphocyte), N (neutrophil), C (CEG), F (FEG)

Table 1: Reference intervals for differential counts of the lemon shark (Negaprion breviostris) peripheral blood leukocytes

	Mean	STD. DEV.	median	MAX	MIN	variance	CV
lymphocyte	80.768	6.313	81.000	92.000	66.000	39.854	1.653
FEG	1.357	1.242	1.000	5.000	0.000	1.543	0.325
CEG	9.286	4.499	9.000	19.000	1.000	20.244	1.178
Neutrophil	6.000	3.110	5.000	15.000	1.000	9.673	0.815
monocyte	2.500	2.676	2.000	12.000	0.000	7.164	0.701
Thrombocyte	29.714	12.812	27.500	60.000	10.000	164.135	3.355
Immature cell	5.400	8.070	3.000	39.000	0.000	65.118	2.501
total granulocyte	16.643	5.754	17.000	29.000	6.000	33.106	1.507
GLR	0.212	0.088	0.210	0.414	0.066	0.008	0.023

Table 9: Leukocyte measurements (in diameter, cm) for Lemon Shark (*N. breviostris*)

	mean	std dev	min	max	med	confidence	variance
large lymphocytes	10.528	0.961	9.200	12.471	10.263	0.411	0.924
small lymphocyte	8.013	0.741	6.342	9.534	8.141	0.189	0.550
FEG	14.345	1.056	12.130	16.080	14.374	0.502	1.115
CEG	14.266	1.313	11.836	16.625	14.244	0.515	1.725
neutrophil	16.522	1.361	13.961	18.926	16.074	0.582	1.852
monocyte	14.513	1.576	11.649	17.850	14.240	0.691	2.484
thrombocyte	10.525	1.812	7.620	16.045	10.160	0.376	3.283
immature cell	11.074	0.713	9.688	12.037	11.097	0.388	0.508
twinning lymphocyte	10.257	2.244	7.640	13.245	10.075	1.967	5.035

*Confidence variable (CV) performed with 95% confidence
** only 4 cells of the twinning leukocyte were counted, but their appearance seemed distinctive enough to present with the remainder of the data

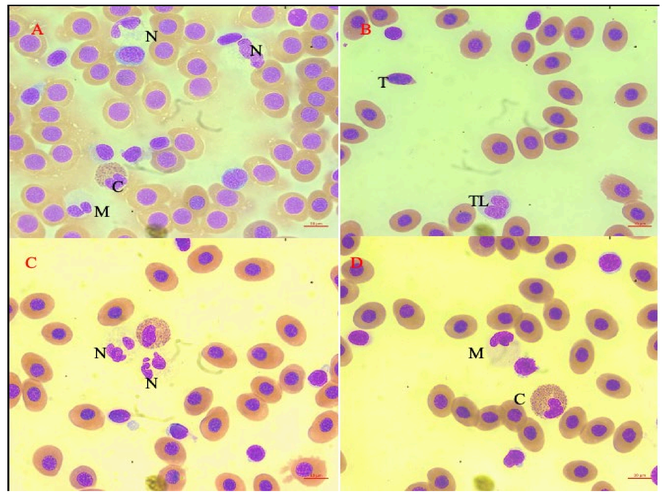


Figure 2:Photos of Giemsa Stained leukocytes of Lemon Shark (*N. breviotris*): TL (twinning Lymphocyte), N (neutrophil), C (CEG), M (monocyte), T (thrombocyte)

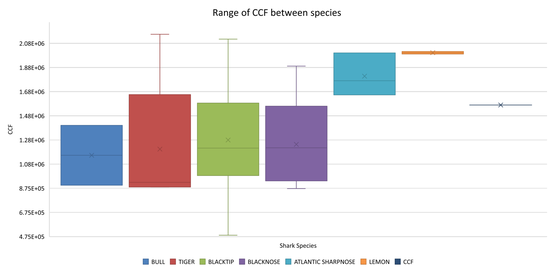


Figure 3: Range of CCF values between the shark species used in this study. The CCF was calculated based on the whole leukocyte count from the Natt Herrick solution sample as compared to number of leukocytes present at 40x on blood smears at 40x.

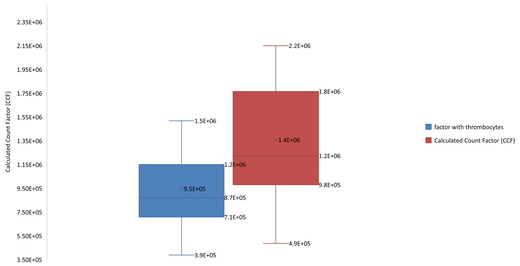


Figure 4: A comparison of the CCF as obtained with or without thrombocytes included in the PBL and Natt & Herrick counts. Discrepancies in the ability to discern thrombocytes from other leukocytes on hemocytometer counts made it difficult to obtain the CCF in the study.

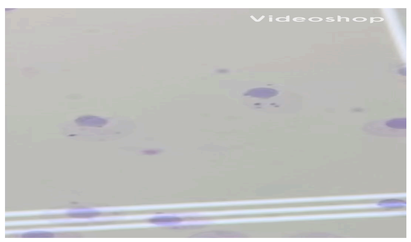
$EQ\ 1$: $TBC = ave.number\ leukocytes\ across\ the\ large\ four\ squares\ in\ the\ hemocytometer\ grid * 200\ (\ the\ dilution\ factor) * 10^4 (\ volume\ available\ on\ hemocytometer)$

$$EQ2: CCF = \frac{\text{TBC}}{\text{ave. number Leukocytes from Peripheral Blood Smear at 40x}}$$

Figure 5:Equation used in the experiment to solve for CCF

Interesting Finding

Tiger shark erythrocyte from the hemocytometer count. The arrow is pointing at one of the structures that was found moving within its cytoplasm.



Discussion

- In comparison to values in the literature for other sharks, the lymphocyte percentage for Lemon sharks is higher
- The granulocytes and monocytes overall had a much larger diameter, which corresponds to the fact that they are likely the main phagocytotic cells in peripheral blood; both coarse eosinophilic and fine eosinophilic granulocytes were present in lemon shark blood
- Hematological reference intervals for lemon sharks in this population are established here for the first time
- Results of the estimation of CBC experiment were mixed; the initial goal was to establish a single number (the CCF) for use as a proxy, facilitating the use of blood smears instead of complete blood counts for wild sharks
- Successfully establishing the CCF would necessitate it having both precision and accuracy
- Results indicate that more research in this area is needed to establish an appropriate CCF for sharks; substantial variation in counts did not yield a CCF that could reliably estimate the CBC from blood smears
- An important drawback to the application of these methods are related to irregularities in counts when identifying cells

References

1. Arnold (2005) 2. Farrel, et al. (2015) 3. Greene, et al(2018) 4.Parkinson, et al.(2017) 5.Dove, et al. (2010) 6.Haman, et al. (2012)

Acknowledgments

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