Hematological findings of free-ranging brown bears (*Ursus arctos*) from eastern Turkey, obtained by blood film evaluation

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ABSTRACT

Hematology is one of the best population health indicators, and the quickest way to gain insight into some hematological parameters is blood film evaluation. Sometimes, due to the inability to store blood, the unavailability of hematological instruments during field work, or the insufficient amount of blood for complete hematological analysis, blood film evaluation could be the only method for obtaining information about hematological changes. The population of brown bears (*Ursus arctos*) is often endangered, and is protected as an important integral species of terrestrial communities. Since any baseline hematological data of free-living endangered species are particularly important, the aim of this study was to test the possibility of using blood film evaluation, as the only source of hematological data, for assessment of an animal's hematological and, consequently, health status. Blood films of seventeen brown bears from eastern Turkey were evaluated to assess the morphology of erythrocytes and leukocytes, estimate the total leukocyte count, determine the differential leukocyte count, and look for the presence of cell inclusions or hemoparasites. Rouleaux formations were present in twelve animals, poikilocytosis in four, while parasitic nematodes, microfilariae, were found in nine out of seventeen bears. The results confirmed that blood film evaluation alone could be of use in assessing an animal's hematological status, but for more accurate assessment of health status, more blood parameters need to be analyzed. New findings in the study, such as the presence of rouleaux formations and microfilaria in brown bears from eastern Turkey, have opened the door for further investigation in this field.

Key words: free-ranging brown bears; hematology; hemoparasites; microfilariae; blood film evaluation; eastern Turkey

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Introduction

Hematology is one of the diagnostic methods used most often for the health assessment of domestic and wild animals. In wild, free-ranging animals, hematological data, when combined with data on body weight, survival and reproduction rate, help to draw conclusions about the animals' physiological status, and consequently species management (MOEN et al., 2010). After collection, blood samples are usually sent for analysis to reference laboratories with large and expensive hematology instruments. Beside analysis using these instruments, an indispensable part of hematology is also a blood film examination which is mainly used to verify the results obtained by means of the hematological instruments, especially differential count, and to determine the morphological features of blood cells and/ or inclusions that cannot be detected by the instruments. Besides, blood film can also be used for estimation of the total leukocyte, erythrocyte and thrombocyte counts (DURBIN et al., 2009). This part of the analysis is usually performed by experienced and trained specialists. During field work with wildlife, especially in remote or rural areas where hematology instruments and proper storage of blood samples are not available, or when the blood volume needed for analysis is limited, blood film evaluation could be the only method for obtaining the information on hematological changes of the animal (ALLISON and MEINKOTH, 2007; DURBIN et al., 2009). In this study, we used blood films from the wild brown bears as the only source of hematological data, since the use of hematology instruments was not available, and the blood for analyses, unlike plasma and serum, cannot be stored for several days during field work.

The brown bear (*Ursus arctos*) is one of the five species of large carnivores in Europe (KACZENSKY et al., 2013) which are an important component of biological diversity (LINNELL et al., 2000). Over the last century the population of brown bears in Europe declined dramatically (KUSAK et al., 2005), but in the last few decades, due to increases in prey availability, forest cover and favorable legislation, the population of brown bears has recovered and increased in size and range (CHAPRON et al., 2014). For successful management and maintenance of populations, regular monitoring of the wild animals' health status is necessary (MÖRNER et al., 2005).

The aim of the study was to evaluate the blood films from brown bears, that is, estimate the total leukocyte and differential count, and determine the morphological features of blood cells and possible inclusions or parasites within or outside the cells. Another goal was to find out whether blood film evaluation alone, as the only source of hematological data, in cases of limited laboratory equipment availability during field work, could be of use in determining an animal's health status

Materials and methods

Animals. Seventeen blood samples from free-ranging brown bears (11 males and 6 females) from north-eastern Turkey were collected during the years 2017 and 2018. The capture and handling of the animals were permitted by the General Directorate of Nature Conservation and the National Parks and Forestry General Directorate of Turkey's Ministry of Forestry and Water Affairs, using approved methods for capturing bears (KACZENSKY et al., 2006), with the addition of the use of trap alarms and surveillance cameras, which enabled prompt response and tranquilization of bears within 20 to 60 minutes after capture. Captured animals were tranquilized using narcotics in the doses recommended by KREEGER and ARNEMO (2009). All the animals underwent external physical examination, with measuring of body temperature, heart and respiration rate, and on the basis of these features they were found clinically healthy at the time of sampling.

The age of the bears was estimated using the method described by STONEBERG and JONKEL (1966). The blood was taken from the femoral vein (*vena femoralis*). Blood samples were collected into EDTA tubes (Becton Dickinson, Vacutainer System USA, Rotherford, New Jersey, USA) within 10 minutes after immobilization. Two blood films for each animal were made immediately after collection, and air dried. Within the next 24 hours the dried blood films were stained with Wright-

Giemsa stain (Merck® Ref no: 1.09204.0500) according to the manufacturer's instructions. The rest of the blood was centrifuged, and plasma was stored at -80°C until further processing.

Laboratory analysis. The stained blood films were sent to the Department of Pathophysiology of the Faculty of Veterinary Medicine, University of Zagreb, Croatia, where the blood films were examined. For each animal, two blood films were examined. The evaluation was performed on a monolayer of each film, under 40x and 100x lenses using an OLYMPUS BX41 microscope. The total white blood cell count (WBC) was estimated from the average leukocyte count on 10 view fields, multiplied by the square of the lens (40x) (HARVEY, 2001; SHELDON et al., 2016). Differential blood count was determined by counting 200 leukocytes on the blood film under the 100x lens. Counting 200 leukocytes ensures and improves the accuracy of the values obtained for the differential blood count.

Statistical analysis. Statistical analysis of the data was performed using the software package SAS 9.4 (Statistical Analysis Software 2002-2012 by SAS Institute Inc., Cary, USA). The MEANS and FREQ procedures were used for descriptive statistics. Data normality was tested using the UNIVARIATE module. The testing of the differences in the individual independent samples between two groups were performed using the Mann-Whitney U test (NPAR1WAY procedure) in cases of non-normally distributed data, while the T-test (TTEST procedure) was used when data sets were normally distributed. Graphs were created using the SGPLOT procedure. The level of statistical significance was set at $P<0.05$.

Results

Estimated total WBC, and relative and absolute differential counts are shown in Table 1. The mean value of total WBC count was 10.1 $x10^3/\mu L$. The differential count revealed that neutrophils were the most represented leukocytes in bears at 64.29% and an absolute count of 6.49 x $10^3/\mu L$. The mean value of band neutrophils was 7.88%, while the absolute count was 0.77 x $10^3/\mu L$. Lymphocytes were the second most common leukocytes, with a mean

percentage of 19.07% and absolute count of 1.96 x $10^3/\mu L$. Monocytes were represented as 3.7 % on average and 0.36 x $10^3/\mu L$ in the count. Eosinophils accounted for 3.82% and 0.41 x $10^3/\mu L$ of all leukocytes. Basophils were the rarest leukocytes in peripheral blood, with only 0.58% and 0.05 x $10^3/\mu L$. Rouleaux formations were present in twelve individuals (Fig. 1), poikilocytosis in four, while microfilariae (Fig. 2.) were found in nine (52.9%) out of seventeen bears.

Table 1. Estimated total WBC count, relative and absolute differential count in brown bears (n=17) from eastern Turkey.

| Parameter (units)	Mean	SD	Range
WBC$_{est}$ (x10³/μl)	10.01	3.47	5.6-16.6
Neutrophils (%)	64.29	14.15	34-86
Neutrophils (x10³/μl)	6.49	2.56	2.3-10.3
Band (%)	7.88	8.02	0-30
Band (x10³/μl)	0.77	0.78	0-2.5
Lymphocytes (%)	19.07	8.1	9-36
Lymphocytes (x10³/μl)	1.96	1.01	0.56-3.68
Monocytes (%)	3.7	2.8	0-12
Monocytes (x10³/μl)	0.36	0.32	0-1.3
Eosinophils (%)	3.82	5.83	0-24
Eosinophils (x10³/μl)	0.41	0.52	0-1.63
Basophils (%)	0.58	0.93	0-3
Basophils (x10³/μl)	0.05	0.09	0-0.35
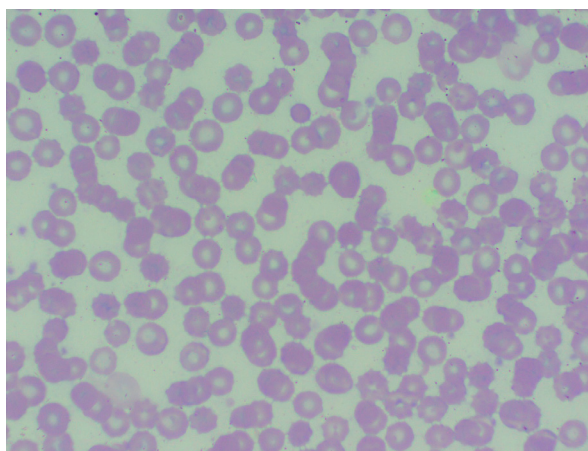
Fig. 1. Rouleaux formation in the blood films (100 x objective) of the brown bears from eastern Turkey.

Fig. 2. Microfilariae and rouleaux formations in the blood films (40 x objective) of brown bears from eastern Turkey.

The statistical differences in total and differential blood count between animals without microfilaria (Group 1) and with microfilaria (Group 2) are shown in Table 2. Bears with microfilariae had a significantly lower (T-test, P=0.01) mean relative number of segmented neutrophils (56.55%) than the bears without microfilariae (73%). However, bears which had microfilariae also had a significantly (T- test, P<0.05) higher relative and absolute mean number of lymphocytes (23.77% and 2.43 x10^3/µL, respectively), (Fig. 3.), as well as a significantly higher (T-test, P<0.05) absolute number of eosinophils (0.65 x10^3/µL) (Fig. 4.).

Table 2. Hematological parameters in bears without microfilariae (Group 1) and bears with microfilariae (Group 2)

Parameter (units)	Group 1 (n=8)		Group 2 (n=9)		
	Mean (±SD)	Range	Mean (±SD)	Range	P-value
WBCest (x10^3/ul)	9.15 (±2.57)	5.6-13.2	10.79 (±4.11)	6-16.6	0.34
Neutrophils (%)	73 (±12.13)	56-86	56.55 (±11.35)	34-73	0.01*
Neutrophils (x10^3/ul)	6.71 (±2.22)	3.2-10.3	6.29 (±2.94)	2.3-9.96	0.75
Band (%)	6.75 (±10.29)	0-30	8.88 (±5.79)	0-18	0.26
Band (x10^3/ul)	0.48 (±0.6)	0-1.7	1.03 (±0.86)	0-2.5	0.15
Lymphocytes (%)	15.12 (±6.59)	9-28	23.77 (±7.3)	13-36	0.02*
Lymphocytes (x10^3/ul)	1.42 (±0.87)	0.56-3.3	2.43 (±0.91)	1.54-3.68	0.01*
Monocytes (%)	3.12 (±2.41)	0-7	4.22 (±3.30)	1-12	0.58
Monocytes (x10^3/ul)	0.3 (±0.3)	0-0.9	0.42 (±0.34)	0.08-1.3	0.28
Eosinophils (%)	1.5 (±1.06)	0-3	5.88 (±7.54)	0-24	0.29
Eosinophils (x10^3/ul)	0.14 (±0.11)	0-0.35	0.65 (±0.63)	0-1.63	0.04*
Basophils (%)	0.5 (±1.06)	0-3	0.66 (±0.86)	0-2	0.54
Basophils (x10^3/ul)	0.05 (±0.12)	0-0.35	0.04 (±0.06)	0-0.16	0.3

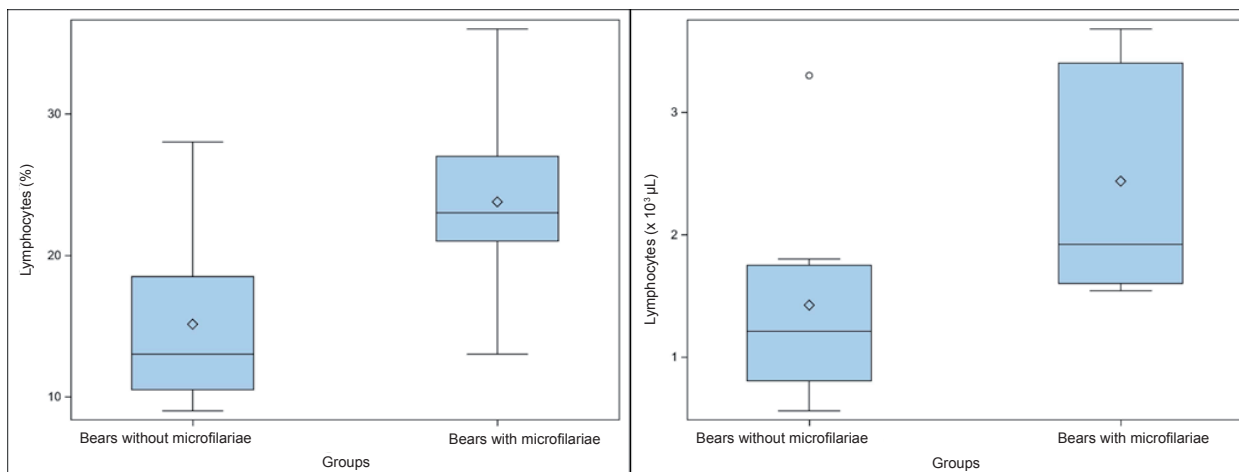*Statistically significant difference between groups

Fig. 3. Differences in the relative and absolute mean numbers of lymphocytes between brown bears
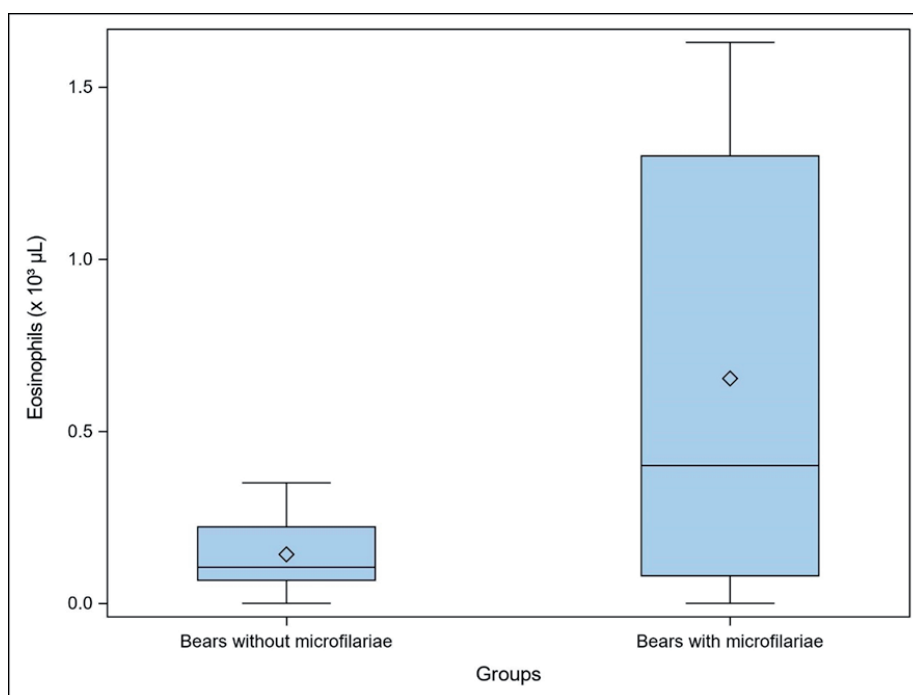from eastern Turkey without and with microfilariae in peripheral blood.

Fig. 4. Differences in the absolute mean numbers of eosinophils between brown bears
from eastern Turkey without and with microfilariae.

Discussion

The mean estimated total WBC count in our study was similar to the previously reported values for brown bears from Croatia (KUSAK et al., 2005; GRÆSLI et al.,2014) as well as for other bear species, such as American black bears (*U. americanus*) (DELGIUDICE et al. 1991) and polar bears (*U. maritimus*) (KIRK et al., 2010). Our results were obtained by estimating the number of

WBC on a blood film, as previously described by HARVEY (2001), ALLISON and MEINKOTH (2007) and DURBIN et al. (2009), while in other studies, the total number of WBC was obtained by analysis using a hematological instrument. We cannot say if the data from those studies can be taken as references, but they can be compared to our results. The mean total WBC count in brown bears from Croatia was similar to the mean total WBC count values found for brown bears in this study (KUSAK et al., 2005) but with a much higher upper range limit. Since the time spent trapped is one of main factors that affects the level of stress and dehydration of captured wild animals (SANTOS et al., 2017; CATTET et al., 2021) the explanation of the authors of the Croatian study was that leukocytosis was caused by the capture method, using foothold snares which were checked only once a day, in the morning. The narrower range and lower upper limit of the WBC count in bears from Turkey can be explained by the improved capturing protocol in which trap alarms were used. That minimized the time between capture and tranquilization, resulting in less strain and stress, and almost no effect on WBC count.

Differential blood count revealed neutrophils as the most represented leukocytes, followed by lymphocytes, monocytes and eosinophils, while basophils were, as in all other mammals, the least represented leukocytes (EBERLE and VOEHRINGER, 2016; MIYAKE and KARASUYAMA, 2017). These findings agree with the literature data for most mammals (SCHALM, 2010).

The most important finding in this study was the detection of microfilariae in the peripheral circulation of studied bears. Nine out of seventeen bears had microfilariae in their blood stream. The microfilaria is an early stage in the life cycle of certain parasitic nematodes, most often *Dirofilaria* spp. The exact species cannot be identified only by its morphological features in the blood film, but more sophisticated methods, such as Knott's technique, immunohistology and PCR, are needed (MORCHÓN et al., 2012; RIMAL et al., 2021). There are about 40 recognized species of *Dirofilari* and at least six of them, i.e., *D. immitis, D. repens,*

D. striata, D. tenuis, D. ursi and *D. spectans,* are known to cause accidental infections in humans (REDDY, 2013). *Dirofilaria ursi*, the bear filarial worm, which causes subcutaneous dirofilariasis in bears, was found in American black bears, brown bears from North America and Asian black bears (*Ursus thiabetanus*) (CRUM et al., 1978.; MICHALSKI et al., 2010; GWYNN et al., 2017). To the author's knowledge no occurrence of *D. ursi* has been reported in European brown bears. Other species of *Dirofilaria*, particularly *Dirofilaria immitis,* were found at necropsy in the heart and pulmonary arteries in one European brown bear from Greece, but no microfilariae were found in the blood stream (PAPANDOPULOS et al., 2017). *D. immitis* is commonly found in dogs in whom it occupies the heart, hence the name heartworm, but it may also be found in a variety of mammals, including humans (REDDY, 2013; MURRAY et al, 2021). We believe that the microfilariae found in our study could also be *Dirofilaria immitis*. However, this presumption has to be tested by further investigations using the above-mentioned methods. We also cannot rule out the possibility that more bears in this study were infected with microfilariae, as they may not always be present in the blood and are found in the lungs and heart during necropsy of the animal. Since the brown bear is an endangered species in Europe, keeping track of the epidemiological occurrence of parasites is an important part of population monitoring and the effective management of wild bears, as well as other wild mammals.

When comparing the blood parameters of the group of bears without microfilariae (Group 1) with the group of bears who had microfilariae in blood films (Group 2), a significant difference was found in neutrophils, lymphocytes and eosinophils. The significantly lower relative number of neutrophils in bears with microfilaria was probably due to the significantly higher relative number of lymphocytes in the same group. Some studies in dogs infected with microfilariae reported lymphopenia (WYSMOŁEK et al., 2020; BEZERRA et al., 2021), while others reported lymphocytosis (SHARMA and PACHAURI, 1981). In our study, the bears with microfilariae

had a significantly higher number of lymphocytes than those without microfilariae. We assume that the higher number of lymphocytes in infected bears could be due to the persistent antigenic stimulation caused by microfilariae (SCHULTZE, 2010). Bears with microfilariae had a significantly higher absolute number of eosinophils (P<0.05) than the bears without microfilariae. Eosinophils, as one of the major effector cells in the immune system, have an important role in anti-parasitic and inflammatory responses. In parasitic invasions, a higher number of eosinophils is usually associated with parasite migration, i.e. extensive parenchymal destruction due to the migration of the parasite through tissues (YOUNG and MEADOWS, 2010). Although eosinophilia in a peripheral blood smear may sometimes be the sole indicator of parasitemia, the absence of eosinophilia does not exclude the presence of parasites (GARG et al., 2019). Only 10% of human patients infected with *D. immitis* have eosinophila (O'CONNELL and NUTMAN, 2015; MURRAY et al., 2021) while in infected dogs, the most common feature of filariasis was regenerative anemia rather than eosinophilia, which was mostly absent (SHARMA and PACHAURI, 1981; KAEWTHAMASORN et al., 2008; WYSMOŁEK et al., 2020; BEZERRA et al., 2021). In our study, the higher number of eosinophils in the group of bears with microfilariae was probably an indicator of anti-parasitic response, but it was also not present in all the infected bears, so further investigations are needed in order to determine eosinophilia as a diagnostic feature of microfilariasis in bears.

Poikilocytes were found in four bears, and were of mostly teardrop shaped (dacrocytes) and oval shaped erythrocytes, which were not described in the previous investigations of these animals. Poikilocytosis occurs in various disorders, but can also be present in clinically healthy goats and young cattle (HARVEY, 2001). Since these cells did not make up more than 10% of the total red blood cells, we consider this finding of non-clinical significance.

Rouleaux formations were found in twelve bears. Usually, rouleaux formation indicates hyperglobulinemia or hypoalbuminemia, but in some species, for example, horses and cats, rouleaux formation is a normal finding because these animals have decreased negative charge on their red blood cells surfaces (eCLINPATH, 2021). In the present study, all the bears with rouleaux formations on the blood films had microfilariae. Only in three blood films where rouleaux formation were present, were no microfilariae found. However, the absence of microfilariae on a blood film does not mean that the animals are not infected with this parasite. SHARMA and PACHAURI (1981) reported hyperglobulinemia and hypoalbuminemia as the most striking changes observed in dirofilaria infected dogs. We assume the findings of rouleaux formations in our investigation could also be due to hyperglobulinemia and hypoalbuminemia caused by the response to a certain pathogen or other inflammatory stimuli, but this presumption needs to be tested with additional laboratory tests. Therefore, in future research, it is necessary to include the biochemical parameters of the blood, and thus determine whether red blood cells in bears normally form stacks due to a low negative charge, or rouleaux formation is a clinically important sign of microfilariosis or some other disorder.

Conclusions

The results of the assessment of WBC count and differential blood count in this study are comparable to and in agreement with the other published hematological values for brown bears. Therefore, blood film evaluation alone could be of use in assessing an animal's hematological status in the absence of laboratory equipment. However, since some pathological conditions are not always followed by alterations in blood cell number or morphology, for determining the health status, additional analyses of hematological and biochemical parameters are needed. The presence of rouleaux formations and microfilaria in the blood of brown bears in eastern Turkey, to the author's knowledge, are the first reported findings of this parasite in the bear population in the Euro-Asian area. These findings not only open possibilities for further investigation, but also contribute to the knowledge of the brown bear's hematology, as well as the spread of microfilarial parasites in Europe and Asia, which helps in monitoring and consequently protecting the wild bear population.

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SAŽETAK

Hematologija je jedan od najboljih pokazatelja zdravlja populacije, a najbrži način dobivanja uvida u neke hematološke parametre je pregledom krvnog razmaza. Katkad, zbog nemogućnosti skladištenja krvi, nedostupnosti hematoloških instrumenata tijekom terenskog rada ili nedovoljne količine krvi za kompletnu hematološku analizu, procjena krvnog razmaza može biti jedina metoda za dobivanje informacija o hematološkim promjenama. Populacija smeđeg medvjeda (*Ursus arctos*) često je ugrožena i zaštićena kao važna integralna, vrsta kopnenih zajednica. Budući da su osnovni hematološki podaci slobodnoživućih ugroženih vrsta posebno važni, cilj je bio ispitati mogućnost korištenja procjene krvnog razmaza kao jedinog izvora hematoloških podataka za određivanje hematološkog, pa tako i zdravstvenog statusa životinje. Pregledani su krvni razmazi sedamnaest smeđih medvjeda iz istočne Turske kako bi se procijenila morfologija eritrocita i leukocita, ukupni i diferencijalni broj leukocita te uočila prisutnost staničnih inkluzija ili hemoparazita. *Rouleaux*-formacije nađene su u dvanaest životinja, poikilocitoza u četiri, dok su parazitske nematode, mikrofilarije, nađene u devet od sedamnaest medvjeda. Rezultati su potvrdili da samostalni pregled krvnog razmaza, bez ostalih hematoloških parametara, može biti koristan u procjeni hematološkog statusa životinje, no za točnije utvrđivanje zdravstvenog stanja potrebna je analiza većeg broja krvnih parametara. Novi nalazi u radu, poput prisutnosti *rouleaux*-formacija i mikrofilarija u smeđih medvjeda iz istočne Turske, otvaraju vrata daljnjim istraživanjima u ovom području.

Ključne riječi: slobodnoživući smeđi medvjedi; hematologija; hemoparaziti; mikrofilarije; procjena krvnog razmaza; istočna Turska