NORTH-WESTERN JOURNAL OF ZOOLOGY 19 (2): 147-154
Article No.: e231507

http://biozoojournals.ro/nwjz/index.html

Blood cell morphology of *Testudo hermanni boettgeri* Mojsisovics 1889 wild populations from Serbia

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Received: 18 March 2023 / Accepted: 03 July 2023 / Available online: October 2023 / Printed: December 2023

Abstract. Examining tortoises' blood cell parameters in natural populations could be useful for monitoring their health status. The current study examines the blood cell morphology of Eastern Hermann's tortoise in populations inhabiting three distinct locations in Serbia: Čermor, Gonjište, and Kunovica. We analyzed the number and morphometric parameters of different types of peripheral blood cells, including their length, width, and cell and nuclear size, and compared blood cell morphology across populations, sexes, and seasons (spring and summer). Our findings revealed significant variations in erythrocyte and lymphocyte morphometry among Eastern Hermann's tortoises from different populations and sexes, highlighting the influence of these factors on blood cell characteristics. Seasonal differences were observed in lymphocyte and thrombocyte counts, emphasizing the dynamic nature of hematological parameters in these tortoises. Additionally, alterations in erythrocyte cytoplasm were observed, suggesting potential pathological conditions. Establishing reference hematological intervals for healthy wild populations is crucial for monitoring health status, identifying early indicators of stress or disease, and guiding conservation efforts for Eastern Hermann's tortoises. Our results contribute to the general knowledge of the blood cell characteristics of Hermann's tortoise and provide insights into potential population-level differences in their physiology.

Keywords: Hermann's tortoise, hematology, leucocytes, thrombocytes, conservation.

Introduction

Hermann's tortoise (*Testudo hermanni*) is a small to medium-sized tortoise species that is autochthonous to the Mediterranean and sub-Mediterranean region of Europe and is considered a flagship species due to its ecological and cultural significance, as well as its conservation status (Đorđević et al. 2013). The Western *T. hermanni hermanni* and the eastern *T. h. boettgeri*, are distributed in southern parts of Europe (Fritz et al. 2006). The eastern subspecies is native to the region of the Balkan Peninsula, including Serbia (Fritz et al. 2006; Golubović et al. 2019), where it is distributed in the south of the Danube and Sava rivers (Golubović et al. 2019).

Despite *T. hermanni* being listed as Near Threatened on the IUCN Red List of threatened species (van Dijk et al. 2004), having cultural importance, being represented in art, mythology, and literature throughout history, its populations have dwindled due to habitat loss, overcollection for illegal trade, and traditional medicine practices (van Dijk et al. 2004, Nikolić et al. 2021). Besides, they are attractive as pets (Bielli et al. 2015), and even if they are bred under strictly controlled conditions on farms (Ljubisavljević et al. 2011), illegal collection from the wild takes place. Many of these animals are returned to the wild when they reach a certain size (Jovanović & Ajtić 2011, Nikolić et al. 2021), but not to their place of origin (Pérez et al. 2004). This could support the spread of various pathogens among local populations and, in combination with other stressors (temperature changes, malnutrition) and their impact on tortoises' immune system, increase their mortality rate (Pérez et al. 2004).

While some studies have compared blood parameters between sexes in the genus *Testudo* (Arikan et al. 2015), there is no published information about blood cell morphology in *T. h. boettgeri* from the Central Balkans. Hermann's tortoises are ectothermic agro-ecosystem residents (Stojadinović et al. 2017) whose activity depends on environmental factors such as temperature and light cycle. The activity level of tortoises influences their metabolic rate, which affects blood cell parameters such as red blood cell count, white blood cell count, and hemoglobin levels (Lawrence 1987, Arikan et al. 2015). Previous studies (Gilles-Baillien 1969, Ljubisavljević et al. 2011) have emphasized the importance of establishing reference values for blood cell parameters, standardizing sampling, and counting procedures in Western Hermann's tortoises. This is because accurate and reliable reference values can provide valuable insights into the health status of populations, which in turn can aid in the detection of any unfavorable conditions affecting them. Additionally, changes in blood cell parameters can be used as an early indicator of stress or disease in these animals (Bergeron et al. 2019). Checking the health status of natural populations of Hermann's tortoises by analyzing their peripheral blood cell parameters could also help to recognize potential priorities for conservation actions.

This study aimed to examine the general morphology of blood cells in Eastern Hermann's tortoise wild populations in Serbia and provide insight into the potential reference intervals related to the number of peripheral blood cells of different types and their morphometric parameters. To address these aims, it was necessary to consider various factors; therefore, we compared values of measured blood cell parameters (1) among different wild populations in Serbia, (2) between sexes, and (3) between two seasons - spring and summer.

Material and methods

Investigated tortoise populations

The analyzed individuals of Herman's tortoise originated from three different wild populations at the following localities:

1. Čermor (44º26′59.56″S; 22º8′43.39″E) is located 2 km from Donji Milanovac, in eastern Serbia. This locality belongs to "Đerdap" National Park. It is mainly covered with oak forest. The size of the investigated area was 3.1 ha within the elevation range from 190 to 263 m above sea level. A total of 17 individuals were examined from this population.

2. Gonjište (44º37′51.48″S; 22º31′35.77″E) is situated 3 km from Kladovo and is also located in eastern Serbia. The size of the investigated area was 7.6 ha within 161-190 m of elevation range. This locality was originally steppe, now converted into vineyards and wheat fields. A total of 18 individuals were examined from this population.

3. Locality in Kunovica (43º18′8.00″S; 22º4′59.00″E) is part of Nature Park "Sićevacka klisura" in south-eastern Serbia. It is predominantly oak forest, and the size of the study area was 23.0 ha within 318-452 m of elevation range. Herman's tortoise in this locality has been regularly monitored since 2010. A total of 19 individuals were examined from this population.

Prior to the blood sample collection, all tortoises were physically examined to determine general health and body condition. Sex and age groups were determined by observing specific morphological characteristics such as tail length, shape of plastron concavity (Đorđević et al. 2011), and growth rings on tortoise shell (scute rings) (Ljubisavljević et al. 2012).

Collection of blood samples

Blood samples were collected under manual restraint with a noninvasive method from the jugular vein by a heparinized syringe with a 26 G needle since other veins are closely associated with lymphatic vessels and carry a risk of collecting mixed blood with lymph (Stahl 2006). In line with the methodology proposed by Sykes & Klaphake (2008), which suggests that up to 10% of the total blood volume can be safely sampled from healthy reptiles (Leineweber et al. 2021), we have collected up to a maximum of 1 ml of blood per individual. The tortoises were released immediately after blood collection procedures at the same place where they were found. Samples were collected in two different seasons, spring (May) and summer (July), during one year of study, following the research dynamics described in Stojadinović et al. (2013, 2017). To prevent any effect of anticoagulant on blood cells, blood smears were made instantly after blood sampling in triplicates, air-dried, and stained with the MayGrünwald-Giemsa method (Piaton et al. 2015). The smears were analyzed under a Leica photomicroscope (Leica® DM 2500), and photos of 10 different fields of view were taken for analysis. The peripheral blood cell morphology was measured using the ImageJ® program (Schneider et al. 2012).

Hematological analysis

As our data did not have a normal distribution, we used non-parametric statistics, i.e., Kruskal-Wallis test for between-gender, among population and seasonal comparisons (STATISTICA v7.0, StatSoft. Inc. 1984-2004) (Hilbe 2007). The number of different types of blood cells was interpreted following 500 counted erythrocytes per individual. Following blood cell determination, their morphometric parameters were evaluated:

- Erythrocytes (length, width, size of a cell; length, width, and size of a nucleus; nuclear-cytoplasmic ratio (NC ratio));
- Leucocytes (diameter (r) and size of lymphocytes, diameter and size of monocytes, diameter and size of eosinophils, diameter and size of basophils, diameter and size of heterophils);
- Thrombocytes (length, width, and size of the cells).

All analyzed parameters were compared between the different localities, between seasons, and between sexes.

Results

All 54 analyzed Eastern Hermann's tortoises (23 males and 31 females) were adult reproductively active animals. Not a single individual showed visible signs of illness.

Morphometry of erythrocytes

In our samples, erythrocytes were observed as ellipsoidal cells with orange cytoplasm. The majority of nuclei were centrally positioned and had round or elliptical shapes, with a few exceptions where the nucleus was irregular. The dense chromatin was dark purple. All measured morphometric parameters of erythrocytes were significantly different among three populations (Čermor, Gonjište, Kunovica) and between sexes ($P<0.05$) (Table 1). The length of erythrocytes was the highest in males from Čermor, while the smallest values were detected in females from Kunovica. In the case of erythrocytes' width, the highest values were observed in females from Čermor and the lowest in females from Kunovica. When measuring the area of the erythrocytes, the smallest red blood cells were found in females from Kunovica, while the highest values of the red blood cell area were measured in males from Čermor. The largest nucleus was found in erythrocytes of females at Gonjište locality, the same as in the case of the NC ratio.

Leucocyte number and morphometry

The number of lymphocytes significantly differed among all three localities ($P<0.05$), with the lowest values in Gonjište and the highest values in Čermor (Table 2). Within the population, differences between sexes were found only for a number of lymphocytes in populations from Čermor and Gonjište (Table 2). The diameter (r) of lymphocytes (Table 3) was significantly shorter in females from Kunovica, while the longest lymphocyte diameter was detected in females from Gonjište. Additionally, similar results were found in the size of the lymphocytes (the lowest value was for females from Kunovica, while the largest value was for females from Gonjište; $P<0.05$).

Monocytes were characterized by light blue cytoplasm with or without vacuoles and nuclei that had a reniform or oval shape and took a larger part of the cell. They were very rare in blood smears, and for some localities and/or gender, there is no data about their size. There was no statistically significant difference in the monocyte count and its morphology among localities, between seasons, or between sexes. Additionally, in males from Gonjište, no monocyte was found.

Only a few eosinophils were found in blood smears, so data on their size cannot be considered valid and generally applicable. They exhibited a round or oval shape, contained red granules that did not completely cover the cytoplasm, and had a typical round or bi-lobular-shaped purple nucleus.

Basophils, filled with round, blue granules, were rarely found; this type of blood cell was not detected in some localities and/or in certain genders. Their number significantly differed when comparing females from Gonjište and Kunovica ($P<0.05$). Basophils were also very rare in examined blood smears, so information about their size was scarce, and it was impossible to analyze their number and morphometric values statistically.

Table 1. Morphometry of erythrocytes. The erythrocyte and its nuclei measurements (μm) established in the peripheral blood of *T. h. boettgeri* from Serbia (interpreted according to 150 measured cells per individual). (average values ± standard deviation with coefficient of variation below and P values of Kruskal-Wallis test, * P values between sexes, ** P values among localities)

Erythrocyte morphometry		**Čermor**	**Gonjište**	**Kunovica**	**P value****
No of individuals		17	18	19	
Length of erythrocytes	Male	18.377±2.164 0.118	17.583±1.364 0.078	18.266±1.538 0.084	<0.0001
	Female	17.666±1.649 0.093	17.677±1.596 0.09	17.257±1.630 0.094	<0.0001
	P value*	<0.0001	0.003	<0.0001	<0.0001
Width of erythrocytes	Male	10.942±1.052 0.096	10.495±1.445 0.138	10.689±1.171 0.11	<0.0001
	Female	11.004±1.077 0.098	10.932±1.107 0.101	10.107±0.909 0.09	<0.0001
	P value*	0.037	<0.0001	<0.0001	<0.0001
Size of erythrocytes	Male	158.553±33.416 0.211	145.245±25.176 0.173	153.863±26.419 0.172	<0.0001
	Female	153.157±24.321 0.159	152.190±25.749 0.169	137.225±20.689 0.151	<0.0001
	P value*	<0.0001	<0.0001	<0.0001	<0.0001
Nucleus length	Male	6.198±0.736 0.119	6.226±1.401 0.225	5.922±0.564 0.095	<0.0001
	Female	6.039±0.831 0.138	6.186±0.719 0.116	5.841±0.593 0.102	<0.0001
	P value*	<0.0001	<0.0001	<0.0001	<0.0001
Nucleus width	Male	4.448±0.599 0.135	4.323±0.626 0.145	4.423±0.406 0.092	<0.0001
	Female	4.311±0.519 0.12	4.495±0.539 0.12	4.306±0.456 0.106	<0.0001
	P value*	<0.0001	<0.0001	<0.0001	<0.0001
Nucleus size	Male	21.744±4.554 0.209	21.396±7.554 0.353	25.594±10.375 0.405	<0.0001
	Female	20.515±4.335 0.211	28.141±27.676 0.983	19.779±3.342 0.169	<0.0001
	P value*	<0.0001	<0.0001	<0.0001	<0.0001
NC ratio	Male	0.139±0.031 0.224	0.156±0.085 0.546	0.169±0.069 0.411	<0.0001
	Female	0.136±0.031 0.231	0.218±1.747 8.006	0.147±0.032 0.22	<0.0001
	P value*	<0.0001	<0.0001	<0.0001	<0.0001

Table 2. Number of different types of peripheral blood cells (numbers interpreted according to 500 counted erythrocytes per individual) (average values ± standard deviation with coefficient of variation below and P values of Kruskal-Wallis test., * P values between sexes, ** P values among localities)

Number of peripheral blood cells		**Čermor**	**Gonjište**	**Kunovica**	**P value****
No of individuals		17	18	19	
Number of lymphocytes	Male	0.203±0.480 2.366	0.102±0.370 3.644	0.276±0.493 1.789	<0.0001
	Female	0.371±0.635 1.711	0.172±0.410 2.379	0.278±0.738 2.655	<0.0001
	P value*	0.004	0.006	0.443	0.658
Number of monocytes	Male	0.024±0.154 6.372	/	0.031±0.173 5.656	0.026
	Female	0.018±0.133 7.416	0.009±0.097 10.279	0.015±0.120 8.226	0.711
	P value*	0.681	/	0.351	0.440
Number of eosinophils	Male	0.024±0.154 6.372	0.004±0.061 16.31	0.020±0.142 6.964	0.146
	Female	0.024±0.153 6.403	0.003±0.056 17.861	0.029±0.196 6.691	0.069
	P value*	0.990	0.898	0.825	0.562
Number of basophils	Male	0.019±0.138 7.141	/	0.010±0.101 9.899	0.081
	Female	/	0.003±0.056 17.861	0.024±0.155 6.34	0.015
	P value*	/	/	0.408	0.529
Number of heterophils	Male	0.101±0.318 3.137	0.030±0.171 5.69	0.112±0.317 2.827	0.003
	Female	0.096±0.295 3.081	0.056±0.244 4.33	0.220±0.470 2.142	<0.0001
	P value*	0.967	0.166	0.063	0.057
Number of thrombocytes	Male	0.560±0.890 1.587	0.248±0.625 2.52	0.694±0.817 1.178	<0.0001
	Female	0.940±1.123 1.195	0.458±0.771 1.685	0.941±1.227 1.304	<0.0001
	P value*	<0.0001	<0.0001	0.182	<0.0001

Heterophils were identified by their spindle-shaped reddish-orange granules in the cytoplasm, and they were observed to be more abundant than other granulocytes. The number of heterophils showed significant differences among all three analyzed localities (P<0.05), with the highest values in Kunovica and lowest values in Gonjište. The significant differences in r and size of heterophils (P<0.05) were observed among males from all analyzed localities, with the lowest values detected in Kunovica and the highest values in Čermor.

When comparing values of the leucocyte morphometric characteristics between sexes (Table 3), a significant difference was observed only at Kunovica locality. The r (and therefore also the size of lymphocytes was significantly lower in females than males (P<0.05). Seasonal differences in the number of lymphocytes were detected between populations from Čermor and Gonjište (Table 4): more lymphocytes were counted in spring (May) than in summer (July) (P<0.05).

Table 3. Morphometry of leucocytes (μm). The leucocytes established in the peripheral blood of *T. h. boettgeri* from Serbia. (average values ± standard deviation with coefficient of variation below and P values of Kruskal-Wallis test, * P values between sexes, ** P values among localities)

Morphometry of leucocytes		**Čermor**	**Gonjište**	**Kunovica**	**P value****
Number of individuals		17	18	19	
r of lymphocytes	Male	4.112±1.212 0.295	3.949±0.605 0.153	3.941±0.717 0.182	0.951
	Female	3.861±0.867 0.224	4.163±0.810 0.195	3.444±0.503 0.146	<0.0001
	P value*	0.134	0.293	0.002	<0.0001
Size of lymphocytes	Male	61.340±47.140 0.769	50.058±15.708 0.314	50.323±19.380 0.385	0.897
	Female	48.677±25.781 0.53	56.443±23.054 0.408	38.017±11.261 0.296	<0.0001
	P value*	0.106	0.293	0.002	<0.0001
r of monocytes	Male	6.222±1.140 0.183	/	6.765±0.171 0.025	0.180
	Female	5.104±0.388 0.076	6.111±1.143 0.187	5.246±0.678 0.129	0.430
	P value*	0.101	/	0.050	0.794
Size of monocytes	Male	124.845±48.150 0.386	/	143.729±7.240 0.05	0.180
	Female	82.103±12.151 0.148	119.991±46.031 0.384	87.383±22.532 0.258	0.430
	P value*	0.101	/	0.050	0.794
r of eosinophils	Male	5.566± 1.046 0.188	5.237±0 1	6.508 ± 3.147 0.484	1.000
	Female	6.023±1.189 0.197	6.553±0 1	6.279±1.503 0.239	0.981
	P value* (gender)	0.773	0.317	1.000	0.932
Size of eosinophils	Male	99.835± 38.686 0.387	86.102±0 1	148.517± 128.632 0.866	1.000
	Female	117.229±42.200 0.36	134.817±0 1	129.725±60.942 0.47	0.981
	P value* (gender)	0.773	0.317	1.000	0.932
r of basophils	Male	5.101± 0.602 0.118	/	4.517±0 1	0.480
	Female	/	6.242±0 1	3.966±0.646 0.163	0.180
	P value* (gender)	/	/	0.180	0.091
Size of basophils	Male	82.565± 18.285 0.221	/	64.052±0 1	0.480
	Female	/	122.342±0 1	50.251±15.536 0.309	0.180
	P value*	/	/	0.180	0.091
r of heterophils	Male	6.823± 1.277 0.187	6.320± 1.020 0.161	5.850± 1.079 0.184	0.033
	Female	6.213±0.743 0.12	6.225±1.130 0.181	5.937±0.974 0.164	0.522
	P value* (gender)	0.058	0.781	0.479	0.013
Size of heterophils	Male	160.713± 58.568 0.364	128.266± 39.437 0.307	110.787± 43.127 0.389	0.033
	Female	122.827±29.354 0.239	125.463±43.666 0.348	113.568±35.542 0.313	0.522
	P value* (gender)	0.058	0.781	0.479	0.013

Table 4. Number of different cell types comparing two seasons and 3 populations (average values ± standard deviation with coefficient of variation below and P values of Kruskal-Wallis test).

Number of different cell types	Čermor		Gonjište		Kunovica	
	May	July	May	July	May	July
No of lymphocytes	0.320±0.626	0.241±0.495	0.285±0.546	0.09±0.310	0.183±0.388	0.36±0.833
	1.955	2.05	1.918	3.445	2.12	2.313
P values	<0.0001		<0.0001		0.065	
No of eosinophils	0.023±0.150	0.025±0.157	0.013±0.115	/	0.021±0.187	0.031±0.174
	6.557	6.245	8.66		8.857	5.603
P values	0.436		/		0.334	
No of basophils	0.023±0.150	/	0.007±0.081	/	0.014±0.118	0.025±0.156
	6.557		12.288		8.396	6.285
P values	/		/		0.503	
No of heterophils	0.08±0.272	0.116±0.336	0.099±0.322	0.025±0.157	0.169±0.376	0.199±0.472
	3.401	2.906	3.237	6.208	2.225	2.375
P values	0.316		0.090		0.927	
No of monocytes	0.046±0.209	/	0.013±0.115	0.002±0.048	0.007±0.084	0.031±0.174
	4.582		8.66	20.833	11.916	5.603
P values	/		0.105		0.135	
No of thrombocytes	0.617±0.963	0.829±1.055	0.841±0.903	0.196±0.549	0.817±0.942	0.901±1.251
	1.56	1.272	1.073	2.801	1.154	1.389
P values	<0.0001		<0.0001		0.955	

Thrombocyte number and morphometry

Thrombocytes were observed as smaller, oval cells with clear cytoplasm. These cells were very elongated with length almost two times greater than their width. Thrombocytes were the least numerous (Table 2) in individuals from Gonjište ($P<0.05$). Sexual difference in the number of thrombocytes (Table 2) was observed in Gonjište and Kunovica, with a higher number of cells in females than males ($P<0.05$).

When morphometric parameters were observed (Table 5), a significant difference was found in the length of thrombocytes among all three localities, but only in females, with the lowest values in Kunovica and highest in Gonjište. The width of thrombocytes differed among analyzed localities both in males and females. The highest width was detected in males from Čermor and the lowest in females from Gonjište. In the case of thrombocyte size, the largest cells were observed in females from Gonjište and the smallest ones in females from Kunovica.

Seasonal differences in the number of thrombocytes were significantly different (Table 4), and the highest values were found in Čermor in July, while in Gonjište, more thrombocytes were counted in May.

Variations in blood cell count and morphometry were observed among all three analyzed factors (localities, season, and sexes). Cell inclusions in the erythrocyte cytoplasm were also noticed in samples from all three localities (Fig. 1).

Discussion

This study revealed significant differences in erythrocyte morphometric parameters (length, width, and area) between the sexes and the three populations studied. These characteristics agree with existing data about the morphology of these types of blood cells (Strik et al. 2007). Erythrocytes were observed in both immature (as polychromatophils) and mature forms. Polychromatophils were smaller, rounder, and more basophilic than mature erythrocytes and had larger, mostly round nuclei. Typically, their presence in peripheral blood is limited to a small fraction, less than 1% (Stacy et al. 2011). Reptilian erythrocytes have an average lifespan of 600-800 days, which is longer than that of mammals due to the slower metabolic rate and red blood cell turnover of reptiles (Stacy et al. 2011). Small vacuoles, observed in erythrocytes of samples analyzed in this study, represent degenerated organelles normally found in healthy Chelonians (Stacy et al. 2011). Detected variation in the size of red blood cells and nuclei is common in healthy reptiles (Stacy et al. 2011). It can be influenced by environmental factors such as temperature, altitude, and air pressure, which could also explain the population-based variability in erythrocytes and nucleus size observed in our study.

It was proposed that sex-related disparities in *T. hermanni* could potentially impact hematological parameters related to the erythrocytic indexes, such as mean corpuscular volume (MCV), which is an indicator of the average size of red blood cells (Bielli et al. 2015). Our study has provided evidence supporting this proposition.

The number of lymphocytes, heterophils, and basophils in blood differed significantly among the three analyzed populations. Our findings regarding leukocyte counts are consistent with previous studies, which identified lymphocytes as the predominant type of white blood cell (Arikan et al. 2015, Bielli et al. 2015) and characterized them as mostly small, round cells with a large nucleus, clumped chromatin, and a small amount of surrounding cytoplasm. Changes in lymphocyte count can indicate inflammatory processes, parasitic infections, and viral diseases (Campbell 2004). Besides, since tortoises are ectotherms and different seasonal temperatures could influence their metabolism (Cloudsley-Thompson 2007), the number of lymphocytes could also be affected. A higher lymphocyte count detected in females from Čermor and Gonjište may be attributed to the inhibitory effect of testosterone on lymphocyte proliferation (Andreani et al. 2014). However, no difference in lymphocyte count was detected between the two sexes in Kunovica. The diameter and size of lymphocytes in females also significantly differed among populations. The morphological parameters of blood cells may vary depending on different factors such as sex, age, nutrition, physiological status, hibernation, habitat, season, temperature, and life in captivity or the wild

(Stacy et al. 2011). The results suggested seasonal variations in the number of lymphocytes in the populations of Eastern Hermann's tortoises from Čermor and Gonjište, with higher counts observed in spring than summer. The probable explanation for all detected variability in the results of lymphocyte analysis (significant variations in lymphocyte count and morphology among populations, including higher lymphocyte count in females compared to males and higher counts observed in spring compared to summer in two localities) could be attributed to Eastern Hermann's tortoises' environmental temperature-dependent metabolism.

Table 5. Morphometry of thrombocytes (µm). The thrombocytes established in the peripheral blood of *T. h. boettgeri* from Serbia (average values ± standard deviation with coefficient of variation below and P values of Kruskal-Wallis test, * P values between sexes, ** P values among localities).

Morphometry of thrombocytes		Čermor	Gonjište	Kunovica	P value**
Number of individuals		17	18	19	
Length of thrombocytes	Male	12.988±2.648 0.204	12.788±1.868 0.146	12.007±2.580 0.215	0.078
	Female	12.423±2.362 0.19	12.635±2.603 0.206	10.779±2.245 0.208	<0.0001
	P value*	0.064	0.642	0.000	<0.0001
Width of thrombocytes	Male	6.459±1.050 0.163	6.062±0.857 0.141	5.999±1.238 0.206	0.002
	Female	6.659±0.941 0.141	6.782±1.051 0.155	5.438±0.836 0.154	<0.0001
	P value*	0.308	<0.0001	0.004	<0.0001
Size of thrombocytes	Male	66.824±20.461 0.306	62.913±20.946 0.333	57.808±20.834 0.36	0.021
	Female	65.295±17.225 0.264	69.096±21.182 0.307	46.434±13.567 0.292	<0.0001
	P value*	0.439	0.024	<0.0001	<0.0001

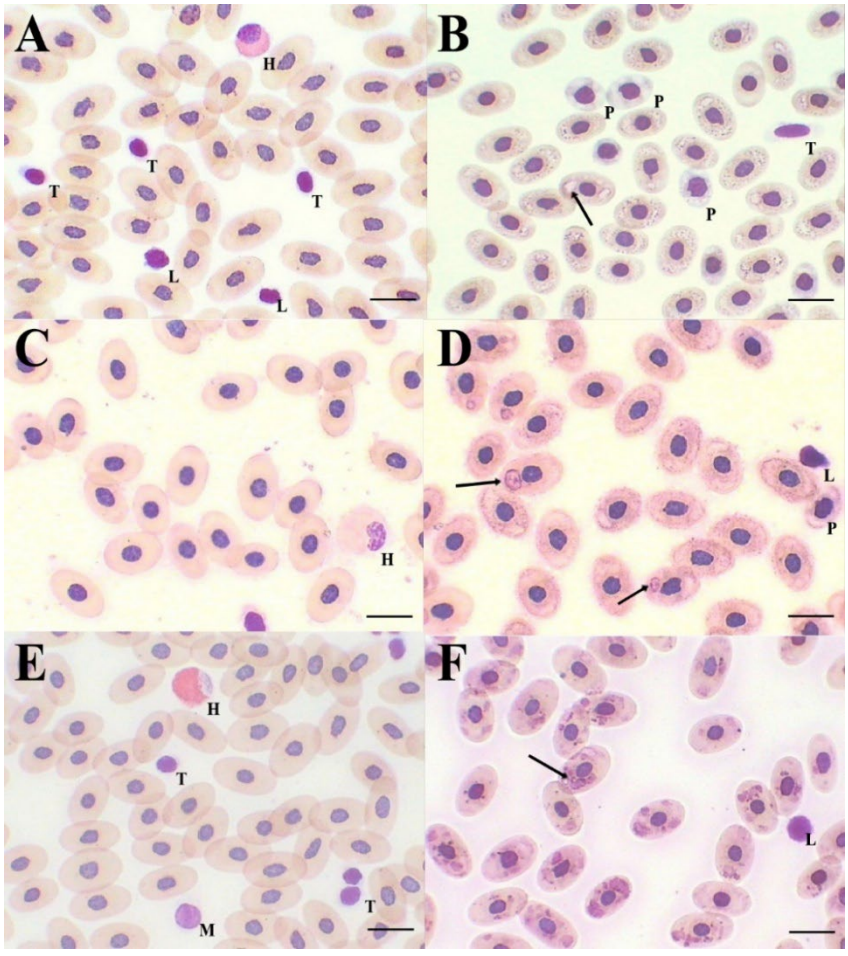

Figure 1. *T. h. boettgeri* peripheral blood cells from A, B – population of Čermor; C, D - population of Gonjište; E, F – population of Kunovica. Black arrows show cytoplasmic inclusions of unknown origin. H: heterophils; L: lymphocytes; M: monocytes; P: immature erythrocytes (polychromatophils); T: thrombocytes. Scale bar: 10 µm.

As described by Redrobe and MacDonald (1999), monocytes appeared similar to lymphocytes. The features of eosinophils observed in our samples were consistent with those previously characterized by Stacy et al. (2011). The same authors identified basophils by their large, basophilic granules stained dark blue and often so numerous that they occlude the whole nucleus (Kassab et al. 2009), also observed in our study.

The identification and general features of heterophils were also consistent with previous findings (Kassab et al. 2009, Bielli et al. 2015). Our study has revealed a significant difference in the number of heterophils among populations. Given that the primary role of heterophils is to eliminate bacteria (Redrobe & MacDonald 1999), an observed increase in the quantity of these cells in one particular population may suggest the presence of infection and lower general health status. Additionally, the number of heterophils can be affected by seasonal factors such as temperature (Campbell 2004); thus, the observed variation in their numbers could be explained by the level of differences in environmental conditions among analyzed localities.

Thrombocytes of tortoises are nucleated and resemble small lymphocytes, so heparin can induce platelet clumping, thereby facilitating their distinction (Stacy et al. 2011). Although a statistically significant difference was observed in the thrombocyte count across analyzed populations, the platelet count appeared to be influenced by sex and season solely in populations from Čermor and Gonjište.

During the examination of blood smears in the current study, distinct changes were observed in the erythrocyte cytoplasm across all localities. These modifications were characterized by cytoplasmic inclusions that resembled those associated with hemoparasitic infection. Basile et al. (2011) also described hemoglobin (Hb) precipitation and Heinz bodies formation in loggerhead red blood cells. In humans and other vertebrates, Heinz bodies have been associated with different pathological conditions, such as hemolytic anemia (Basile et al. 2011). The percentage of tortoises with erythrocyte alterations in one locality was calculated in relation to the total number of individuals from which the blood was taken. The tortoises with the abovementioned alterations were present in Čermor and Gonjište at a frequency of 11.76% and 16.66%, respectively, whereas a higher incidence of such individuals was observed in Kunovica (31.57%). Upon examining the population-specific variability of the leukocyte count, it was noted that the Kunovica population exhibited a significant increase in the numbers of both lymphocytes and heterophils, in addition to a higher incidence of tortoises with erythrocyte alterations. These findings suggest that one of the pathological conditions mentioned before could explain structures in the erythrocyte cytoplasm. This information raises new questions that will require further research in the field and laboratory to determine the cause of the observed changes in erythrocyte cytoplasm.

Research has shown captive reptiles have distinct hemogram patterns compared to their wild counterparts (Stacy et al. 2011). While captive species have been studied in this regard, little is known about the hematology of wild reptile populations. Thus, determining reference values for peripheral blood cells of wild populations, such as populations of *T. h. boettgeri*, is of utmost importance. This is crucial because any deviations from these reference values could indicate the compromised health status of the population, which can indicate stress, disease, or other environmental factors. Establishing reference hematological intervals can be crucial in preventing the continued exploitation of Hermann's tortoise populations in Serbia. By providing new evidence on the hematological parameters of wild populations, these reference intervals can support the implementation of appropriate conservation measures.

In conclusion, the results of this study revealed differences in various blood cell parameters among analyzed population samples of Eastern Hermann's tortoises. They pointed to the possible lower health status of one of them due to the indication of the presence of hemoparasites. This highlights the importance of establishing reference values for blood cell parameters in Eastern Hermann's tortoises, particularly in wild populations. The findings also revealed that variations in peripheral cell number and morphometric parameters can be influenced by population, sex, and season. Therefore, it is crucial to consider these factors when establishing comprehensive reference hematological intervals in healthy wild populations of *T. h. boettgeri*. These reference intervals can serve as a baseline for monitoring the health status of wild populations, identifying early indicators of stress or disease, and guiding conservation efforts. Overall, this study also contributes to the general understanding of the hematological parameters of Hermann's tortoises and provides valuable insights into their conservation.

Acknowledgments

This work was funded by Rufford Small Grants No. 18761-1 and 22238-2 for MN, JS, and DSZ and Grant No. 173025 for the Ministry of Education, Science and Technological Development of the Republic of Serbia for DS and JCI. We owe a debt of gratitude to members of the Biological Society "Dr. Sava Petrovic" from the Faculty of Sciences and Mathematics University of Niš in Serbia for participating in the fieldwork. We thank the Savić family for their lovely hospitality during our fieldwork.

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