REPTILES & AMPHIBIANS

Clinical Hematology of the Nose-horned Viper, *Vipera ammodytes* (Linnaeus 1758)

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Abstract.—The establishment of hematological profiles of animals taken from their natural environment or kept in captivity has great importance in determining the health status and biology of these species. Pollution in the habitat of reptiles and the impact of various diseases can be determined by analyzing blood parameters. In this study, the clinical hematology (erythrocyte and leukocyte count, erythrocyte types, differential blood formula, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration) of three Nose-horned Vipers (*Vipera ammodytes*) captured from Vize (Kırklareli, Türkiye) were examined. We detected the frequency of nuclear abnormalities in this species for the first time. We also provide the first erythrometric measurements of *Vipera ammodytes*, and found that the erythrocyte sizes of this species were larger compared to other species in Viperidae. Knowledge of the general health status of animals is important in species conservation action plans and monitoring studies.

With the monitoring of the health status of wildlife species, early detection of changes occurring in the ecosystem are possible such as habitat fragmentation (Johnstone et al. 2012), the presence of pathogens (Conrad et al. 2005; Childs et al. 2007; Yockney et al. 2013; Bagamian et al. 2014; Carson et al. 2014), and environmental pollution (Guillette et al.1995; Fox, 2001; Pitman et al. 2011; Lloyd et al. 2016). Habitats are increasingly fragmented over the years, thus affecting the health of animals and potentially their adaptative success (Martínez-Mota et al. 2007; Cottontail et al. 2009).

Hematological analyses are of great importance in determining the biology and health of both wild and captive species (Canfield 1998; Campbell and Ellis 2007; Tumkiratiwong et al. 2012). Reptiles, which are poikilothermic vertebrates, can be used as non-standard bioindicators for investigating the presence of pollutants and mutagens in the environment and for analyzing natural populations (Matson et al. 2005; Strunjak-Perovic et al. 2010). Determination of blood profiles in reptiles is also important for detecting the characteristic blood values of species, as well as identifying the impact of factors such as pollution and various diseases on their health (Dessauer 1970; Campbell 2006). In addition, blood parameters in reptiles may vary depending on age, sex, season and reproductive status (Dessauer 1970; Duguy 1970; Frye 1991; Wilkinson 2003; Hidalgo-Vila et al. 2007).

Some environmental effects can cause damage to the genetic material of organisms (Lee and Steinert 2003). Therefore, it is also of great importance to evaluate, monitor and investigate the effects of external (environmental) factors on the genetic material. The micronucleus test is one of the most widely used biological markers for on-site monitoring of genotoxic pollution in natural environments (Al-Sabti 1994, 1995; Al-Sabti and Metcalfe 1995; Bolognesi et al. 2006; Udroiu 2006; Strunjak-Perovic et al. 2010; Schaumburg et al. 2012; Çördük et al. 2019). In addition, detection of erythrocyte types to determine the cytotoxic effects of pollution is also a method used to predict hematological abnormalities (Guilherme et al. 2008; Pollo et al. 2019; Dönmez 2021). A number of hematological studies have been conducted on venomous snakes (Hattingh and Willemse 1976; Troiano et al. 1997; Dutton and Taylor 2003; Allender et al. 2006; Santos et al. 2008), but many species remain to be assessed using clinical methods.

Snakes have a well-developed hematopoietic and immune system that reacts to all environmental factors, making them unique organisms for environmental research. The assessment of the immune status of snakes under the effect of anthropogenic pressure located in different environments is not fully understood (Romanova et al. 2021) for a variety of species. The Nose-horned Viper (*Vipera ammodytes*) is a venomous snake species found in Eurasia, and characterized by its distinctive fleshy horn at the tip of the nose. It is commonly found in dry and rocky areas (Arnold and Ovenden 2002), and is considered one of the most dangerous snakes in Europe

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with its potent venom that often results in mortality in human envenomation cases (Boulenger 2000; Proverbio et al. 2012). *Vipera ammodytes* is listed in Appendix II (Strictly protected fauna species) of the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Anonymous 1982; Lisicic et al. 2013), and as LC (Least Concern) on the IUCN Redlist (International Union for Conservation of Nature) (IUCN 2022). In Türkiye, hematological studies are usually performed on the size and number of blood cells (Arıkan et al. 2004; Tok et al. 2006; Çiçek et al. 2009), but a detailed clinical hematology study of *V. ammodytes* has not been conducted.

Determining the clinical hematology values of *V. ammodytes* is useful not only for understanding general aspects of the ecology of this species in nature but it may reveal information about the health of wild populations. Alterations in some hematological parameters compared with the reference values in previous studies can be used as evidence of disturbances in physiological state, such as diseases or stress in reptiles (Bloom and Brandt 2008; Davis et al. 2008; Kitana et al. 2016). In this study, we conducted the first hematology study on a population of *V. ammodytes* from Türkiye (Vize, Kırklareli) and discuss nuclear abnormalities and erythrometric measurements found therein.

Methods

We collected three female *Vipera ammodytes* (TBL = 57 cm, 64 cm and 55 cm) from Göztepe in Vize District, Kırklareli Province, Türkiye (41.584364°N, 27.796861°E; Fig. 1). The necessary permits for this study were issued by the Ethics Committee of Animal Experiments of Çanakkale Onsekiz Mart University (decision number: 2021/10-05). Morphological measurements were taken with digital calipers (Mitutoyo, accuracy: 0.01 mm) (Table 1). Snakes were released after data and blood sample acquisition.

We extracted 1 ml of blood from the postorbital sinus of the captured snakes using heparinized hematocrit tubes (Bush and Smeller 1978; Tosunoğlu et al. 2004) and determined erythrocyte counts, erythrocyte types, leukocyte counts, differential blood formulas, hemoglobin (Hb), hematocrits (HCT), mean corpuscular volumes (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentrations (MCHC). Erythrocytes and leukocyte counts were performed using the Olympus CX21 microscope and a Neubauer Hemocytometer (Gül et al. 2011). Hemoglobin determination was performed using the Sahli method. For the hematocrit determination, blood samples were centrifuged at 1,000 rpm for 5 minutes in a microhematocrit centrifuge device (Electro-Mag M19) and calculated as a percentage (Tanyer 1985). The

Table 1. General morphological parameters of *Vipera ammodytes* (ML: Mouth Length, HL: Head Length, HW: Head Width, SVL: Snout Vent Length, TBL: Total Body Length).

	I	II	III
ML (mm)	20.51	20.73	20.77
HL (mm)	26.53	28.84	28.11
HW (mm)	21.44	18.43	21.26
SVL (cm)	49	56	51
TBL (cm)	57	64	55

Fig. 1. A Nose-horned Viper (*Vipera ammodytes*), the habitat, and the location near Vize/Kırklareli, Türkiye, where it was caught. Photographs by Bengi Baycan.

mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values were calculated mathematically in accordance with the results obtained from the analyses (Tanyer 1985). Blood smears prepared from blood samples were stained with Wright's Stain for erythrocyte counts and differential blood formulas, and Giemsa Stain for nuclear abnormality detection (Josende et al. 2015; Çördük et al. 2019).

We made four measurements (at 1,000x) of 40 randomly selected erythrocytes from each blood smear using an Olympus 1–15X micrometric eyepiece: erythrocyte length (EL), erythrocyte width (EW), nuclear length (NL), and nuclear width (NW). Shapes of erythrocytes and nuclei were determined by EL/EW and NL/NW ratios, and the shape of the nucleus/cytoplasm was determined by the NS/ES ratio. Erythrocyte size (ES) and nuclear size (NS) were calculated mathematically in accordance with the results obtained from the measurements (Atatür et al. 1999). The differential blood formulas were generated from blood smears of each individual (Tanyer 1985).

For determining erythrocyte types, we searched for enucleated erythrocytes (EE), mitotic erythrocytes (ME), pyknotic erythrocytes (PE), and immature erythrocytes (IE) while counting 1,000 erythrocytes on blood smears (Pollo et al. 2019; Dönmez 2021). Micronucleus (MN) and other nuclear abnormalities such as kidney-shaped nuclei, lobed nuclei, notched nuclei, blebbed nuclei, and binucleated cells also were detected by counting 1,000 erythrocytes in the blood smears at 1,000x magnification. Micronucleus was defined as follows: a) MN should be less than one-third of the main nucleus, b) MN should not be in contact with the main nucleus, c) MN should be the same color and density as the main nucleus and should not be refractive (Heddle and Countryman 1976; Fenech 2000; Çördük et al. 2018). The standard values of the obtained data were evaluated by using Microsoft Excel and IBM SPSS Statistics 20 programs.

Results

Erythrocyte and leukocyte counts were 600,000–694,000/mm^3 (6.0–6.94 × 10^{11}/L) and 740–1.300/mm^3 (0.74–1.3

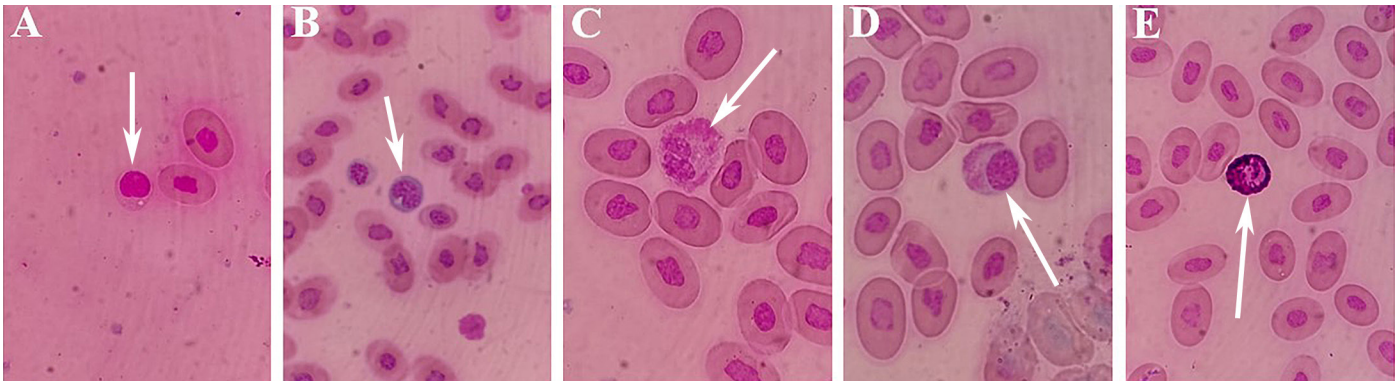

Fig. 2. Leukocytes of *Vipera ammodytes*: lymphocyte (A), monocyte (B), eosinophil (C), heterophil (D), and basophil (E).

Table 2. Hematological parameters of *Vipera ammodytes* (MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration).

	I	II	III
Erythrocyte Count (1 mm^3)	600000	613000	694000
Leukocyte Count (1 mm^3)	1300	870	740
Lymphocyte (%)	50	45	62
Monocyte (%)	6	10	5
Eosinophil (%)	27	19	22
Heterophil (%)	14	15	9
Basophil (%)	3	11	2
Hemoglobin (g/dL)	5.4	5.2	5.4
Hematocrit (%)	25	39	34
MCV (fl)	833.33	1270.35	979.82
MCH (pg)	180	169.38	155.61
MCHC (%)	21.6	13.33	15.88

× 10^9/L), respectively. Among leukocytes, lymphocytes were most abundant, followed by eosinophils, heterophils, monocytes, and basophils, respectively (Fig. 2). Hemoglobin values were 5.2–5.4 g/dL (52–54 g/L) and hematocrit values were 25–39%. We observed ranges of 833.33–1270.35 femtoliters (fL) for MCV, 155.61–180 picograms (pg) for MCH, and 13.33–21.6% for MCHC (Table 2). Mean erythrocyte sizes were 158.79–180.83 µm, and mean nuclear sizes were 28.83–30.82 µm.

The ratios of EL/EW and NL/NW indicated that erythrocytes (EL/EW = 1.55–1.57) and nuclei (NL/NW = 1.18–1.48) were ellipsoidal (Table 3). Percentages of total nuclear abnormalities were 7.4–9.2% (Fig. 3; Table 4). Immature erythrocytes were most abundant, followed by pyknotic

Table 3. Descriptive statistics of erythrometric measurements of *Vipera ammodytes* specimens (n: Measured Erythrocyte Number, SE: Standard Error, SD: Standard Deviation, EL: Erythrocyte Length, EW: Erythrocyte Width, NL: Nuclear Length, NL: Nuclear Width, ES: Erythrocyte Size, NS: Nuclear Size, EL/EW: Erythrocyte Length/Erythrocyte Width, NL/NW: Nucleus Length/Nucleus Width, NS/ES: Nucleus Size/ Erythrocyte Size).

I						
	n	**Minimum**	**Maximum**	**Mean**	**SE**	**SD**
EL (µm)	40	16.5	22.0	19.03	0.21	1.36
EW (µm)	40	10.0	13.0	12.10	0.10	0.68
NL (µm)	40	6.0	9.0	7.61	0.10	0.66
NW (µm)	40	4.5	6.5	5.16	0.06	0.41
ES (µm²)	40	149.15	207.24	180.83	2.61	16.51
NS (µm²)	40	23.55	40.03	30.82	0.54	3.42
EL/EW (µm²)	40	1.30	1.91	1.57	0.02	0.14
NL/NW (µm²)	40	1.07	1.88	1.48	0.02	0.17
NS/ES (µm²)	40	0.13	0.20	0.17	0.00	0.01
II						
	n	**Minimum**	**Maximum**	**Mean**	**SE**	**SD**
EL (µm)	40	16.0	21.5	17.75	0.17	1.09
EW (µm)	40	9.0	13.0	11.37	0.13	0.87
NL (µm)	40	5.0	7.5	6.56	0.10	0.63
NW (µm)	40	4.5	6.5	5.58	0.07	0.46
ES (µm²)	40	113.04	210.96	158.79	2.94	18.65
NS (µm²)	40	19.62	38.26	28.83	0.63	4.04
EL/EW (µm²)	40	1.33	2.00	1.56	0.02	0.12
NL/NW (µm²)	40	1.00	1.55	1.18	0.02	0.14
NS/ES (µm²)	40	0.13	0.24	0.18	0.00	0.02
III						
	n	**Minimum**	**Maximum**	**Mean**	**SE**	**SD**
EL (µm)	40	15.5	22.0	18.70	0.19	1.23
EW (µm)	40	10.0	14.0	12.07	0.14	0.90
NL (µm)	40	6.0	8.5	7.02	0.09	0.60
NW (µm)	40	4.5	6.5	5.23	0.06	0.42
ES (µm²)	40	133.84	211.95	177.36	2.92	18.50
NS (µm²)	40	23.55	35.71	28.85	0.50	3.17
EL/EW (µm²)	40	1.28	2.00	1.55	0.02	0.14
NL/NW (µm²)	40	1.07	1.77	1.35	0.02	0.16
NS/ES (µm²)	40	0.13	0.20	0.16	0.00	0.01

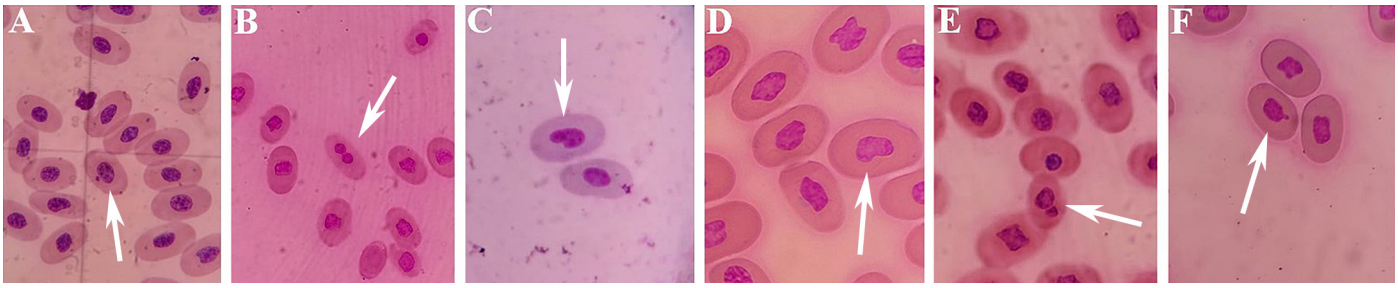

Fig. 3. Micronucleus and other nuclear abnormalities of *Vipera ammodytes*: Micronucleus (A), lobed nucleus (B), notched nucleus (C), kidney-shaped nucleus (D), binucleate cell (E), blebbed nucleus (F).

Table 4. Frequency (%) of micronucleus and other nuclear abnormalities of *V. ammodytes*.

	I	II	III
Micronucleus (%)	0	0.1	0.1
Lobed Nucleus (%)	0.5	0.3	0.8
Notched Nucleus (%)	3.2	4.6	3.5
Blebbed Nucleus (%)	3.4	3.9	2.6
Binucleate Cells (%)	0	0	0.1
Kidney-shaped Nucleus (%)	0.4	0.3	0.3
TOTAL NUCLEAR ABNORMALITY (%)	**7.5**	**9.2**	**7.4**

Table 5. Frequency (%) of different erythrocyte types on *V. ammodytes* species.

	I	II	III
Enucleated Erythrocyte (%)	0.4	0.6	1.5
Mitotic Erythrocyte (%)	0.2	0.1	0
Immature Erythrocyte (%)	4.6	8.0	4.0
Pyknotic Erythrocyte (%)	1.4	4.8	1.7

erythrocytes, enucleated erythrocytes, and mitotic erythrocytes, respectively (Fig. 4; Table 5).

Discussion

Hematological parameters may vary depending on external factors such as sex, age, reproductive status, activity, stress, altitude, and captivity conditions (Hartman and Lessler 1964; Wojtaszek 1991, 1992; Palenske and Saunders 2003; Santos et al. 2008; Gül et al. 2011). When our findings on clinical hematology were compared with a previous study conducted on a Croatian population of *V. ammodytes* (Lisicic et al. 2013), the hematocrit value of one individual (Individual II) was higher; MCV and MCH values were higher than the reference range. All other values were between referenced ranges in the literature.

When comparing our results to previous studies, the erythrocyte count we observed was congruent with what is found in the literature, but the number of leukocytes were below the reference ranges (Babudieri 1930; Lisicic et al. 2013). According to Fierascu et al. (2018), erythrocytopenia and leukocytopenia may be observed in individuals that prefer pesticide-exposed habitats. The individuals in this study were collected from habitat in a region that has extensive sheep and goat farming in agricultural areas. Thus, it is possible that the observed low number of leukocytes in the blood of the snakes in our study may be due to agricultural activities, pesticide-use, and habitat fragmentation. Future studies are needed to verify this.

In the study of Lisicic et al. (2013), lymphocytes had the highest frequency followed by heterophils, eosinophils and

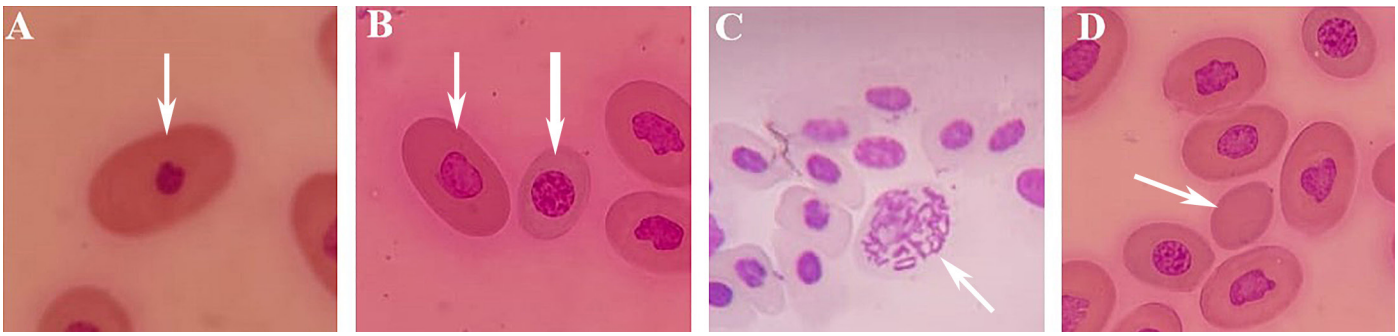

Fig. 4. Erythrocytes of *Vipera ammodytes*: Pyknotic erythrocyte (A), mature erythrocyte (thin arrow) and immature erythrocyte (thick arrow) (B), mitotic erythrocyte (C), enucleated erythrocyte (D).

basophils in female *V. ammodytes* individuals. Our results on lymphocyte and heterophil frequency corroborate this, but our eosinophil and basophil frequencies were higher than what has been calculated in other studies. Monocyte frequency was 5–10 % in our individuals. In Lisicic et al. (2013), they evaluated azurophils instead of monocytes because these two leukocyte types showed similar cytochemical properties; however, we cannot draw comparisons as we did not look at azurophils.

Higher eosinophil counts (19–27%) were seen in two individuals and higher basophil frequency (2–11%) was seen in one individual when compared to the literature values (Lisicic et al. 2013). The number of eosinophils in the circulating blood of reptiles can vary depending on species and seasonal factors (Campbell 2004). Eosinophils range from 7–20% of total leukocytes in healthy reptiles. Although the eosinophil function has not been well studied in reptiles, abnormally high eosinophil counts have been associated with parasitic infections (e.g., protozoa, helminths) and other types of antigenic stimulation (Mead and Borysenko 1984; Campbell 2004; Stacy et al. 2011). Basophil numbers also vary by species but are usually low. However, basophils in some reptilian species can vary from 0–40% of total leukocytes (Duguy 1970; Jacobson et al. 1990; Marks and Citino 1990; Campbell 2004). Likely, the basophils of reptiles do not change according to season like other granulocytes (Saint Girons 1970; Campbell 2004). Considering the previous studies conducted on venomous snakes, some reports indicate that the number of basophils may increase depending on the geographical region, age of the animal (Work et al. 1998) and blood parasites or viral infections (Sypek and Borysenko 1988; Vasaruchapong et al. 2014). No external signs of illness or infection were observed in the individuals of this study.

Some studies have been done on micronucleus and other nuclear abnormalities (such as notched nuclei, blebbed nuclei, lobed nuclei, kidney-shaped nuclei, and binucleate cells) on snake species (Strunjak-Perovic et al. 2010; Tok et al. 2014), but studies showing nuclear abnormalities in *V. ammodytes* have yet to be done prior to this study. Frequency of micronucleus and other nuclear abnormalities in snakes can vary depending on environmental pollutants (Tok et al. 2014), seasons, biological cycle characteristics of the species and sex (Strunjak-Perovic et al. 2010). In previous studies, findings have shown a positive correlation between genotoxic damage and deformation of environmental quality due to agricultural practices (Ossana and Salibian 2013; Josende et al. 2015; Babini et al. 2015; Pollo et al. 2019). We hypothesize that the reason for other nuclear abnormalities we observed may have originated from the presence of agricultural areas around the studied animals' habitats.

The observed imbalance in the ratio of immature and mature erythrocytes detected in blood may indicate that the individual has a disease or infection (Campbell 2006; Oliveira-Junior et al. 2009; Lisicic et al. 2013). When studies conducted on different vertebrate animal groups were examined, the frequency of immature erythrocytes was also evaluated in order to clarify the results of nuclear abnormalities (Minissi et al. 1996; Pacheco and Santos 2002; Guilherme et al. 2008). The immature erythrocyte frequency of the three *V. ammodytes* individuals in our study was higher than the literature (Lisisic et al. 2013).

The ratio between different types of erythrocytes gives information about the normal activity of erythropoiesis, but could also be evidence that pathological changes have occurred (Lisicic et al. 2013). A high incidence of immature erythrocyte frequency is associated with erythropoiesis and pyknotic erythrocyte frequency is associated with apoptosis (Saquib et al. 2012; Peltzer et al. 2013). The increases in these frequencies are thought to be related to the response to cell damage or stress (Ray et al. 2005). Also, in studies conducted on amphibians, the presence of enucleated and mitotic erythrocytes in blood may represent a short-term mechanism developed to increase oxygen transport capacity, especially in conditions of water pollution (Barni et al. 2007; Peltzer et al. 2013; Pollo et al. 2019).

Studies of erythrometric characteristics of some viperids include Çiçek et al. (2009), who examined the morphology of blood cells of different snake families in Türkiye and reported that the largest erythrocytes in viperids were in *Macrovipera lebetinus* and the smallest were in *Montivipera xanthina*. Also, Arıkan et al. (2004) examined the morphology and size of blood cells of some viperid snakes, finding that the largest erythrocytes were in *Vipera wagneri*, and the smallest erythrocytes were in *Montivipera xanthina*. However, in the present study, we observed that erythrocyte sizes of *V. ammodytes* were larger (113–211 µm) than those of other species.

In addition, as mentioned in Arıkan et al. (2004), the erythrocytes and nuclei of *V. xanthina*, *V. wagneri*, and *Vipera eriwanensis* were ellipsoidal, which is also what we found in our study on *V. ammodytes*. In a study of *Natrix tessellata*, Gül et al. (2019) reported a negative correlation between body size and erythrocyte size, and a decrease in erythrocyte size as body size increases. We found that erythrocyte size of the largest *V. ammodytes* we studied was smaller than that of the other two individuals.

The clinical hematology parameters and reference values of Nose-horned Viper (*Vipera ammodytes*) distributed in Vize (Kırklareli/Türkiye) were determined, and nuclear abnormality analyses were performed for the first time in this species. This study will be a reference for future projects on this species and the study of how environmental factors affects the hematology and genotoxicology of these snakes.

Acknowledgments

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