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HEMATOLOGIC PARAMETERS OF CAPTIVE *Bothrops atrox* (SQUAMATA: VIPERIDAE)

Heloísa Castro PEREIRA[1], Letícia Prata Juliano Dimatteu TELLES[2], Líria Queiroz Luz HIRANO[2], Maísa Paschoal RIOS[1], André Luiz Quagliatto SANTOS[1]

[1] Faculty of Veterinary Medicine and Zootechnics, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil.
[2] Faculty of Agronomy and Veterinary Medicine, Universidade de Brasília (UnB), Brasília, Distrito Federal, Brazil.

Corresponding author:
Heloísa Castro PEREIRA
helo_ca@hotmail.com

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Abstract

The breeding of venomous snakes in captivity for research purposes and mainly as a source of pharmaceutical products highlights the need to determine hematological parameters for monitoring and ensuring a healthy breeding populationThe complete blood count is used to help diagnose alterations such as anemia, inflammatory diseases, parasitemia, hematopoietic disorders, hemostatic and toxicological changes, as well as bacterial and viral inclusions. Thus, the objective of this study was to define reference parameters for complete blood count in *Bothrops atrox* snakes. Blood samples were collected from 20 specimens of *B. atrox* from the Pentapharm do Brasil commercial breeding facility for laboratory examination. Mean values and standard deviation were: hematocrit 33.6 ± 5.47%, hemoglobin 10.81 ± 2.07g/dL, total number of erythrocytes 0.59 ± 0.1 x 106/mm^3, leukocytes 11387.5 ± 3279.2/mm^3 and thrombocytes 28175 ± 6320/mm^3. No significant difference was observed between males and females and heterophils were the predominant leukocyte cell type.

Keywords: Blood Count. Clinical Pathology. Leukogram. Reptile.

1. Introduction

The snake *Bothrops atrox* Linnaeus 1758, popularly known as the common lancehead, is bred in captivity for extraction of its venom. Such byproduct is used in the global pharmaceutical industry as raw material for drugs to treat cardiovascular disorders (Sajevic et al. 2011; Jacob-Ferreira et al. 2017). This species of snake belongs to the class Reptilia, order Squamata, suborder Ophidia, family Viperidae and subfamily Crotalinae (Bernarde 2011; Costa and Bérnils 2018).

The interest in captive breeding of snakes has increased worldwide. However, specialized veterinarians often encounter difficulties in obtaining laboratory reference values for the prophylactic management of diseases in these reptiles. In the literature, there is little information on the hematological, biochemical and clinical parameters of the species *B. atrox*, and the characterization of such data is important in the evaluation of the health of the breeding population, diagnosis and therapeutic follow-up (Trujillo et al. 2016; Kindlovits et al. 2017; Troiano, 2018).

In members of the Reptilia class, the total blood volume is estimated to be between 5 and 8% of body weight, and most animals can efficiently tolerate acute blood loss of up to 10% of this amount (Nardini et al. 2013). In addition, the lymphatic vessels of reptiles are more superficial and closer to the blood circulation,

which can cause errors in puncture resulting in dilution of the blood sample with lymph, and the need to obtain a new sample (Nardini et al. 2013; Heatley and Russel 2019).

The anticoagulants most commonly used in animal blood processing are lithium heparin and ethylenediaminetetraacetic acid (EDTA). The former is the first choice for some reptile species, because of the hemolysis seen with the use of EDTA, which is common in testudines. On the other hand, heparin can promote leukocyte and thrombocyte aggregation, with blue staining in the smear. Thus, whenever possible, blood smears without anticoagulants are recommended (Nardini et al. 2013; Campbell 2015).

Blood cells found in reptiles include erythrocytes and nucleated thrombocytes, heterophils, eosinophils, basophils, monocytes, lymphocytes, and azurophils (Campbell 2014; Heatley & Russel, 2019). According to Heatley & Russel (2019), eosinophils are rare or absent in most snakes and their presence is controversial, although it has been previously described in recent work such as that of Kindlovits et al. (2017) with the genus *Bothrops* (*B. atrox* and *B. jararacussu*) and *Corallus hortulanus*, and the research of Quadrini et al. (2018), with *Python bivittaus*. Reptile erythrocytes are ellipsoid, permanently nucleated, and larger than those of birds and mammals (Campbell 2014).

The complete blood count (CBC) is used to help diagnose alterations such as anemia, inflammatory diseases, parasitemia, hematopoietic disorders, hemostatic and toxicological changes, as well as bacterial and viral inclusions (Nardini et al. 2013; Campbell 2014). Some examples of parasites found in snake blood are those of the genera *Haemogregarina*, *Hepatozoon*, and *Serpentoplasma* (Nardini et al. 2013). The detection and causes of anemia in these reptiles are similar to those in mammals and birds, with markers of regeneration characterized by the presence of basophilic cytoplasmic stippling, polychromasia, binucleation, increased anisocytosis, and increased erythroid precursors (Heatley and Russel 2019). In snakes, increased azurophils have been associated with acute cases of inflammation and infectious disease (Heatley and Russel 2019).

To ensure the success in the prophylactic management of snakes, focusing on the maintenance of a healthy breeding population, with longer life span and welfare, reference laboratory data are important regarding several variables such as species, age, origin, housing characteristicsand study region. Therefore, the objective of this study was to define reference parameters for blood count of the *Bothrops atrox* species.

2. Material and Methods

The research was authorized by the Authorization and Information System on Biodiversity (SISBIO), number 69416-2, National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), registration AF8A99F, and also had a favorable opinion number 040/19 from the Ethics Committee on Animal Use of the Federal University of Uberlândia. Samples were collected from snakes from the Pentapharm do Brasil commercial breeding facility in Uberlândia, Minas Gerais, Brazil, with registration number 4.644.776 at the Brazilian Institute for Environment and Renewable Natural Resources (IBAMA).

A total of 20 born in captivity adult *Bothrops atrox* were used, ten (50%) of them male and ten (50%) female, with an approximate age of six years, kept in vivariums in pairs, in an environment of controlled temperature and humidity, between 25 to 30ºC and 60 to 80%, respectively, and photoperiod of 12 hours light and 12 hours dark. The animals were fed with mice every 21 days, and water was available ad libitum. The animals were clinically healthy.

Blood collections were performed during the month of March 2019. Snakes were physically restrained using a hook and a clear plastic tube, to avoid direct contact with the animal's head. Samples of approximately 0.5 mL of blood were collected, punctured in the caudal vein, with disposable 3 mL syringes and disposable 13 x 0.3 mm (30G) hypodermic needle, which were packed in tubes with ethylenediaminetetraacetic acid (EDTA) (Troiano 2018). At the time of obtaining the blood samples, blood smears were also made without anticoagulant.

The snakes did not suffer any changes in their routine, and the samples were collected together with the routine procedures of the breeding center, which follows all animal welfare, biosafety, and federal laws. The samples were transported in a Styrofoam box to a clinical pathology laboratory and processed within four hours after collection.

Hematocrit was determined using a microhematocrit centrifuge. The concentration of hemoglobin was performed by the hemoglobin cyanide (HiCN) method in a semi-automatic biochemical analyzer model BIO-200®. The hemoglobin kit and standard used were from Labtest Diagnóstica® and the methodology was performed according to the manufacturer's instructions for use (Troiano, 2018).

To determine the total number of erythrocytes, leukocytes, and thrombocytes, a 1:100 dilution was performed (10 µl of blood to 1 mL of Natt-Herrick's solution), with cytological counting in a mirrored Neubauer hemocytometer, under a microscope with 400x magnification. The total numbers of each cell (erythrocytes, leukocytes, and thrombocytes) in the 25 central quadrants were obtained, and the value found was multiplied by a factor of 1000. The formula to obtain the factor is: % counted (1/1) x dilution (1:100) x constant (10) (Almosny et al. 2000). The hematimetric indexes were calculated according to the original formulas of Wintrobe (1993).

Differential leukocyte count was performed on blood smears stained with Fast Panoptic®, viewed on an optical microscope for observation at 1000x magnification, with immersion oil. A total of 100 cells were counted, determining the percentage of each (relative value), for subsequent determination of the absolute value of each. For absolute value, the relative value multiplied by the number of total leukocytes and divided by 100 was used, this was done for each blood cell.

Due to the inconsistency in terminology, the nomenclature proposed by Hawkey and Dennet (1989) was used. Thus, the leukocyte types were described as mononuclear cells (lymphocytes, monocytes and azurophils) or as granulocytes (heterophils, basophils and eosinophils).

The statistics were performed using Microsoft Excel Professional Plus 2019 and Bioestat 5.0 (Ayres et al. 2007). Initially, the normality pattern of the data distribution was evaluated using the Shapiro-Wilk test and the occurrence of extreme values based on the variances. Additionally, descriptive statistics were performed for the data and, in cases of parameters with normal distribution, the lower and upper limits of the 95% confidence interval for the mean were determined. For the values that did not present normality of distribution, the Bootstrap method was applied, with 1000 simulations, and 95% confidence interval.

Additionally, the Bioestat 5.0 program was used for comparison of means between male and female test results. For parametric data, Analysis of Variance and Tukey's T-test were used, with a significance level of $p<0.05$. For non-parametric data, the Mann Whitney test was used, also with a significance level of $p<0.05$.

3. Results

There was no significant difference ($p>0.05$) in the comparison for the means of the blood count parameters between males and females (Table 1).

The results that showed normal distribution were: total number of red blood cells ($p=0.66$), hemoglobin ($p=0.65$), hematocrit ($p=0.95$), mean hemoglobin concentration (MHC; $p=0.49$), mean corpuscular hemoglobin concentration (MCHC; $p=0.19$), relative heterophils ($p=0.08$), absolute ($p=0.30$) and relative azurophils ($p=0.08$), absolute ($p=0.16$) and relative ($p=0.33$) monocytes, absolute ($p=0.39$) and relative ($p=0.21$) lymphocytes and platelets ($p=0.12$). In contrast, mean corpuscular volume (MCV; $p=0.009$), total leukocytes ($p=0.049$), absolute heterophils ($p=0.03$), absolute ($p=0.009$) and relative eosinophils ($p=0.009$), and relative azurophils ($p=0.048$) did not show normal distribution pattern.

In the analysis of extreme values, only one data from the MCV (n=538fL) was considered an outlier, as well as two data from the relative azurophil count (n= 0 and n= 20%). All extreme results were disregarded for the descriptive analysis.

4. Discussion

The collection of blood from snakes is usually performed from the tail vein because it is a safer and easier method to perform, especially for venomous animals. However, a caution to be observed is the possibility of contamination of the sample by lymph (Gillett et al. 2015), which dilutes the content, leaving it clearer and altering the results (Troiano et al. 2000). In the present study, this occurrence was not observed, which was avoided by firmly immobilizing the post-cloacal region of the animals and introducing the needle through the ventral face of the body.

Table 1. Values of mean, standard deviation (SD), standard error (SE), median (Med), minimum and maximum (Min-Max) for blood count of male (M) and female (F) captive *B. atrox* (n=20).

Parameter		Mean	SD	EP	Med	Min-Max	IC (95%)*	p (MxF)
Erythrocytes (millions/mm^3)	M[a]	0.6	0.11	0.02	0.61	0.45-0.83	0.53-0.65	0.6593
	F[a]	0.58	0.09	0.02	0.59	0.37-0.69	0.51-0.62	
Hemoglobin (g/dL)	M[a]	11.26	2.53	0.57	11.65	7.90 – 15.6	9.8-12.5	0.6544
	F[a]	10.36	1.47	0.33	10.15	7.4-12.4	9.5-11.1	
Hematocrit (%)	M[a]	34.5	6.08	1.36	34.5	26 – 46	31.1-37.5	0.5167
	F[a]	32.7	4.95	1.11	33	22-38	29.7-35	
MCV (fl)	M[a]	574	19.75	4.42	580	547-596	561.5-584.1	0.5815[a]
	F[b]	566.5	18.11	4.05	555.5	549-595	556.3-575.9	0.5340[b]
MCH (pg)	M[a]	186.94	14.75	3.3	190.90	157.7 – 201.8	177.8-193.6	0.2096
	F[a]	179.87	8.98	2.01	179.05	167.2-200	175.1-184.4	
MCHC (%)	M[b]	32.39	2.33	0.52	33.75	27.9-34.7	30.9-33.5	0.5276[a]
	F[a]	31.76	1.31	0.29	31.9	29.5-33.6	31-32.3	0.1405[b]
Total Leukocytes	M[a]	12175	3242.62	725.42	11500	8750-19500	10450.7-13850.9	0.2953
	F[a]	10600	3287.6	735.48	9250	6750-17750	8851-12300.6	
Absolut Leukogram (/mm^3)								
Heterophiles	M[a]	5125	2600.84	581.84	4665	2082-10140	3740.1-6479.1	0.2134
	F[a]	3900	1229.77	275.12	4275	1980-5390	8875.2-12300.6	
Eosinophils	M[b]	63	90.90	20.33	0	0-230	10.5-110	0.1616[a]
	F[a]	125	100.23	22.42	97.5	0-285	3142.8-4529.7	0.1736[b]
Basophils	M	0	-	-	0	0-0	-	-
	F	0	-	-	0	0-0	-	
Azurophiles	M[a]	1336.67	418.51	93.85	1265	700-2050	1078.4-1550.1	0.8372
	F[a]	1264.5	549.18	122.86	1062.5	666-2280	964.1-1551.2	
Monocytes	M[a]	522.5	335.02	74.95	548.75	0-1040	326.8-689.8	0.5253
	F[a]	420.56	154.92	34.66	437.5	180-630	325.3-499.7	
Lymphocytes	M[a]	4729	1475.96	330.19	4380	2340-7020	3872.2-5479.3	0.6092
	F[a]	4211.75	1456.87	325.92	4005	2640-6840	3391.4-4918.5	
Relative Leukogram (%)								
Heterophils	M[a]	41.6	14.73	3.3	47.5	16-57	32.4-48.7	0.9296
	F[a]	41.1	10.84	2.42	42	22-54	34.6-46.3	
Eosinophils	M[b]	0.6	0.84	0.19	0	0-2	0.1-1	0.0950[a]
	F[a]	1.3	0.95	0.21	1	0-3	0.8-1.7	0.1124[b]
Basophils	M	0	-	-	0	0-0	-	-
	F	0	-	-	0	0-0	-	
Azurophils	M[a]	10.2	5.03	1.13	9.5	0-20	7.2-12.6	0.5828
	F[a]	11.7	2.58	0.58	11.5	8-16	10.2-13	
Monocytes	M[a]	4.4	2.63	0.59	4	0-8	2.8-5.7	0.7675
	F[a]	4.7	1.83	0.41	4.5	2-8	3.7-5.5	
Lymphocytes	M[a]	38.8	7.73	1.73	37	26-54	34.2-42.8	0.7148
	F[a]	40.4	11.2	2.5	38	29-64	34.5-46.1	
Thrombocytes (/mm^3)	M[a]	30900	6458.41	1444.84	28500	23500-44500	27427-34226.8	0.0660
	F[a]	25450	5110.99	1143.4	25250	16000-360000	22526-28002	

- : Not evaluated. [a]: Data with normal distribution by Shapiro Wilk test (Analysis of Variance and Tukey's T-test, p<0.05); [b]: Data without normal distribution by Shapiro Wilk test (Mann Whitney test, p<0.05); MCHC: Mean corpuscular hemoglobin concentration; MCHC: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; n: sample number. *ICI(95%): for data with a normal distribution, represents the lower and upper bounds of the 95% confidence interval for the mean. For data without a normal distribution, it is the Boostrap. p: p value of Analysis of Variance and Tukey's T-test (parametric data) or Mann Whitney test (non-parametric data). Significance level of p<0.05.

Lithium heparin is a widely used anticoagulant in reptile hematological samples, especially in Testudines (Campbell 2015). In the research of Harr et al. (2005), with *Python molurus bivittatus*, minimal differences were observed in the results of hemograms performed with EDTA and lithium heparin. In this study, we chose to make the smears at the time of collection, without any type of anticoagulant in the sample, however, the authors point out that in previous experiences with the species, EDTA allows obtaining smears with better quality for differential counting, because the staining promoted by heparin results in dysmorphic and unclear cells.

The erythrocyte count of *B. atrox* was close to those found by Troiano et al. (1999) for *Bothrops jararacuçu* (642.3±4.23 x 10^9/L) and *B. moojeni* (543.1±9.2 x 10^9/L) and by Trujillo et al. (2016) for *B. atrox*

(555.42±1.62 x 10^3/uL). The hemoglobin concentration obtained similar result to that reported by Troiano et al. (1997) for *Crotalus dissucus terrificus* which was 11.5±4.3 g/dL and by Trujillo et al. (2016) for *B. atrox* (7.48±.11 g/dL). However, the hematocrit was higher than those reported in previous works and in those by Glaser et al. (2013) and Gomez et al. (2016) for *B. jararacussu* (24.71 ± 9.55%) and *Bothropoides jararaca* (23.12 ± 5.99%) and *B. asper* (22 ± 7%) and *Crotalus simus* (27 ± 6%), respectively.

The number of total leukocytes was close to the values found for the species *Crotalus dissucus terrificus*, *B. ammodytoides* and *B. leucurus* (Troiano et al. 1997; 1999; Grego et al. 2006). But it was higher when comparing with *B. jararacuçu* and *B. moojeni*, (Troiano et al. 2000), and lower than the values found for *B. alternatus*, *B. neuwiedii disporus*, *B. asper* and *C. simus* (Troiano et al. 2000; Gomez et al. 2016).

Regarding snake leukocytes, in this study, heterophils were the most commonly found leukocyte cell type, followed by lymphocytes, azurophils, monocytes, eosinophils, and basophils. However, lymphocytes were the predominant cell group in studies with the species *C. dissucus terrificus* (56.25 ± 12.25%), *B. ammodytoides* (52.2 ± 6.87%), *B. alternatus* (51.4 ± 6.75%), *B jararacussu* (50.6 ± 3.27%), *B. moojeni* (51.05 ± 8.21%), *B. newiedi diporus* (52 ± 6.85%), *B. leucurus* (4.35 ± 2.58x10^3/mm^3 for females and 7.49 ± ,56x103/mm^3 for males), *B. asper* (66 ± 11%) and *C. simus* (70 ± 14%) (Troiano et al. 1997; 1999; 2000; Gomez et al. 2016; Grego et al. 2006).

The second predominant cell type was variable among the papers. Azurophils followed lymphocytes for *C. dissucus terrificus* (18.05 ± 3.5%) and *B. leucurus* (1.57 ± 1.28x10^3/mm^3 for females and 2.36 ± 2.75x10^3/mm^3 for males) in the studies by Troiano et al. (2006), respectively. In the studies by Troiano et al. (1999; 2000) with *B. ammodytoides* (16.3 ± 1.85%), *B. alternatus* (15.2 ± 2.01%), *B jararacussu* (16.5 ± 0.95%), *B. moojeni* (15.7 ± 2.1%) and *B. newiedi diporus* (14.95 ± 3.01%), eosinophils were the second predominant cell group. In the work of Gomez et al. (2006) with *B. asper* (25 ± 11%) and *C. simus* (14 ± 7%), it was monocytes, but in this research the second most predominant type were lymphocytes followed by azurophils.

Although basophils were not found, these cells have been reported in small quantities in other snake species. Studies have demonstrated the presence of this cell type in *C. dissucus terrificus* (1.5 ± 0.8%), *B. ammodytoide*s (1 ± 0.3%), *B. alternatus* (1 ± 0.3%), *B jararacussu* (0.5 ± 0.2%), *B. moojeni* (0.9 ± 0.2%), *B. newiedi diporus* (0.75 ± 0.1%), *B. leucurus* (0.49 ± 0.63x10^3/mm^3 for females and 0.46 ± 0.31x10^3/mm^3 for males), *B. asper* (3 ± 4%) and *C. simus* (1 ± 1%) (Troiano et al. 1997; 1999; 2000; Grego et al. 2006; Gomez et al. 2016).

In the study by Trujillo et al. (2016), also conducted with *B. atrox*, 24 adult snakes maintained in captivity at the Instituto Nacional de Salud (INS) and the Oswaldo Meneses Serpentarium (UNMSM) located in Lima, Peru were selected. Thirteen specimens were male and eleven were female, and these samples collected in November and December 2008. The samples were packaged in sodium heparin bottles. The values found by Trujillo et al. (2016495.70 – 639.00 103/ul eritrócitos; 6.40 – 9.00 g/dl hemoglobina; 18.14 – 25.00 hematócrito in the erythrogram were similar to those found in this research. On the other hand, in the leukogram, the snakes from Peru had the lowest number of total leukocytes (4.45 x 10^3 /uL). Lymphocytes (75.04 ± 7.45%) were the predominant cell group of white cells found by Trujillo et al. (2016), followed by azurophils (12.5 ± 6.82%), heterophils (5.63 ± 3.82%), eosinophils (2.96 ± 1.99%), monocytes (1.96 ± 1.4%) and basophils (1.63 ± 1.5%), differently from that found in the present study. These variations demonstrate the importance of performing reference parameters for the same species from different sites, especially in the case of reptiles that have the environmental factor as a strong determinant in their metabolism.

In Brazil, Kindlovits et al. (2017) investigated the cytochemical and morphological aspects of blood cells of snakes of the genus *Bothrops* and *Crotalus*. Blood was collected from 50 captive animals from the Instituto Vital Brasil, and eight specimens of the species *B. atrox* showed parasites of the genus *Hepatozoon*, which did not occur in this study. Although *B. atrox* is a species present in Brazilian breeding centers and is widely used in research about the properties of its venom for the pharmaceutical industry (Sajevic et al. 2011; Jacob-Ferreira et al. 2017), there are few studies regarding its hematological parameters that can guide information regarding its physiological state (Troiano et al. 2000; Trujillo et al. 2016). As in the study by Trujillo et al. (2016) and in the present work, eosinophils were also found in the specimens of *B. atrox*, in small quantities. There is great difficulty in establishing hematological parameters for reptiles since they are

ectothermic animals and are influenced by intrinsic and environmental factors (Nardini et al. 2013). Thus, it is important the characterization of regional values and the evaluation of environmental, seasonal aspects, the nutritional status, housing characteristics, age and origin of the animal during the evaluation (Almosny 2014). In the work of Gomez et al. (2016), a statistical difference was observed for the number of heterophils for *B. asper* and of eosinophils, lymphocytes, and monocytes for *C. simus* between free-living and captive animals. In the study by Troiano et al. (1997) with *C. dissuus terrificus*, no significant variation between male and female parameters was observed, but the influence of seasonal variation was reported, which was not evaluated in the present study.

5. Conclusions

No significant difference was observed in hematological values between males and females of *Bothrops atrox*. Mean and standard deviation values for hematocrit (33.6 ± 5.47%), hemoglobin (10.81 ± 2.07g/dL), total number of erythrocytes (0.59 ± 0.1 x 10^6/mm^3), leukocytes (11387.5 ± 3279.2/mm^3) and thrombocytes (28175 ± 6320/mm^3) were determined, with results close to those reported for other *V iperidae* species. However, the predominant leukocyte cell types were different from other studies, in which we observed a higher number of heterophils (45.5 ± 13.72%), followed by lymphocytes (38.63 ± 8.39%), azurophils (11.202 ± 3.61%), monocytes (4.89 ± 2.68%), eosinophils (1 ± 1.02%) and basophils (0 ± 0%).

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