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Short Communications

PRELIMINARY EVALUATION OF COMPLETE BLOOD COUNT AND DIURNAL VARIATION OF HEMATOLOGICAL PARAMETERS IN BLACK RAT SNAKE *(PANTHEROPHIS OBSOLETUS)*

BELIĆ Maja[1*], LUKAČ Maja[2], DVOJKOVIĆ Nika[3], GALEŠIĆ Tena[3], ROBIĆ Mirna[1], TURK Romana[1]

1University of Zagreb, Faculty of Veterinary Medicine, Department of Pathophysiology, Heinzelova 55, 10000 Zagreb, Croatia;
2University of Zagreb, Faculty of Veterinary Medicine, Department of Poultry Diseases with Clinic, Heinzelova 55, 10000 Zagreb, Croatia;
[3]University of Zagreb, Faculty of Veterinary Medicine, Heinzelova 55, 10000 Zagreb, Croatia

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Abstract

Introduction. The purposes of this study were to determine the presence of daily variations in hematological parameters of the black rat snake (*Pantherophis obsoletus*) and to compare them with the reference interval values of its subspecies, yellow rat snake (*Pantherophis obsoleta quadrivittata*).

Materials and Methods. Blood from four black rat snakes was collected in the morning and in the evening, and after each collection, blood smears, packed cell volume (PCV), red blood cell count (RBC) and hemoglobin concentration (HGB) were determined, while erythrocyte indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated and differential leukocyte counts were evaluated.

Results and Conclusions. There were no statistically significant differences in any of the blood parameters in the morning and in the evening. Most of the complete blood count values in the black rat snakes were lower, but still within the reference range of those

*Corresponding author – e-mail: mbelic@vef.hr

found in the literature for the yellow rat snake. The results of hematological parameters of the black rat snake could be beneficial for further research of physiological and pathological variations in complete blood count of these snakes, for diagnosing health conditions and for detecting various diseases.

Key Words: daily variation, hematology, interspecies differences, snake

INTRODUCTION

During the past several decades, the number of endangered species has been increasing rapidly, as has the number of exotic animals kept as pets. Reptiles constitute the majority of animals in both of these categories. To assure welfare and health of the animals through proper management, protection and veterinary care, it is very important to have as much knowledge as possible about their physiology and biology. One useful tool for assessing the physiological and health conditions of animals is hematologic evaluation, a mainstay of diagnosis (Parida et al., 2014; Jenkins-Perez, 2012; Tosunoğlu et al., 2011).

Hematologic evaluation in reptiles is a challenging procedure, since some of the tests need to be processed manually and the reliability of the results is dependent on the skill and experience of the person performing the evaluation. The reason for manual processing of reptilian blood is the fact that all reptile blood cells have a nucleus, which is problematic for automatic differentiation of blood cells using commercial hematological analyzers (Canfield, 1998; Stacey et al., 2011). There is also great diversity in the number and morphology of blood cells among more than 8000 described reptilian species (Claver and Quaglia, 2009) and even among species within the same genus (Stacy et al., 2011). Besides, the hemogram of reptilian patients is influenced by many internal (gender, age, species) and external factors (season, environment, temperature, diet, stress, etc. (Campbell and Ellis, 2007). All these factors can significantly affect the hematological parameters in healthy animals during the year. Some external factors such as venipuncture site, temperature and diet can be controlled and influenced by people and so variations in hemogram results that occur due to these factors can be prevented. Other factors, such as age, gender, animal species or season cannot be influenced, but knowing them, their effect on quantitative changes of some hematological parameters can be explained (Žilčnik and Halán, 2013; Tosunoğlu et al., 2011). There are many studies published on the hematology and biochemistry of snakes (Rousselet et al., 2017; LaGrange et al., 2014; Sykes and Klaphake, 2008; Dabrowski et al., 2007; Strik et al., 2007; Harr et al., 2008; Alleman et al., 1999), but some snake species remain for which hematological and biochemical data are insufficient or do not exist at all.

The black rat snake (*Pantherophis obsoletus*) belongs to the genus *Pantherophis*, family *Colubridae*; all snakes in this family are nonvenomous. This is the largest order of snakes, representing two-thirds of all known snake species. Members of this family are found on all continents except Antarctica, and are widespread from the Arctic Circle

to the southern tips of South America and Africa (Blouin-Demers et al., 2007; Blouin-Demers et al., 2002). Besides the black rat snake, the subspecies called yellow rat snake (*Pantherophis obsoleta quadrivittata*) is also very popular among snake breeders. Differences between these two subspecies are the color of the skin and temperament. The yellow rat snake is considered as passive, while the black rat snake is more aggressive when in danger (LeClere, 2005). Both subspecies of rat snake are often held in captivity as pets and so are common patients in veterinary clinics.

To the authors' knowledge, there are hematologic reference values for yellow rat snake in the literature (Carpenter, 2013; Bounous et al., 1996), but none for black rat snake. Therefore, the aims of this study were to: 1) investigate the diurnal variation of hematological parameters in the black rat snake, as an external factor that could possibly influence its hemogram, and; 2) compare the determined hematological parameters of the black rat snake with literature data for the yellow rat snake.

MATERIALS AND METHODS

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Zagreb.

Animals and blood sampling

Four black rat snakes from the same litter and kept by the same owner were clinically evaluated at the Faculty of Veterinary Medicine University of Zagreb. All snakes were four years old, mean body weight (±SE) 317 (±3) g. Three snakes were males and one was female. They were all hatched and raised in captivity and kept in a terrarium under conditions required for this snake species. All snakes were clinically examined and were determined to be physiologically normal. Also, parasitological examination of the animals revealed no parasites were present.

During the blood sampling trial, the animals were housed individually in plastic tanks, dimensions 70x30x30 cm, with secure latch lids. Every tank contained a water bowl big enough for the snake to fit into. Peat was used as a suitable substrate to allow the animals to exhibit burrowing behavior, and a piece of wood bark was placed in every tank as a hiding place. The temperature ranged from 25 to 30°C during the day, and heating was provided by placing a heating mat (Exo terra, Hagen Inc, Canada) under one side of the tank, with a thin cotton pad between the tank and the heating mat to protect the animals from burns. During the night, the temperature ranged from 22 to 24°C, ensured by switching off the heating mats. Ultraviolet B (UVB) light was not provided since scientific reports about the need of this species for UVB light are lacking.

Blood was collected twice on the same day, morning and evening at 07:00 and 19:00 h. Blood was taken from the ventral tail vein (*v. coccygea ventralis*) using disposable sterile plastic syringes with 23-gauge needles. From each snake, 0.4 ml of blood was taken

in total on one day in both venipunctures i.e. 0.2 ml in the morning and 0.2 ml in the evening (the maximum allowable blood volume that can be taken from a snake weighing 300 g is 1.5-2.4 ml).[7]

Three blood smears for each snake at each venipuncture time were made immediately with blood taken directly from the syringe without any anticoagulant. The remainder of the blood was stored in lithium-heparinized tubes for further analysis that followed immediately.

Hematological analyses

After the each venipuncture, the following hematological parameters were determined: packed cell volume (PCV), red blood cell count (RBC) and hemoglobin concentration (HGB). From these parameters, the erythrocyte indices, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were calculated. Finally, the differential leukocyte number was determined on blood smears as well as erythrocyte, leukocyte and thrombocyte morphology. The blood analysis was always performed by the same person.

PCV was obtained by a microhematocrit method. The microhematocrit tubes were centrifuged at 10,000 *g* for 5 minutes and the result was obtained on PCV reader.

HGB was determined by the cyanmethemoglobin method on a UV-VIS spectrophotometer (Helios Gamma, Thermo Spectronic). Before measuring the optical density, the blood samples mixed with reagent were centrifuged so that the free nuclei from lysed erythrocytes would not affect the optical density of the sample (Campbell and Ellis, 2007).

RBC was performed in a Neubauer hemocytometer using 1:200 dilution with the Natt and Herrick solution as described in Campbell and Ellis (2007). The measurements were repeated three times and the mean value was used as the result. The red cell indices, MCV, MCH, and MCHC were calculated using standard formulae (Strik et al., 2007).

Since there are several methods for determining the white blood cell count in reptiles manually, none of which is highly accurate, reliable and consistent (Jenkins-Perez, 2012; Rovira, 2010), we only determined differential white blood cell counts on blood smears. Six types of leukocytes were determined: heterophils, eosinophils, basophils, lymphocytes, monocytes and azurophils.

Blood smears were prepared immediately, air-dried and stained with May Grünwald-Giemsa stain and examined under a light microscope OLYMPUS BX 41 TF using the oil immersion (1000 x) objective. The percentages of different leukocytes were determined after counting a total of 100 cells (Bell and Gregory, 2014).

Statistical analysis

For statistical analysis, SigmaStat 3.0 for Windows (Jandel Corporation, San Rafael, CA, USA) was used. Descriptive statistics, including mean, standard error and minimum

and maximum values were calculated for each parameter. The Kolmogorov-Smirnov test was used to determine whether the data was normally distributed. Statistical comparisons between hematological parameters in the morning and in the evening were made by one-way ANOVA test. Statistically significant differences between values were set at $P<0.05$.

RESULTS AND DISCUSSION

Mean±SE, minimum and maximum determined levels for all hematological parameters in the four snakes in the morning and in the evening are shown in Table 1. There was no statistically significant difference between morning and evening values ($P>0.05$).

Table 1. Hematological values in the morning and in the evening in four black rat snakes, mean ±SE (min-max)

Hematological parameters	Morning	Evening
Packed cell volume (%)	17.8 ± 1.6 (14-21)^{NS}	18 ± 1.4 (16-22)^{NS}
Hemoglobin concentration (g/dl)	2.4 ± 0.2 (2.1-2.8)^{NS}	3.0 ± 0.2 (2.7-3.6)^{NS}
Red blood cell count (x106/μL)	0.35 ± 0.0 (0.34-0.35)^{NS}	0.44 ± 0.1 (0.24-0.63)^{NS}
Mean corpuscular volume (fL)	513.2 ± 43.8 (412-600)^{NS}	445.7 ± 79.8 (286-666)^{NS}
Mean corpuscular hemoglobin (pg)	70.2 ± 4.1 (62-80)^{NS}	76.0 ± 15.6 (46-120)^{NS}
Mean corpuscular hemoglobin concentration (mg/dl)	13.8 ± 0.8 (13-16)^{NS}	16.8 ± 0.5 (16-18)^{NS}
Heterophils (%)	18 ± 1.7 (14-22)^{NS}	20 ± 2.7 (14-26)^{NS}
Lymphocytes (%)	53 ± 4.6 (44-64)^{NS}	50 ± 5.8 (37-60)^{NS}
Monocytes (%)	4 ± 0.9 (2-6)^{NS}	3 ± 1.1 (1-6)^{NS}
Azurophils (%)	24 ± 3.7 (16-34)^{NS}	26 ± 2.9 (20-32)^{NS}
Eosinophils (%)	-	-
Basophils (%)	3 ± 0.6 (0-4)^{NS}	2 ± 0.9 (0-4)^{NS}

NS - not significant

Table 2 presents the literature reference interval values for yellow rat snake and the mean values (min-max) of all parameters measured in one day (morning and evening) in our four black rat snakes. In the black rat snakes, mean values of PCV, HGB, RBC, MCH, MCHC, heterophils, monocytes, azurophils and basophils were lower than the mean values of these parameters in yellow rat snake. Moreover, mean values of HGB, MCH and MCHC in the black rat snakes were lower than the reference range for these parameters in yellow rat snake. MCV and lymphocytes were the only two parameters for which mean values were higher in our black rat snakes than in the yellow rat snake, but these values were still within the reference range for the yellow rat snake.

Table 2. Hematological reference values of tellow rat snake according to Carpenter (2013) and mean hematological values (min–max) of four black rat snakes in our study

Hematological parameters	Yellow rat snake (Carpenter, 2013)	Black rat snake (our study)
Packed cell volume (%)	24 (9-46)	18 (14-22)
Hemoglobin concentration (g/dl)	8.3 (2.8-15.2)	2.7 (2.1-3.6)*
Red blood cell count (x106/μL)	0.77 (0.21-1.34)	0.35 (0.24-0.36)
Mean corpuscular volume (fL)	361 (198-765)	480 (286-666)
Mean corpuscular hemoglobin (pg)	121 (90-175)	73 (46-120)*
Mean corpuscular hemoglobin concentration (mg/dl)	32 (26-54)	16 (13-18)*
Heterophils (%)	21 (16-42)	19 (14-26)
Lymphocytes (%)	44 (37-58)	52 (37-64)
Monocytes (%)	16 (3-39)	4 (1-6)
Azurophils (%)	33 (16-33)	25 (16-34)
Eozinophils (%)	1 (0-1)	0
Basophils (%)	3 (1-7)	3 (0-4)

* Values that are not within the reference interval of the yellow rat snake

Comparison of hematological values of black rat snakes taken in the morning and in the evening revealed there was no statistically significant difference. However, PCV, HGB, RBC, MCV, MCH, MCHC, heterophils, azurophils and eosinophils were lower in the morning than in the evening, while lymphocytes, monocytes and basophils were lower in the evening.

Due to the fact that our study was performed on four snakes only, we cannot firmly state that diurnal variations in hematological parameters values in those animals do not exist. However, since the conditions of venipuncture were the same, the animals were blood relatives, of the same age and from the same owner, and the blood analysis was performed by the same person, we consider our results valuable.

Since there are no data showing the normal hematological parameters of black rat snakes, we compared the results obtained with available data for the yellow rat snake (Carpenter, 2013). The reference intervals for yellow rat snake are wide, since values are influenced by many external and internal factors. Hematological values for PCV, RBC, MCV, and all the leukocytes determined in our study on black rat snakes were within the reference intervals determined for yellow rat snake. However, HGB, MCH and MCHC determined in our black rat snakes were not within the reference range of the yellow rat snake. HGB was very close to the lowest level of the reference interval and, therefore, we do not consider our results to be in aberrance. HGB is usually low in anemic animals due to hemolysis, bleeding or bone marrow dysfunction (Saggese, 2009), but the animals in our study were clinically healthy and there were no

signs of anemia (paleness of mucosa, anorexia, lethargy, decreased RBC, PCV and HGB, polychromasia more than 1%, poikilocytosis, anisocytosis, mitotic figures, and there were no signs of inflammation or chronic/degenerative diseases) and, therefore, these conditions were ruled out. Since RBC indices were calculated from HGB levels (which were low), RBCs were also low. The predominant leukocytes in snake blood are lymphocytes. The mean lymphocyte count in out black rat snakes was higher than the mean literature values for yellow rat snake. Comparison of our hematological values for black rat snake with those of Indian cobra and royal cobra showed no significant aberrance (Parida, 2014; Salakij, 2002). Compared with literature data values for corn snake (*Pantherophis guttatus*) (Campbell and Ellis, 2007), RBC and HGB in our black rat snakes were slightly higher. Overall, since most hematological values for our black rat snakes were lower compared to the yellow rat snake, but still fell within the reference interval for yellow rat snake, we cautiously conclude that the literature data values for yellow rat snakes can be applied to black rat snake.

CONCLUSION

This is a preliminary study on black rat snake hematology, but further studies on a larger number of animals should be performed in order to the effect of diurnal variation on the black rat snake hematogram. Nonetheless, these preliminary results on hematological parameters of black rat snake could be helpful for future studies on physiological and pathophysiological variations in blood tests of these snakes to establish their health status, diagnose diseases, and as an aid in the preservation and protection of their population.

Acknowledgment

The results of the study in this article were also presented in the form of poster presentation at the 3rd International Conference on Avian, Herpetological and Exotic Mammal Medicine, Venice, Italy, 2017.

Authors contributions

BM, LM, ND and TG have made conception, design and drafting of the manuscript and have been involved in acquisition of data. Maja L. performed physical examination of the animals and venipuncture. Maja B., DjN and GT carried out the laboratory work and made substantial contributions to data analysis. BM and TR performed the statistical analysis and interpretation of data. BM, LM, RM and TR have revised manuscript critically for important intellectual content.

Conflict of interest statement

None of the authors of this manuscript has declared any conflict of interest.

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PRELIMINARNO ISPITIVANJE KOMPLETNE KRVNE SLIKE I DNEVNIH VARIJACIJA HEMATOLOŠKIH PARAMETARA KOD *PANTHEROPHIS OBSOLETUS*

BELIĆ Maja, LUKAČ Maja, DVOJKOVIĆ Nika, GALEŠIĆ Tena, ROBIĆ Mirna, TURK Romana

Kratak sadržaj

Uvod. Cilj ovog istraživanja je bio da utvrdi prisustvo dnevnih varijacija hematoloških parametara kod vrste *Pantherophis obsoletus* i da ih uporedi sa referentnim vrednostima kod podvrste *Pantherophis obsoleta quadrivittata*.

Materijal i metode. Krv od četiri zmije je uzorkovana ujutru i uveče. Posle svakog uzorkovanja određeni su hematocrit, broj eritrocita, koncentracija hemoglobina i hematološki indeksi, dok je sa krvnog razmaza određena leukocitarna formula.

Rezultati i zaključak. Jutarnje i večernje vrednosti crvene i bele krvne loze se nisu međusobno razlikovale. Većina dobijenih vrednosti kod *Pantherophis obsoletus* su se

nalazile u okviru donjih granica referentnih vrednosti publikovanih za *Pantherophis obsoleta quadrivittata*. Rezultati hematoloških parametara za *Pantherophis obsoletus* mogu biti korisni za dalje istraživanje fizioloških i patoloških varijacija kompletne krvne slike zmija u svrhu procene zdravstvenog stanja kao i detektovanja različitih oboljenja.

Ključne reči: hematologija, dnevne varijacije, interspecijske razlike, zmija