JAES
Journal of Anatolian Environmental and Animal Sciences
(*Anadolu Çevre ve Hayvancılık Bilimleri Dergisi*)
DOI: https://doi.org/10.35229/jaes.1398305
Year: 9, No: 1, 2024 (110-118)
AÇEH
Yıl: 9, Sayı: 1, 2024 (110-118)
ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Blood Cell Types and Abnormalities in Free Ranging Hermann's Tortoise (*Testudo hermanni*) Populations from Different Habitats

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Received: *30.11.2023* ***Accepted:*** *19.03.2024* ***Puplished:*** *21.03.2024*

How to cite: **Boran, B., Baycan, B., Gül, Ç. & Tosunoğlu, M. (2024).** Blood Cell Types and Abnormalities in Free Ranging Hermann's Tortoise (*Testudo hermanni*) Populations from Different Habitats. *J. Anatolian Env. and Anim. Sciences*, ***9***(1), 110-118. https://doi.org/10.35229/jaes.1398305

Atıf yapmak için: **Boran, B., Baycan, B., Gül, Ç. & Tosunoğlu, M. (2024).** Farklı Habitatlardaki Trakya Tosbağası (*Testudo hermanni*) Popülasyonlarında Kan Hücre Tipleri ve Anomalileri. *Anadolu Çev. ve Hay. Dergisi*, ***9***(1), 110-118. https://doi.org/10.35229/jaes.1398305

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Abstract: *Testudo hermanni* is considered near threatened globally in the wild. In this study differential leukocyte percentages, nuclear abnormalities, and erythrocyte morphology abnormalities of *Testudo hermanni* populations from two different habitat types in the Thrace Region were determined to whether habitats have a potential effect on blood cells. A total of 37 tortoises, 18 from an open habitat in Kırklareli/Karakoç, and 19 from an isolated habitat in Edirne/Keşan were studied. According to the results obtained from the Micronucleus Test, which detects genotoxic damage, there was no statistically significant difference between the two habitats. There were statistically significant differences in erythrocyte morphology abnormalities and differential leukocyte count between two populations, such as frequencies of anucleated erythrocyte, nuclear shift, elliptical shape distortion, monocyte and eosinophile percentages. This is the first study examined nuclear abnormalities of *Testudo hermanni* in Türkiye.

Keywords: Hematology, blood cells, micronucleus test, *Testudo hermanni*, thrace region.

Farklı Habitatlardaki Trakya Tosbağası (*Testudo hermanni*) Popülasyonlarında Kan Hücre Tipleri ve Anomalileri

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Öz: *Testudo hermanni*, doğada küresel olarak nesli tehlike altında olarak kabul edilmektedir. Bu çalışmada, Trakya Bölgesi'nde iki farklı habitat tipinde bulunan *Testudo hermanni* popülasyonlarının lökosit formülleri, nüklear anormallikleri ve eritrosit morfolojisi anormallikleri tespit edilerek habitatların kan hücreleri üzerinde potansiyel bir etkiye sahip olup olmadığı belirlenmiştir. Kırklareli/Karakoç'taki açık bir habitattan 18 ve Edirne/Keşan'daki izole bir habitattan 19 olmak üzere toplam 37 kaplumbağa örneği üzerinde çalışılmıştır. Genotoksik hasarı tespit eden Mikronukleus Testi'nden elde edilen sonuçlara göre iki habitat arasında istatistiksel olarak anlamlı bir fark bulunmamıştır. Ancak, çekirdeksiz eritrosit, nüklear kayma ve eliptik şekil bozukluğu gibi eritrosit morfolojisi anormalliklerinde ve monosit ile eozinofil yüzdesinde iki popülasyon arasında anlamlı farklar olduğu tespit edilmiştir. Vücut büyüklükleri ile diğer parametreler arasında korelasyonlar tespit edilmiştir. Bu çalışma, ülkemizde *Testudo hermanni*'nin nüklear anormalliklerinin incelendiği ilk çalışmadır.

Anahtar kelimeler: Hematoloji, kan hücreleri, mikronükleus testi, *Testudo hermanni*, trakya bölgesi.

INTRODUCTION

The detection of genetic damage serves as a valuable biomarker, utilized as an effective tool in environmental monitoring (Park & Choi, 2007; Zapata et al., 2016). Genotoxicity tests have various endpoints such as DNA strand breaks, chromosomal aberrations, point mutations, including micronuclei formation (Ng et al., 2010). Using similar endpoints in different species enables inter-species comparison (Dusinka et al., 2012). With its easy applicability Micronucleus Test (MN) conducted on peripheral blood erythrocytes is one of the widely used standard in vitro mutagenicity test within the genetic toxicology (Fenech, 2000). The Micronucleus Test can be used to determine the number of micronuclei in cells, originating from complete chromosomes or acentric chromosome fragments not included in the main nucleus, and allows for the detection and monitoring of genotoxic effects (Şekeroğlu & Atlı-Şekeroğlu, 2011). This method enables the identification of early biological responses occurring prior to genetic damage, showing instability in the organism's health status (Carballo & Mudry, 2006; Latorre et al., 2015).

Due to their diverse reproductive modes, variations in thermal regulation, wide habitat diversity, diverse dietary habits, and long lifespan, reptiles serve as excellent models for studying genotoxicity induced by contaminants (Gardner & Oberdorster, 2005). In recent years, the MN has been utilized to identify nuclear abnormalities in peripheral blood cells, aiming to determine the effects of environmental pollutants on certain freshwater terrapin species (Gül et al., 2019; Boran, 2020; Frossard et al., 2021; Çördük et al., 2022). Furthermore, there were studies using this method on some terrestrial reptile species such as lizards (Schaumburg et al., 2012; 2014; Aroutiounian et al., 2019; Freire et al., 2021; Silva et al., 2021) and snakes (Strunjak-Perovic et al., 2010; Baycan et al., 2022), but there was no study encountered so far addressing the determination of nuclear abnormalities in *Testudo hermanni* (Hermann's Tortoise).

The preparation of blood smears is an invasive yet easily applicable procedure that has been successfully used to assess the health and physiological status of animals in wild populations (Arıkan & Çiçek, 2010; van der Horst et al., 2013). The evaluation of the morphologies of red and white blood cells is necessary for a comprehensive hematological assessment (Campbell, 2004). Acquiring information about erythrocyte development and morphology enables us to understand the physiology of tortoises. Additionally, it can assist in health assessments of populations under stress or threatened species (Walton & Hofmeyr, 2017). The changes observed in erythrocyte shape could be a complementary approach in predicting genotoxicity (Pollo et al., 2019).

The Hermann's tortoise is one of the endangered species due to reasons such as pollution (Mingo et al., 2016), habitat fragmentation (Guyot & Clobert, 1997), habitat destruction from agricultural activities (Matache et al., 2006; Rozylowicz & Dobre, 2009), forest fires (Hailey, 2000) and collection as a pet (Cheylan, 2001; Fernández-Chacón et al., 2011). According to the International Union for Conservation of Nature's (IUCN) Red List, *Testudo hermanni* is categorized as 'Near Threatened' (NT) globally, and its population status is decreasing (van Dijk et al., 2004). Additionally, this species is listed in Appendix II (Strictly protected fauna species) of the Bern Convention on the Conservation of European Wildlife and Natural Habitats and also in "Appendix II includes species not necessarily threatened with extinction, but in which trade must be controlled in order to avoid utilization incompatible with their survival" of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. General distribution area of this tortoise includes regions in Europe where the Mediterranean and semi-Mediterranean climates prevail (Cheylan, 2001; Karaman, 2017). In Türkiye, this species is only found in the Thrace Region (Baran et al., 2021). It prefers habitats with Mediterranean vegetation and typically favors semi-open formations on sun-exposed hills (Karaman, 2017).

Hematological studies conducted on *Testudo hermanni* include plasma biochemistry (Scope at al., 2013; Isani et al., 2014; Özdamar, 2014; Hetényi et al., 2016; Leineweber et al., 2019; 2021; Kirchner et al., 2023), erythrocyte morphology (Uğurtaş et al., 2003; Çiçek et al., 2015) and hematological values (Lawrance and Hawkey, 1986; Muro et al., 1998; Tosunoğlu et al., 2005). Although there are studies that calculate the differential leukocyte counts (Tosunoğlu et al., 2005; Neiffer et al., 2005; Petersen, 2016), there was no study about nuclear and erythrocyte morphology abnormalities in *Testudo hermanni* in Türkiye.

In this study differential leukocyte counts, nuclear abnormalities, and erythrocyte morphology abnormalities of *Testudo hermanni* populations distributed in two different habitats in the Thrace Region were determined to investigate whether habitat types have a potential effect on blood cells.

MATERIAL AND METHOD

Study sites: The Thrace Region is an important gateway area located in the northwestern part of Türkiye, connecting Asia and Europe. The Çanakkale and İstanbul Straits, along with the Sea of Marmara, separate Thrace from the Anatolian part. Two different habitat types of *Testudo hermanni* populations from Thrace Region were selected as study sites (Figure 1). Population I were collected from a sparsely wooded grassland (EUNIS code: E7) in Kırklareli/Karakoç (41°47'5.62"N, 27°12'33.07"E), which was an open habitat. Through observations made during fieldwork, it was determined that small cattles were grazed twice a day, in the morning and evening, in this area. Population II was collected from a lines of trees area (EUNIS code: G5.1) in Edirne/Keşan (40°45'16.29"N, 26°42'53.24"E), which was an isolated habitat. According to observations, it was determined that tortoises in this habitat was more isolated from human impact.

Figure 1. The study sites [A: Map view of study sites, B: Kırklareli/Karakoç, C: Edirne/Keşan (https://maps.google.com)].

Data collection: Field studies were conducted at each locality between April and September 2023. A total of 37 tortoise specimens, 18 from Kırklareli/Karakoç, and 19 from Edirne/Keşan were collected by hand for analyses. Sex was distinguished by observing external characteristics of tortoises, such as the concavity of the plastron and the length of tails, which were indicative of males (Willemsen and Hailey, 2003). The straight carapace length (SCL) of the tortoises were measured with tortometer and body weight (BW) were determined with digital scale (Weightlab Instruments, accuracy: 0.01g). The necessary permissions have been obtained from the Ethics Committee of Animal Experiments of Çanakkale Onsekiz Mart University (decision no: 2023/01-06) for the studies that carried out. A total of 2 mL of blood was taken from dorsal caudal vein by 5 mL syringe with a diameter of 21-gauge needle (Ballard & Cheek, 2003; Thrall et al., 2004). Blood smears were prepared from the collected blood samples and were examined by microscope (Olympus CX21). The individuals were released back to their habitats after collecting blood samples.

Examination of blood smears: For differential leukocyte count, 100 leukocyte cells were identified and recorded in Wright-stained blood smears. The percentages of five different leukocyte types in peripheral blood-lymphocytes, monocytes, eosinophils, heterophils, and basophils-were determined through mathematical calculations (Arıkan et al., 2012). Additionally, 1000 erythrocytes were counted on the blood smears and the frequency of immature erythrocyte index (IE) was calculated (Guilherme et al., 2008). Micronucleus Test was performed on Giemsa-stained blood smears, 1000 mature erythrocytes were counted to detect nuclear abnormalities such as micronucleus, lobbed, notched, blebbed, kidney-shaped nucleus and binucleate (Josende et al., 2015; Gül et al., 2019). Also, erythrocyte morphology abnormalities were used in predicting hematological fluctuations (Pollo et al., 2019). Frequencies of pyknotic erythrocytes (PE), mitotic erythrocytes (ME), anucleated erythrocytes (AE), and nuclear shift (NS) were determined as nuclear shape abnormalities, while frequencies of elliptical shape distortion (ESD) and vacuolization (V) in erythrocytes were determined as erythrocytic morphology abnormalities.

Statistical analyses: The standard values of the obtained data were evaluated by using softwares (Microsoft Excel, IBM SPSS Statistics 26 and R Project for Statistical Computing). Kolmogorov-Smirnov Test for normality was used in analysis of the data. The Mann-Whitney U Test for non-parametric data and Student-T Test for parametric data were used to determine whether there was a difference in all values between populations. The Spearman correlation test was used to determine whether there were correlations between body sizes and parameters obtained by blood smears such as differential leukocyte percentages, nuclear abnormalities, and erythrocyte morphology abnormalities. In all cases, $p \leq 0.05$ value was considered statistically significant. As a result of the analyses, no significant difference was found between sexes; therefore, female, and male specimens were evaluated together.

RESULTS

In this study differential leukocyte percentages, nuclear abnormalities, and erythrocyte morphology abnormalities of *Testudo hermanni* populations distributed in two different habitat types in the Thrace Region were determined. Parameters belong to erythrocyte and leukocyte types examined from blood smears under microscope were given in Figure 2.

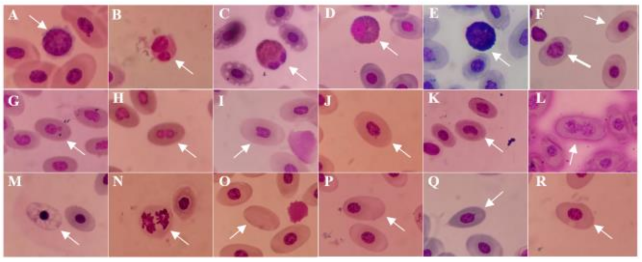

Figure 2. Blood cells examined by microscope [A: Lymphocyte, B: Monocyte, C: Eosinophile, D: Heterophile, E: Basophile, F: Mature (thin arrow) and Immature (thick arrow) Erythrocytes, G: Micronucleus, H: Lobbed Nucleus, I: Notched Nucleus, J: Blebbed Nucleus, K: Kidney-Shaped Nucleus, L: Binucleate, M: Pyknotic Erythrocyte, N: Mitotic Erythrocyte, O: Anucleated Erythrocyte, Q: Elliptical Shape Distortion, R: Vacuolization].

When examining BW of the samples, it was determined that the average BW of Population I was 1054.44±103.35 g, and BW of Population II was 960±78.49 g. Regarding SCL, Population I had an average of 17.12±0.69 cm, and Population II had an average of 15.92±0.43 cm.

The differential leukocyte count and IE of two *Testudo hermanni* populations are given in detail in Table 1. In terms of the differential leukocyte formula, the percentages of monocytes, heterophils, and basophils were higher in Population I, while the percentages of lymphocytes and eosinophils were higher in Population II. According to the results obtained from the immature erythrocyte index in this study, the mean immature erythrocyte frequency in the peripheral blood of individuals in Population I, found in an open habitat, was 33.11‰, while in Population II individuals found in an isolated habitat, this value was determined to be 27‰. There were statistically significant differences between the two populations in terms of monocyte (t=2.764, df=35, p=0.009) and eosinophile (U=93.000, p=0.017) percentages.

When examining the frequencies of nuclear abnormalities detected by the MN, it was seen that Population I had higher values in all parameters except for the notched nucleus (Table 2). However, no statistically significant difference was found between the two populations (p>0.05).

In terms of erythrocyte morphology abnormalities, Population I had higher values in all parameters (Table 3). There were statistically significant differences of anucleated erythrocyte (U=123.000, p=0.032), nuclear shift (U=51.500, p<0.001), and elliptical shape distortion (U=93.500, p=0.017) frequencies between the two populations.

Table 1. The differential leukocyte count and immature erythrocyte indices of two *Testudo hermanni* populations (* Statistically significant differences).

Bood Cells	POPULATION I		POPULATION II	
	Minimum-Maximum	Mean±SE	Minimum-Maximum	Mean±SE
Lymphocyte (%)	36-78	54.56±2.73	19-81	56.26±3.63
Monocyte (%)*	3-21	11.06±1.23	2-12	7.16±0.71
Eosinophile (%)*	0-14	6.06±1.04	3-24	9.79±1.18
Heterophile (%)	15-47	26.44±2.27	4-45	25.37±2.85
Basophile (%)	0-6	1.89±0.47	0-5	1.42±0.32
Immature Erythrocyte Index (‰)	2-116	33.11±6.84	0-77	27.00±4.27

Table 2. Frequencies (%) of mean nuclear abnormalities in erythrocytes with standard error.

POPULATIONS	Micronucleus (%)	Lobbed Nucleus (%)	Notched Nucleus (%)	Blebbed Nucleus (%)	Kidney-Shaped Nucleus (%)	Binucleate (%)	Total Nuclear Abnormalities (%)
I	0.072±0.026	0.039±0.018	1.839±0.280	2.383±0.351	0.089±0.022	0.072±0.033	4.494±0.529
II	0.021±0.009	0.032±0.017	2.005±0.242	2.205±0.261	0.068±0.027	0.005±0.005	4.337±0.476

Table 3. Frequencies (%) of mean erythrocyte morphology abnormalities in erythrocytes with standard error (* Statistically significant differences).

POPULATIONS	Nuclear Shape Abnormalities				Erythrocytic Shape Abnormalities	
	Pyknotic Erythrocyte (%)	Mitotic Erythrocyte (%)	Anucleated Erythrocyte (%)*	Nuclear Shift (%)*	Elliptical Shape Distortion (%)*	Vacuolization (%)
I	0.206±0.055	0.044±0.020	0.033±0.011	1.817±0.277	0.483±0.079	0.194±0.060
II	0.100±0.030	0.005±0.005	0.005±0.005	0.816±0.171	0.268±0.064	0.121±0.027

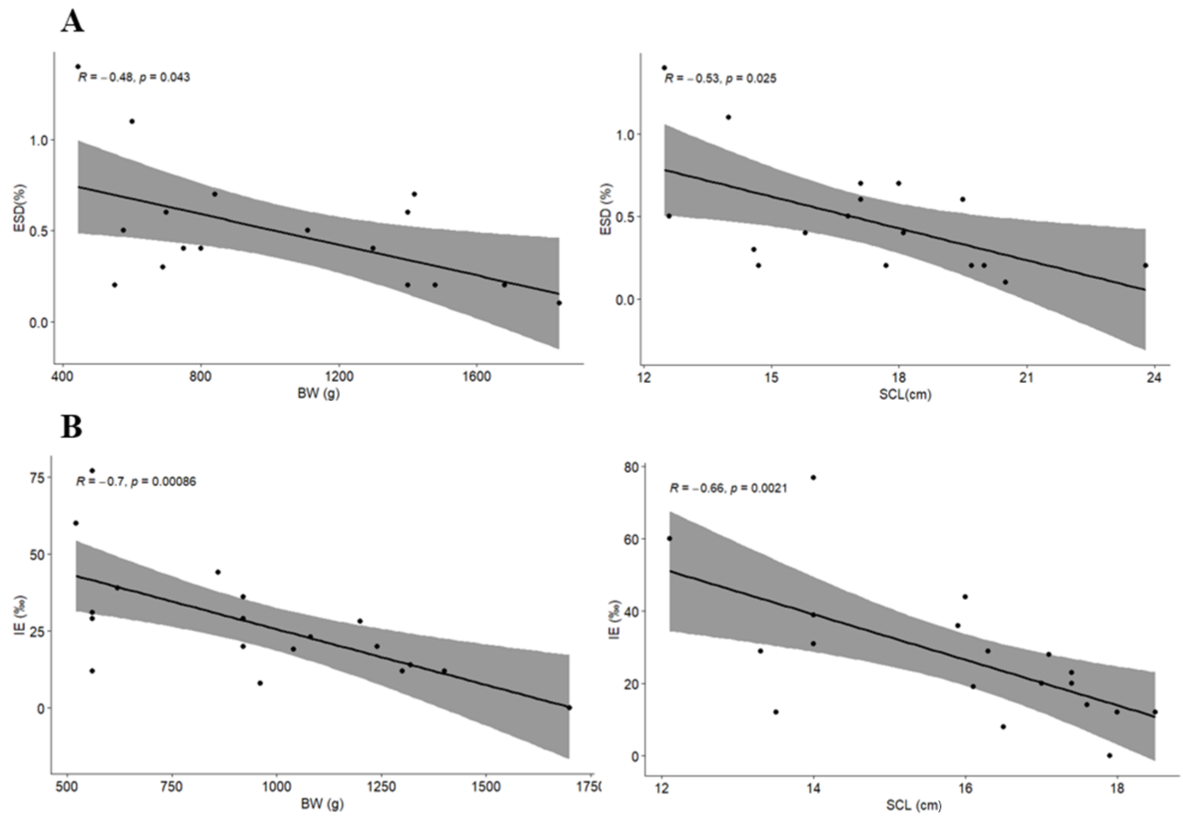

Figure 3. Scatter point graph of the correlation between A: BW and ESD (left), SCL and ESD (right) in Population I, B: BW and IE (left), SCL and IE (right) in Population II.

The Spearman correlation test was used to determine whether there were correlations between body sizes and other parameters, statistically significant results were found for both populations (Figure 3). When correlation analyses were conducted between body sizes and other parameters in Population I, a positive correlation was found between BW and SCL (r=0.937, p<0.001), while a negative correlation was found between BW and elliptical shape distortion (r=-0.481, p=0.043). Additionally, a negative correlation was observed between SCL and elliptical shape distortion (r=-0.526, p=0.025). In Population II, a positive correlation was observed between BW and SCL (r=0.960, p<0.001), and a negative correlation was found between BW and IE (r=-0.700, p=0.001). Furthermore, a negative correlation was identified between SCL and IE (r=-0.661, p=0.002).

DISCUSSION

According to the results of MN, which detects genotoxic damage, there was no statistically significant difference between the two habitats. But there were statistically significant differences in terms of differential leukocyte count and erythrocyte morphology abnormalities between two populations, such as frequencies of anucleated erythrocyte, nuclear shift, elliptical shape distortion, monocyte and eosinophile percentages.

Accurate interpretation of the leukogram in reptile species requires manual counting techniques and the evaluation of blood smears because nucleated erythrocytes in reptiles interfere the functionality of automated blood analyzers (Isani et al., 2014). In previous studies it was observed that heterophils were the dominant leukocyte type in blood smears of *Testudo hermanni* (Çiçek et al., 2015), but our study indicates lymphocytes as the predominant leukocyte type. Tosunoğlu et al. (2005) were studied differential leukocyte types of *Testudo hermanni* individuals from Thrace Region and determined percentages of lymphocytes as 42-50%, monocytes as 20-28%, eosinophils as 17-23% and basophils as 8-15%. When our data was compared with the previous study, it can be observed that the percentages of leukocyte types, except for basophil percentage, were similar. While the immune capacity of basophils in turtle species is known, similar to basophils or mast cells in mammals, the numbers of basophils in the peripheral blood vary among many species (Zhang et al., 2011).

Determining the frequency of immature erythrocytes provides important information about hematological dynamics, but because it reflects the balance between various factors, an isolated analysis may not precisely identify the specific cause (Guilherme et al., 2008). In a study examining erythrocyte morphologies in a tortoise species, *Psammobates geometricus*, based on season, sex, and age, it was concluded that the highest frequency of immature erythrocytes was observed in both males and females during winter, and the lowest was in autumn (Walton & Hofmeyr, 2017). The low frequency of immature erythrocytes can be explained by nutritional deficiencies during the dry season, which may down-regulate erythropoiesis in tortoises (Walton & Hofmeyr, 2017). In present study it was found that immature erythrocyte frequency of Population I, found in an open habitat, was higher than Population II found in an isolated habitat. As mentioned in the literature, the inability to reach food due to movement restriction in isolated habitat or nutrient deficiency may have caused an increase in the number of immature erythrocytes in Population II.

Assessing the genotoxicity levels in wild populations provides information about the status of the ecosystem and serves as a foundation for estimating the risks of environmental pollution to wildlife and indirectly to humans (Sebbio et al., 2014; Aroutiounian et al., 2019). The number of micronuclei is a marker of genetic damage in adults, and an increase in their number indicates impaired cell health (Zúñiga-González et al., 2000). External chemical genotoxic agents can trigger the formation of micronuclei and other nuclear abnormalities in terrestrial animals (Fenech et al., 2011; Freire et al., 2021). Also, previous research has linked genotoxic damage to environmental degradation from agricultural practices (Ossana and Salibian, 2013; Josende et al., 2015; Babini et al., 2015; Pollo et al., 2019). However, Zúñiga-González et al. (2000) suggested that the presence of micronucleus in erythrocytes may increase in young individuals of some species due to immature reticuloendothelial system. In this study when nuclear abnormalities detected by the MN were examined, it was determined that Population I from a habitat with grazing activities had higher values in all parameters except for the notched nucleus.

Morphology of erythrocytes, in terms of characteristics such as size and shape, have crucial importance in hematological studies due to the central role of blood cells in animal physiology (Walton & Hofmeyr, 2017). These morphological differences observed in erythrocytes have been studied more extensively in amphibians, and there is a lack of comparative studies in terrestrial organisms. Frequency of all erythrocyte morphology abnormalities that observed in this study were higher in Population I from an open habitat with grazing activies. Erythroblasts reflect increased erythropoiesis, while pyknotic cells are associated with apoptosis (Saquib et al., 2012; Peltzer et al., 2013). Increases in the frequencies of these cells are generally linked to a response to stress conditions or cellular damage and may indicate a greater ability to eliminate damaged cells before they enter circulation (Natale et al., 2018). The high frequencies of anucleated and mitotic cells may represent a short-term mechanism for increasing oxygen-carrying capacity (Barni et al., 2007; Peltzer et al., 2013; Pollo et al., 2019). Additionally, erythrocytic vacuoles have been associated with iridoviral infections in amphibians and reptiles (Johnsrude et al., 1997).

When correlation analyses were examined, in Population I negative correlations was found between BW and ESD and SCL and ESD. In Population II, there were also negative correlations between BW and IE and SCL and IE. It has been observed in a study, poikilocytosis that erythrocytes with different morphologies are produced was particularly high in young individuals. Furthermore, nutritional deficiencies can also lead to the low frequency of immature erythrocytes (Walton & Hofmeyr, 2017).

CONCLUSION

As a result, there were statistically significant differences between populations in open and isolated habitats in terms of percentages of monocyte and eosinophile, frequencies of anucleated erythrocyte, nuclear shift, and elliptical shape distortion. Nuclear abnormalities in *Testudo hermanni* were studied first time and there was no statistically significant difference between habitat types. The effects of environmental influences on species in different habitats should be assessed with all aspects. This will lead to new conservation measures, especially for endangered species. Monitoring and conservation plans should be established based on these findings.

ACKNOWLEDGEMENTS

This study was supported by Çanakkale Onsekiz Mart University, The Scientific Research Coordination Unit, Project Number: FDK-2021-3555.

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