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Effects of Altitude and Temperature on Erythrocyte Morphology of *Emys orbicularis* (Linnaeus, 1758) and *Mauremys rivulata* (Valenciennes, 1833)

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Abstract: The decrease in erythrocyte size of animals live at high altitudes yields an evolutionary advantage to survive by providing adaptation to colder temperatures and low partial oxygen pressures. We examined the effect of geographical changes on the erythrocyte morphology of two terrapins, *Emys orbicularis* and *Mauremys rivulata*, and analyzed whether such erythrocyte characteristics as size and volume changed at high altitudes and different temperatures. We found out that the erythrocyte characteristics varied both within and between the populations. They varied depending on altitude for *E. orbicularis* and on temperature for *M. rivulata*. However, the erythrocyte characteristics were not correlated with the environmental parameters, except between sunshine duration and erythrocyte length, size, and nucleus volume for *E. orbicularis*.

Keywords: Hematology, blood cell, European pond turtle, Caspian turtle.

Yükseklik ve Sıcaklığın *Emys orbicularis* (Linnaeus, 1758) ve *Mauremys rivulata* (Valenciennes, 1833)'nın Eritrosit Morfolojisi Üzerine Etkisi

Öz: Yüksek rakımlarda yaşayan hayvanlarda eritrosit boyutlarındaki azalma, düşük sıcaklıklara ve düşük kısmi oksijen basıncına adaptasyon sağlayarak hayatta kalmalarına evrimsel bir avantaj kazandırmaktadır. Coğrafik değişikliklerin eritrosit morfolojisi üzerindeki etkilerini iki tatlısu kaplumbağası türü olan *Emys orbicularis* ve *Mauremys rivulata*'da inceledik ve boyut ve hacim gibi eritrosit özelliklerinin yüksek rakım ve farklı sıcaklıklarda değişip değişmediğini analiz ettik. Eritrosit özelliklerinin hem populasyon içerisinde hem de populasyonlar arasında farklılık gösterdiğini belirledik. *E. orbicularis* için yüksekliğe bağlı olarak, *M. rivulata* içinse sıcaklığa bağlı olarak değişiklik göstermektedir. Bununla birlikte, *E. orbicularis* için güneşlenme süresi ile eritrosit uzunluğu, büyüklüğü ve nukleus hacmi arasındaki korelasyon dışında, eritrosit özellikleri ile çevresel parametreler arasında korelasyon görülmemektedir.

Anahtar kelimeler: Hematoloji, kan hücresi, Benekli Kaplumbağa, Çizgili Kaplumbağa.

1. Introduction

High-altitude habitats force animals to adapt to low oxygen levels (Lu et al., 2015). These animals must maintain the balance between O_2 supply and O_2 demand (Ramirez et al., 2007; Storz et al., 2010). When O_2 is lower than needed, all vertebrates face death owing to its essential role for brain functions (Lutz & Kabler, 1997). Lower vertebrates are prone to survive at low O_2 levels due to the efficient anaerobic periods in short terms; however, turtles are even more resilient since their anaerobic periods can be much longer than those of the other animals (Jackson, 2002). High-altitude environments lead to physiological difficulties for animals due to the harsh conditions particularly colder temperatures and low partial oxygen pressures (P_{O2}) as compared to low-altitude environments (Storz & Moriyama, 2008; Su et al., 2018). High-altitude hypoxia leads to an increase in the number of erythrocytes (Su et al., 2018). Vertebrates have a capacity to endure high altitudes and manage to survive despite the decreases in O_2 tension that potentially restrict the aerobic life (Samaja et al., 2003; Weber, 2007). Although some investigations stated slight or no correlation between blood parameters and altitudinal distribution in reptiles (e.g. Dessauer, 1970; Ruiz et al., 1993), others showed higher hematologic values in upland-distributed species (Vinegar & Hillyard, 1972). The variation in erythrocyte sizes with regard to altitudinal differences may be explained by the effect of surface on gas exchange; for example, a small blood cell permits more gas exchange than a larger one. As in anurans, the ones that live at higher altitudes have smaller erythrocytes (Baraquet et al., 2013). Temperature is another important factor that affects the blood parameters and the number of the red blood cells. Hemoglobin contents increase in cold environments whereas they decrease at warmer temperatures (Washburn & Huston, 1968; Moye et al., 1969).

Haematology of Anatolian terrapins were studied previously (e.g., Yılmaz & Tosunoğlu, 2010; Tosunoğlu et al., 2011). Some of these studies focused on the erythrocyte morphology (e.g., Metin et al., 2006; Arıkan & Çiçek, 2010; Çiçek et al., 2015), but none of them discussed the effects of environmental factors. Herein, we aimed to understand the relationship between geographical and environmental factors, such as altitude, temperature, precipitation, and sunshine duration differences, and the erythrocyte morphology of *E. orbicularis* and *M. rivulata*.

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2. Material and Methods

We conducted field surveys at seven localities in southern Anatolia (Table 1). All the populations of *E. orbicularis* varied by altitude [0-1193 m above sea level (asl)] as well as mean temperature, annual precipitation, and sunshine duration (between 1940 and 2019; the Turkish State Meteorological Service). The three populations of *M. rivulata* were similar in terms of altitude (0 and 11 m asl); therefore, we compared them with regard to other environmental variables.

Table 1. Environmental characteristics of study sites and the number of specimens sampled for each location.

Loc. no	Loc. name	Coordinate	Altitude	Annual Mean Temperature (°C) (1940-2019)	Annual Mean of Daily Sunshine Duration (h) (1940-2019)	Total Monthly Precipitation Average (mm) (1940-2019)	Species	Number F	M	J	Total
1	Muğla/ Dalaman	36°47'29"N 28°48'49"E	0 m	15.5	87	1214.8	*E. orbicularis* *M. rivulata*	3 3	3 4	- -	6 7
2	Hatay/ Tekebaşı	36°03'18"N 36°02'03"E	0 m	18.3	86	1168.2	*E. orbicularis* *M. rivulata*	4 4	2 3	1 -	7 7
3	Mersin / Silifke	36°36'39"N 33°57'21"E	11m	19.1	89.5	615.8	*E. orbicularis* *M. rivulata*	1 4	- 1	- -	1 5
4	Isparta/ Barla	38°00'32"N 30°47'6"E	917 m	12.2	85	570.2		2	6	-	8
5	Burdur/ Gölhisar	37°08'14"N 29°30'26"E	935 m	13.2	89	428	*E. orbicularis*	4	4	-	8
6	Konya/ Karapınar	37°42'54"N 33°35'36"E	1093m	11.6	88.7	327.7		6	4	-	10
7	Konya/ Suğla	37°20'25"N 32°02'22"E	1193m	12	94.4	340.7		1	-	-	1

The current study was conducted in the context of the project that was confirmed by Animal Ethics Committee of Ege University (Approval number: 2010-013). We collected blood samples from the caudal vein by using heparinized glass capillaries. Five to ten blood smears were prepared immediately for each individual and all individuals were released after the blood samples had been collected. The prepared blood smears were stained with Wright's stain. For each blood smear, 40 erythrocytes were chosen randomly and measured by using an eyepiece ocular micrometer (Olympus CX31). Erythrocyte length (EL), erythrocyte width (EW), nuclear length (NL), and nuclear width (NW) were measured. The volumes of erythrocytes (EV) and their nuclei (NV) were calculated according to the following formulae: $EV = (EL \times EW^2) \times (\pi/6)$ [μm^3] and $NV = (NL \times NW^2) \times (\pi/6)$ [μm^3]. In addition, the nucleocytoplasmic ratios (NR) were calculated according to the following formula: $NR = NV / (EV - NV)$ (Uca, Arıkan, & Çiçek, 2017). The blood smears of 41 *E. orbicularis* and 19 *M. rivulata* specimens were examined.

All statistical analyses were performed by PAST vers.3 (Hammer et al., 2001). Normality of the measurements was tested by using the Kolmogorov-Smirnov D test. If the data set was distributed normally (Kolmogorov–Smirnov D test, $P \geq 0.05$), then parametric tests were used for comparison. The correlations between erythrocyte characteristics and environmental parameters were compared via the Spearman correlation test.

3. Results

The erythrocytes of both *E. orbicularis* and *M. rivulata* are ellipsoidal and have an ellipsoidal nucleus located at the center of the cell (Fig. 1 and 2). The cytoplasms are yellowish pink and the nuclei are dark purple due to Wright's stain.

The comparison of *E. orbicularis* populations showed that the mean EL, EW, and EV were minimum at Loc3 which was the hottest locality with the annual mean temperature of 19.1°C. The mean NL, NW, NS, and NV were minimum while the mean EW, EV, and ES were maximum at Loc4 which had the minimum sunshine duration.

Loc6 and Loc7 have similar environmental characteristics such as temperature, precipitation, and altitude because of their proximity. They are both situated in the same province, Konya. They have the minimum annual mean temperature and the total monthly precipitation average while having the maximum altitude among the study sites. All the mean nuclear measurements were maximum at these two localities (Table 2). However, there is only one environmental parameter, sunshine duration, correlated to EL, ES, and NV. While ES and NV were positively affected from sunshine duration, EL was affected negatively. Apart from this, there is no statistically significant correlation detected between erythrocyte morphology and the other environmental parameters (Table 4).

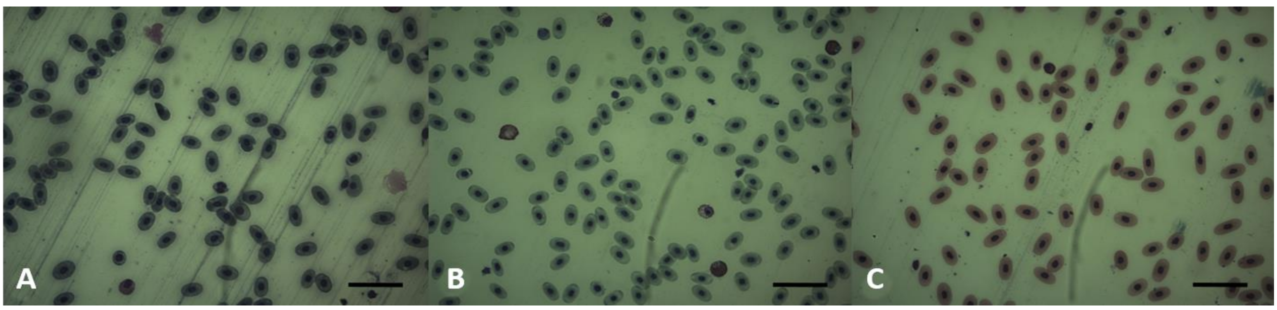

Figure 1. Photomicrographs of *Emys orbicularis* erythrocytes from A. Loc7 (1193 m asl); B. Loc1 (0 m asl) and C. Loc2 (0 m asl). Horizontal bar: 20 μm.

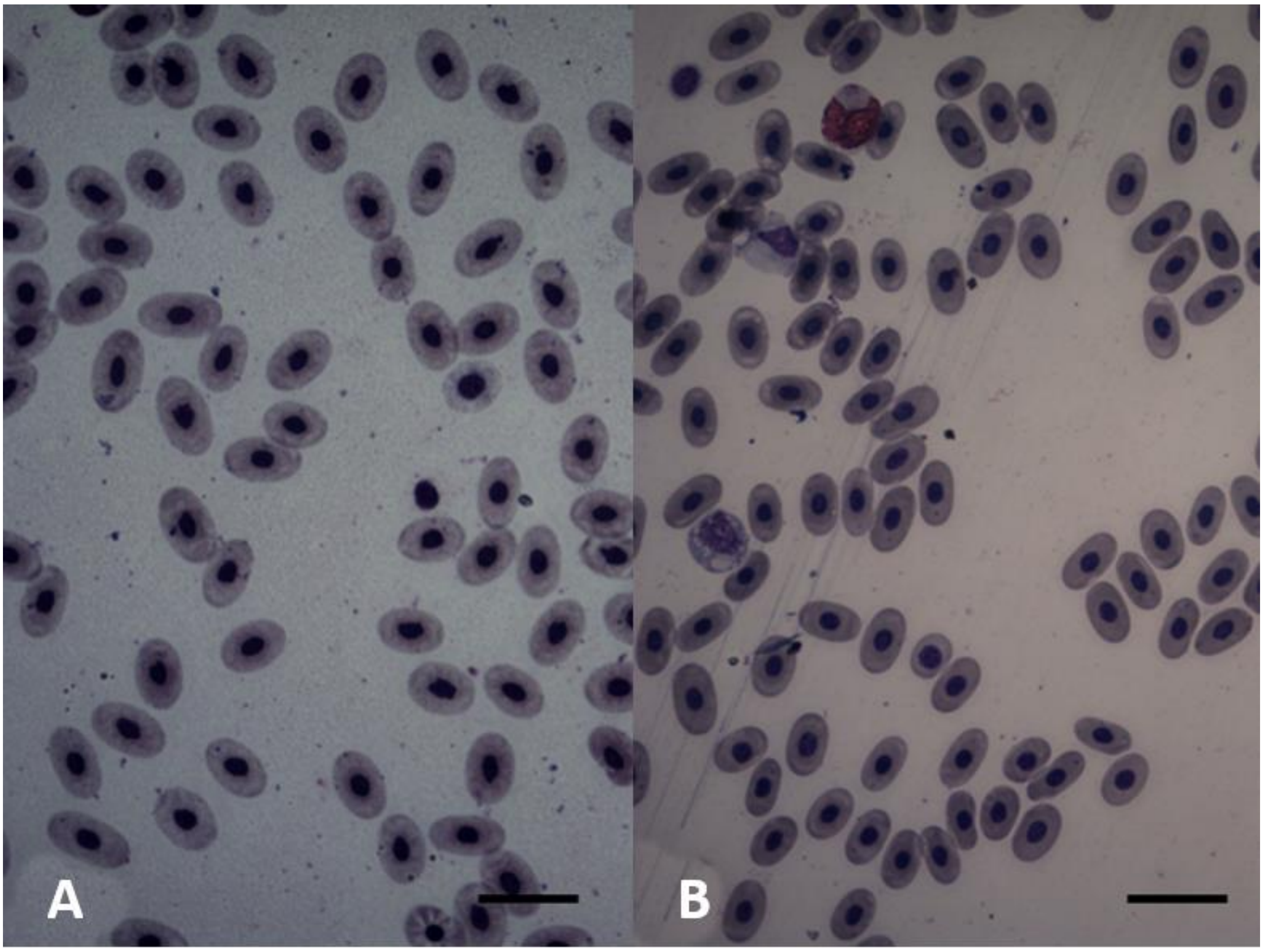

Figure 2. Photomicrographs of *Mauremys rivulata* from A. Loc1 and B. Loc3. Horizontal bar: 20 µm.

Because of all the populations of *M. rivulata* studied were at sea level, we did not compare them in terms of altitude. All measurements except NR were maximum at Loc1 which was the westernmost locality with the minimum annual mean temperature among the study sites. Moreover, all the values except EW were minimum at Loc3 which have the maximum annual mean temperature but the minimum sunshine duration and annual mean precipitation. Although Loc2 was the easternmost locality, measurements from this population have average results except EW and NR (see Table 3). Furthermore, there is no statistically significant correlation detected between erythrocyte characteristics and the environmental parameters for *M. rivulata*.

4. Discussion

Environmental conditions like season, geographical position, mean temperature, and precipitation influence many physiological processes such as age, sex, reproduction, and blood parameters of vertebrates (Moye et al., 1969; Jacobson, 2007). Erythrocytes are the transporters of oxygen and carbon dioxide.

Table 2. The mean erythrocyte measurements of *Emys orbicularis*.

Measurement* Locality	EL (µm)	EW (µm)	EL/EW	NL (µm)	NW (µm)	NL/NW (µm)	NS (µm²)	ES (µm²)	NS/ES (µm²)	NR (µm³)	EV (µm³)	NV (µm³)
Loc1	19.56	11.15	1.76	5.59	4.29	1.32	19.20	174.71	0.11	0.047	1291.25	57.72
	±1.84	±1.04	±0.19	±0.76	±0.76	±0.15	±5.77	±26.11	±0.03	±0.022	±300.68	±28.71
Loc2	20.37	11.25	1.83	5.72	4.23	1.38	19.05	180.34	0.10	0.044	1373.56	55.26
	±1.73	±1.29	±0.25	±0.77	±0.68	±0.29	±4.09	±27.6	±0.02	±0.018	±352.99	±19.48
Loc3	18.09	10.12	1.80	5.50	4.28	1.30	18.58	143.9	0.13	0.0105	928.02	54.42
	±1.26	±1.05	±0.24	±0.73	±0.62	±0.22	±4.14	±19.29	±0.03	±0.003	±227.81	±19.19
Loc4	19.91	11.87	1.69	5.46	3.94	1.39	16.94	186.09	0.09	0.0326	1493.71	45.30
	±1.57	±1.28	±0.19	±0.77	±0.47	±0.24	±3.23	±27.82	±0.17	±0.0112	±369.68	±13.77
Loc5	19.08	11.80	1.63	5.53	4.32	1.30	18.80	177.35	0.10	0.0425	1418.32	55.48
	±1.58	±1.35	±0.20	±0.74	±0.65	±0.25	±3.92	±28.79	±0.02	±0.0168	±379.38	±19.13
Loc6	19.81	11.08	1.80	5.92	4.25	1.41	19.90	172.96	0.11	0.0489	1302.81	58.00
	±1.86	±1.40	±0.22	±0.71	±0.62	±0.22	±4.38	±30.29	±0.02	±0.0186	±426.42	±21.18
Loc7	18.81	11.68	1.62	6.00	4.53	1.35	21.34	173	0.12	0.0525	1367.46	65.62
	±1.32	±1.28	±0.18	±0.70	±0.61	±0.25	±3.84	±26.02	±0.02	±0.0175	±359.24	±18.65
Total	19.67	11.42	1.74	5.65	4.21	1.36	18.81	176.94	0.10	0.0423	1370.02	54.45
	±1.77	±1.34	±0.22	±0.77	±0.65	±0.24	±4.39	±28.95	±0.02	±0.0191	±380.36	±21.04

Table 3. The mean erythrocyte measurements of *Mauremys rivulata*.

Measurement* Locality	EL (µm)	EW (µm)	EL/EW (µm)	NL (µm)	NW (µm)	NL/NW (µm)	NS (µm²)	ES (µm²)	NS/ES (µm²)	NR (µm³)	EV (µm³)	NV (µm³)
Loc1	20.47 ±1.49	11.58 ±1.17	1.78 ±0.19	6.49 ±0.92	4.65 ±0.62	1.41 ±0.27	23.80 ±4.96	186.44 ±25.77	0.12 ±0.02	0.0563 ±0.0185	1456.85 ±342.87	75.44 ±23.53
Loc2	18.62 ±1.52	10.69 ±1.09	1.76 ±0.23	5.85 ±0.82	4.47 ±0.67	1.33 ±0.25	20.66 ±4.65	156.29 ±20.16	0.13 ±0.03	0.0627 ±0.0259	1125.48 ±246.90	63.28 ±21.81
Loc3	17.50 ±1.27	10.93 ±0.99	1.61 ±0.18	5.74 ±0.75	4.23 ±0.63	1.37 ±0.22	19.20 ±4.36	150.30 ±18.09	0.12 ±0.03	0.0555 ±0.0248	1105.36 ±222.94	55.76 ±20.75
Total	18.83 ±1.84	11.02 ±1.15	1.72 ±0.22	6.01 ±0.89	4.45 ±0.67	1.37 ±0.25	21.15 ±5.00	163.40 ±26.18	0.13 ±0.03	0.0587 ±0.0238	1217.02 ±313.27	64.65 ±23.29

Table 4. Correlation between erythrocyte morphology and environmental parameters according to spearman rho test (Bold values indicate statistically significance).

		EL (µm)	EW (µm)	EL/EW (µm)	EV (µm³)	ES (µm²)	NL (µm)	NW (µm)	NL/NW (µm)	NV (µm³)	NS (µm²)	NS/ES (µm²)	NR (µm³)
Altitude	**cc**	-0.342	0.234	-0.618	0.414	0.414	0.218	0.414	-0.288	0.183	0.450	0.198	0.541
	p	0.452	0.613	0.139	0.355	0.355	0.638	0.355	0.531	0.694	0.310	0.670	0.210
Annual Mean	**cc**	-0.143	-0.321	0.468	-0.464	-0.107	-0.631	-0.500	0.000	0.145	-0.643	-0.321	-0.571
Temperature (°C)	**p**	0.760	0.482	0.289	0.294	0.819	0.129	0.253	1.000	0.756	0.119	0.482	0.180
Annual Mean of Daily	**cc**	**-0.893**	-0.321	-0.396	0.393	**0.821**	-0.523	0.429	**-0.750**	**0.800**	0.250	-0.464	0.536
Sunshine Duration (h)	**p**	**0.007**	0.482	0.379	0.383	**0.023**	0.229	0.337	**0.052**	**0.031**	0.589	0.294	0.215
Total Monthly	**cc**	0.179	-0.143	0.396	-0.393	-0.214	-0.360	-0.357	0.286	-0.091	-0.393	-0.214	-0.464
Precipitation Average (mm)	**p**	0.702	0.760	0.379	0.383	0.645	0.427	0.432	0.535	0.846	0.383	0.645	0.294

*EL: Erythrocyte Length; EW: Erythrocyte Width; NL: Nucleus Length; NW: Nucleus Width; NS: Nucleus Size; ES: Erythrocyte Size; NR: Nucleocytoplasmic Ratio; EV: Erythrocyte Volume; NV: Nucleus Volume.

** cc: correlation coefficient; p: significance level

The size and shape of erythrocytes influence the efficiency and rate of gas exchange (Hartman & Lessler, 1964). Small cells have a larger surface/volume ratio than large cells have; thus, they have a greater rate of oxygen exchange than larger ones do (Stacy et al., 2011; Javanbakht et al., 2013).

Because erythrocytes play an essential role in vertebrate physiology, it is important to understand their morphological variations to be able to identify changes depending on the environmental factors. Changes in temperature and rainfall affect food availability which influences the metabolism of ectotherms (e.g., Litzgus, & Hopkins, 2003; Setlalekgomo et al., 2012).

The increase in the number of red blood cells depending on altitude is an adaptation to be able to provide more gas exchange in a relatively short time. Therefore, the size and volume of the erythrocytes decrease at higher altitudes (Ramirez et al., 2007) whereas the number increases. Lu et al. (2015) reported that *Phrynocephalus erythrurus*, a reptile that lives at high altitudes, has more erythrocytes with less cell volume than those living at low altitudes. Accordingly, similar results were found for the Tibetan chicken (Su et al., 2018). Temperature is another factor that affects cell morphology. It has been known that the erythrocyte volumes and sizes increase at colder temperatures (e.g., Washburn & Huston, 1968; Moye et al., 1969). In parallel with these results, we calculated the minimum erythrocyte sizes and volumes from Loc3.

We found out that the mean values of erythrocyte sizes and volumes of *E. orbicularis* varied among the study sites; however, these variations did not correlate with environmental changes. There was a difference of 1193 m between the lowest and highest localities; yet, we discovered that the minimum and maximum values of the measurements were at Loc3 and Loc4, respectively. Altitude difference between these localities is 906 m, nearly the same between Loc1 and Loc7, but the annual mean temperature difference is nearly twice than those between Loc1 and Loc7 (3.5°C between Loc1-Loc7, 7.1°C between Loc3-Loc4). Thus, we concluded that the temperature also affects the erythrocyte sizes but we do not have correlation on erythrocyte morphology for our sample.

Making a comparison of erythrocyte sizes with the literature is not straightforward because few studies indicate in which altitude the samples were taken and the others do not provide any information about environmental parameters. For this reason, it is not possible to discuss all environmental factors with the published literature. Maximum values for EL and ES were reported 22.5 µm, 249.4 µm² respectively for *Emys trinacris* (Arizza et al., 2014). Maximum ES for Anatolian populations of *E. orbicularis* is 225.1 µm² reported from captive specimens (Metin et al., 2006).

Our results indicated that sunshine duration positively correlate with ES and NV whereas negatively correlate with EL for *E. orbicularis*. This means the turtles have longer sunshine duration, have much bigger spherical erythrocytes, and have larger nucleus volume. Basking is a common behavior in ectotherms and many freshwater turtles bask either at the surface of the water or on the substrates (Bulté & Blouin-Demers, 2010). Sunshine duration becomes crucial for regulation of metabolic rate to this respect. Bulté & Blouin-Demers (2010) suggested that basking behavior allows turtles to increase their metabolic rate by up to 30.1%.

Erythrocytes of *M. rivulata* were large both in size and in volume at Loc1, while they were small at warmer Loc3. Because of the altitudes were the same among the localities of *M. rivulata*, the differences between the Loc1 and Loc3 were temperature which could be an effective environmental variable on erythrocyte morphology and precipitation. Loc1 had been a cooler habitat with more rainfall for the turtles in which we measured larger cells and nuclei.

In conclusion, our results support the literature knowledge that the temperature and the altitude affect the erythrocyte sizes. The turtles living in the higher temperature or/and elevation are tend to have smaller erythrocyte sizes. However, these variations on erythrocyte sizes do not correlate with environmental parameters in all studied populations. Further studies with more populations that have various intermediate environmental characteristics are needed for a better point of view.

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Ethics committee approval: This study was performed in accordance with ethical standards of animal experiments. Legal research ethics committee approval permissions for the study were obtained from the Ege University, Animal Experiments Local Ethics Committee (No: 2010-013).

Conflict of interest: The authors declares that there is no conflict of interest.

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