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A comparative haematological research on *Bufo bufo* (Linnaeus, 1758) and *B. verrucosissimus* (Pallas, 1814) in Türkiye

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Abstract

In this study, it is aimed to contribute to the systematic situation of *Bufo bufo* and *B. verrucosissimus* by comparing some blood parameters for the first time. For that, fieldwork was done in Lake Borçka Karagöl, Artvin for *B. verrucosissimus* and Lake Uzungöl, Trabzon for *B. bufo* in 2022. A total of 6 adult individuals (3 males, 3 females) were captured per species. For each individual, 1 ml blood sample was acquired from spontaneously pulsating heart ventricle and blood smears were prepared. Under a microscope, 40 randomly chosen erythrocytes from each smear were measured to acquire 9 distinct blood cell parameters. Additionally, erythrocyte and leukocyte counts were calculated using Neubauer hemacytometer. It was found that female individuals had larger values for measurement of blood cell parameters. Mean erythrocyte and leucocyte numbers were higher in the blood of *B. bufo* than *B. verrucosissimus* but there was not a significant difference between species for erythrocyte and leukocyte counts. In females, erythrocytes were larger and narrower in *B. verrucosissimus* than *B. bufo* species. Alike, nuclei were larger and narrower as observed in *B. verrucosissimus*, similar to erythrocytes. However, *B. bufo* had larger and wider erythrocytes but larger and narrower nuclei in males. Given the differences between sexes, these characters thought as not diagnostic. In addition, PCA analysis showed overlapped position of species in morphospace and supported the weak discrimination power of the characters. Our findings will contribute to the future studies as a reference source for basic blood parameters.

Keywords: blood cell, Common Toad, Caucasian Toad, erythrocyte, morphology

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Türkiye'deki *Bufo bufo* (Linnaeus, 1758) ve *B. verrucosissimus* (Pallas, 1814) üzerine karşılaştırmalı bir hematolojik araştırma

Özet

Bu çalışmada, bazı kan parametrelerini ilk kez karşılaştırılarak *Bufo bufo* ve *B. verrucosissimus* türlerinin sistematik durumuna katkı sağlanması hedeflenmiştir. Bu amaçla, 2022 yılı içerisinde *B. verrucosissimus* için Artvin Borçka Karagöl ve *B. bufo* için Trabzon Uzungöl mevkisinde saha çalışması yapılmıştır. Tür başına toplam 6 yetişkin birey (3 erkek, 3 dişi) yakalanmıştır. Her birey için atmaya devam eden kalp ventrikülünden 1 ml kan örneği alınmış ve yayma preparatlar hazırlanmıştır. Mikroskop altında, 9 farklı kan hücresi parametresine ait veriyi elde etmek için her yaymadan rastgele seçilen 40 eritrosit ölçülmüştür. Ayrıca Neubauer hemasitometresi kullanılarak eritrosit ve lökosit sayıları hesaplanmıştır. Kan hücresi parametrelerinin ölçüm sonuçlarına göre dişilerin erkeklerden daha büyük değerlere sahip olduğu belirlenmiştir. *B. bufo* türünün kanındaki ortalama eritrosit ve lökosit sayıları *B. verrucosissimus* türüne göre daha fazla olsa da eritrosit ve lökosit sayıları açısından türler arasında anlamlı bir farklılık tespit edilmemiştir. Dişiler için eritrositler *B. verrucosissimus* türünde *B. bufo* türüne göre daha büyük ve daha dar şekle sahiptir. Benzer şekilde, *B. verrucosissimus* türünde çekirdekler, eritrositlerde gözlendiği gibi daha büyük ve daha dar şekillidir. Öte yandan, erkekler için *B. bufo* türüne ait eritrositler daha büyük ve daha geniş şekilli fakat çekirdekleri daha büyük ve daha dar şekillidir. Cinsiyetler arasındaki farklılıklar göz önüne alındığında, bu karakterlerin diyagnostik olmadığı

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düşünülmüştür. Ayrıca PCA analizi türlerin morfouzaydaki örtüşen konumlarını göstermiş ve karakterlerin zayıf ayırt etme gücünü desteklemiştir. Bulgularımız temel kan parametreleri için referans kaynağı olarak gelecek çalışmalara katkı sağlayacaktır.

Anahtar kelimeler: kan hücreleri, Siğilli Kurbağa, Kafkas Siğilli Kurbağası, eritrosit, morfoloji

1. Introduction

Haematological parameters allow the inference of physiological information specific to a species and offer valuable data for ecological studies on amphibian species [1]. In addition, the characteristics are evaluated as environmental indicators that react rapidly to changes in the surrounding environment [2, 3]. As a result, it is often used for assessing environmental stress. The systematic research on amphibian species benefits from the utilization of haematological markers besides their ability to detect changes in physiological, pathological, ecological, and environmental factors [4, 5]. It is feasible to draw conclusions regarding internal (gender, species-specific characteristics, age) and external (season, habitat, trophic) elements through the comparison of these metrics.

Amphibian blood cells are composed of leukocytes, platelets, and erythrocytes. Leukocytes are more resemble to those in human blood. Thrombocytes are nucleated spindle cells that fulfil the same function as mammalian platelets. Large erythrocytes of amphibians emerged through evolutionary processes distinguish them from other vertebrates. Compared to birds and mammals, their erythrocytes are more robust and persistent. The shape of erythrocyte cells is oval, nucleated, and biconcave [6, 7]. The number of erythrocytes varies with body size, age, gender, season, and environmental factors in addition to species and individuals within a population. Furthermore, the quantity of erythrocytes in amphibians differs greatly throughout species and presents useful data for systematic evaluations [8].

The *Bufo bufo* species group refers to a taxonomic group of toads within the genus *Bufo* distributing in Western Palearctic realm. The species group includes four closely related taxa namely Eichwald's toad *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov and Rosanov, 2008, Spiny toad *Bufo spinosus* Daudin, 1803, Common toad *Bufo bufo* (Linnaues, 1758) and Caucasian toad *Bufo verrucosissimus* (Pallas, 1814). In the recent molecular studies, *Bufo bufo* and *B. verrucosissimus* have been assessed as sister species, and the broad distribution of both species have overlapped in Türkiye. Besides, the systematic situation of these taxa has become a subject in various studies based on molecular and morphological data [9, 10]. Lately, the presence of a narrow hybrid zone between these species has been reported in the northeastern Anatolia [11]. Unlike the presence of numerous comparative studies, there is no comparison in terms of serological characters and blood parameters between these species. Previous studies have focused only on the blood parameters of *B. bufo* species from Türkiye and described the characteristic of blood cells [12-15]. As a comparative study, Tosunoğlu and Taskavak [16] investigated 10 samples obtained from Manyas (Balıkesir) and Çamlıhemşin (Rize) districts in terms of blood-serum proteins. As a result of the study, they reported that there was no qualitative or quantitative difference in serum protein phenograms. Regarding their findings, the researchers reported that the samples from both localities should be considered as *Bufo bufo spinosus* subspecies and that the taxon called *B. bufo verrucosissimus* in Çamlıhemşin district could be synonymous. Given the current geographic distributions and presence of the hybrid zone between species, the comparison remained blur and did not adequately describe the haematological patterns.

The lack of a comparative study between *B. bufo* and *B. verrucosissimus* regarding blood parameters is an important gap in the literature that needs to be resolved. In addition, the absence of serological data on the *B. verrucosissimus* species is an essential taxonomic deficiency. In this study, it is aimed to contribute to the systematic situation of both species by comparing some blood parameters for the first time.

2. Material and method

Fieldwork was done in Lake Borçka Karagöl, Artvin (41.386124 N, 41.854107 E; 1450 m) for *B. verrucosissimus* and Lake Uzungöl, Trabzon (40.622108 N, 40.285267 E; 1100 m) for *B. bufo* in September 2022. For each species, a total of 6 adult individuals (3 males, 3 females) were captured. Adult samples were sexed following the external sexual characteristics: densely melanized fingers and presence of nuptial pad in males, and the opposite in females. Fieldwork and sampling were done with the permission of the Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Fisheries and Aquaculture Sampling (number: E-21264211-288.04-6387153), and the local ethics committee for animal experiments (Republic of Türkiye Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments, approval reference number: 2022/19).

The specimens were taken to the laboratory alive and snout-vent length (SVL) was measured using a digital calliper to the nearest 0.01 mm. Before sampling blood, individuals were anesthetized in 250 mg/L MS222 solution. For each individual, 1 ml blood sample was acquired from spontaneously pulsating heart ventricle using 21-gauge needle and 5 ml syringe. Afterwards, 4 different blood smears were fixed using methanol and was exposed to Wright’s stain

for 15 min. For each smear, a total of 40 randomly selected erythrocytes were measured using Olympus BX51 microscope at 200x and 400x magnifications for following characters: erythrocyte length (EL), erythrocyte width (EW), nucleus length (NL), nucleus width (NW), erythrocyte shape (ESh: EL/EW), nucleus shape (NSh: NL/NW), nucleus/cytoplasm shape (NCSh: NSh/Esh), erythrocyte size (ES: ELEWπ/4) and nucleus size (NS: NLNWπ/4). The erythrocyte (EN) and leukocyte (LeuN) counts were calculated by diluting Hayem and Turck solutions and using Neubauer hemacytometer.

Descriptive statistics were calculated using obtained measurements. Normality assumption was controlled using Kolmogorov-Smirnov test. The measurement differences between the sexes and species were compared using Student's t test and Mann-Whitney U test. Univariate analyses were run using the *stats* package. To reduce the dimension of dataset and to explain variable contribution, principal component analysis (PCA) was performed using log10-transformed data. For that, all specimens were used without grouping. The relationships between SVL and blood parameters were investigated using correlation analysis. All analyses were executed in R Programming Language v4.1.2 [17].

3. Results

Erythrocyte shape is oval and resemble to amphibian blood cell characteristic. Nuclei are generally elliptical and located at the centre of the erythrocytes. Cytoplasm is stained light purple whereas chromophilic nuclei are dark blue and purple. Descriptive statistics indicating measurements of blood cell characters were presented in Table 1.

For the variables showed non-parametric distribution ($p<0.05$), pairwise comparisons between sexes and species were carried out using Mann-Whitney U test. Regarding sexual comparison of whole data, significant differences were found in EL ($Z= -4.426$; $p <0.01$), EW ($Z= -7.132$; $p <0.001$), NL ($Z= -3.420$; $p <0.01$), NW ($Z= -8.334$; $p <0.001$), ESh ($Z= -8.129$; $p <0.001$), NSh ($Z= -9.496$; $p <0.001$), NS ($Z= -3.715$; $p <0.001$), NCSh ($Z= -2.927$; $p <0.01$) between sexes. When comparing species, significant differences were found in EL ($Z= -2.880$; $p <0.05$), EW ($Z= -4.284$; $p <0.001$), NW ($Z= -6.149$; $p <0.001$), ESh ($Z= -5.145$; $p <0.001$), NSh ($Z= -5.514$; $p <0.001$), NS ($Z= -4.371$; $p <0.001$), NCSh ($Z= -2.838$; $p <0.05$) for females and in EW ($Z= -13.594$; $p <0.001$), NL ($Z= -3.750$; $p <0.001$), NW ($Z= -4.859$; $p <0.001$), ESh ($Z= -10.698$; $p <0.001$), NSh ($Z= -6.464$; $p <0.001$), ES ($Z= -8.426$; $p <0.001$), NCSh ($Z= -5.219$; $p <0.001$) for males. In the variables showing normal distribution, there was only a significant difference between sexes in terms of SVL ($t= 7.33$; $df=10$; $p<0.001$). The distribution of data classified by species and sex was displayed using boxplots in Figure 1.

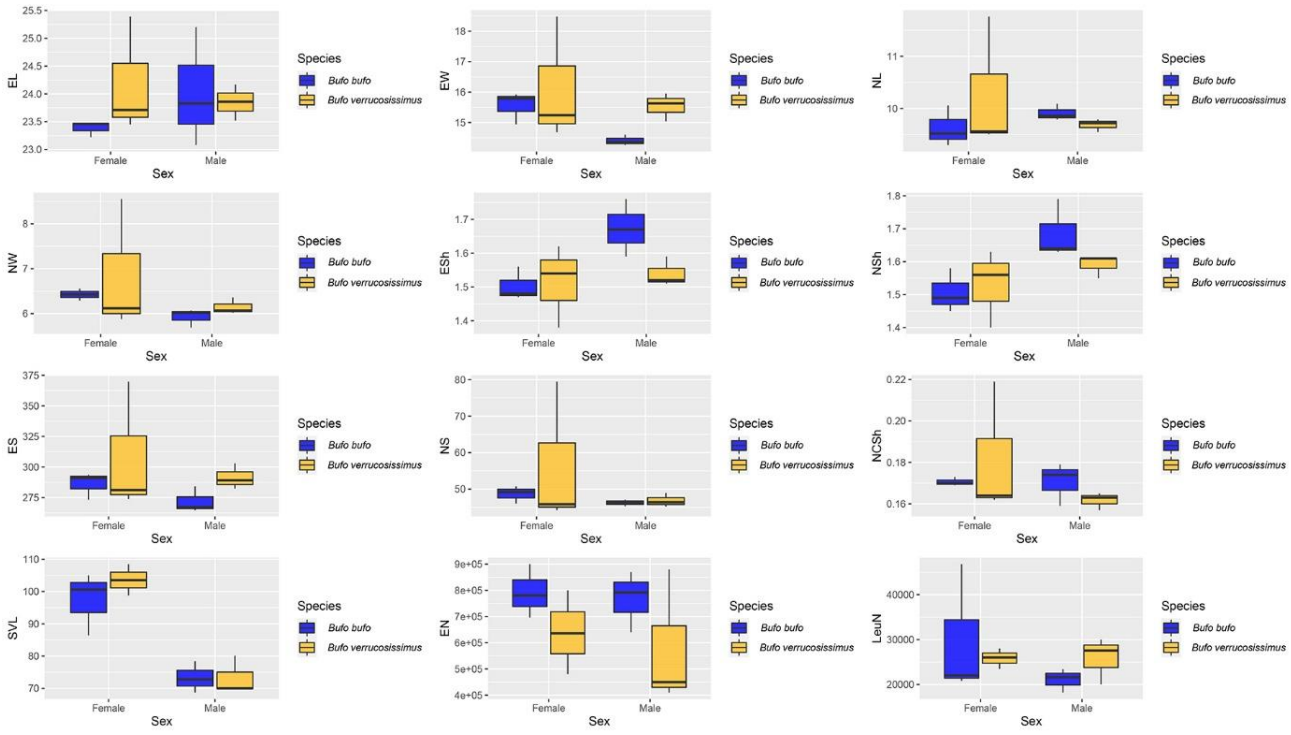

Figure 1. Boxplots representing differences in blood cell parameters between species based on sexes. The tick line in box is showing median. The lines positioning under and on the box are whiskers

Table 1. Descriptive statistics of SVL and blood parameters of *B. bufo* and *B. verrucosissimus*, respectively (mm: millimetre; µm:micrometre)

	Bufo bufo (Uzungöl) / Females				
Variable	N	Mean	St. Error	Min	Max
SVL (mm)	3	97.33	5.61	86.40	105.00
EN	3	792000	59194.59	696000	900000
LeuN	3	29866.67	8473.75	20800	46800
EL (µm)	480	23.38	0.07	18.89	28.17
EW (µm)	480	15.55	0.05	11.22	19.43
NL (µm)	480	9.63	0.04	6.82	12.87
NW (µm)	480	6.43	0.02	4.66	8.62
ESh (µm)	480	1.51	0.00	1.14	2.20
NSh (µm)	480	1.51	0.00	1.00	2.56
ES (μm^2)	480	285.98	1.54	190.69	394.74
NS (μm^2)	480	48.64	0.29	29.02	80.19
NCSh (µm)	480	0.17	0.00	0.10	0.29
	Bufo bufo (Uzungöl) / Males				
Variable	N	Mean	St. Error	Min	Max
SVL (mm)	3	73.30	2.81	68.70	78.40
EN	3	767333.33	67531.06	640000	870000
LeuN	3	21066.67	1524.613	18200	23400
EL (µm)	480	24.04	0.10	18.14	29.88
EW (µm)	480	14.41	0.04	11.47	20.58
NL (µm)	480	9.91	0.04	7.39	12.54
NW (µm)	480	5.92	0.03	4.36	7.96
ESh (µm)	480	1.67	0.00	1.17	2.50
NSh (µm)	480	1.6	0.01	1.06	2.46
ES (μm^2)	480	271.98	1.45	182.98	438.13
NS (μm^2)	480	46.25	0.35	26.05	67.88
NCSh (µm)	480	0.17	0.00	0.10	0.28
	Bufo verrucosissimus (Karagöl) / Females				
Variable	N	Mean	St. Error	Min	Max
SVL (mm)	3	103.60	2.80	98.80	108.50
EN	3	638666.67	92385.66	480000	800000
LeuN	3	25820.00	1313.67	23460	28000
EL (µm)	360	23.78	0.10	16.24	30.18
EW (µm)	360	15.35	0.09	11.06	25.01
NL (µm)	360	9.78	0.06	6.75	13.79
NW (µm)	360	6.29	0.06	4.26	17.58
ESh (µm)	360	1.56	0.00	1.03	2.22
NSh (µm)	360	1.58	0.01	0.69	2.39
ES (μm^2)	360	287.68	2.58	180.01	562.87
NS (μm^2)	360	48.88	0.74	30.08	168.23
NCSh (µm)	360	0.16	0.00	0.10	0.43
	Bufo verrucosissimus (Karagöl) / Males				
Variable	N	Mean	St. Error	Min	Max
SVL (mm)	3	73.30	3.40	69.80	80.10
EN	3	580050.00	150422.19	410000	880000
LeuN	3	25853.33	3010.23	20000	30000
EL (µm)	480	23.85	0.08	14.34	30.04
EW (µm)	480	15.54	0.05	10.97	19.32
NL (µm)	480	9.69	0.04	6.58	12.95
NW (µm)	480	6.15	0.03	3.74	8.37
ESh (µm)	480	1.54	0.00	1.00	2.11
NSh (µm)	480	1.59	0.01	0.79	2.41
ES (μm^2)	480	291.47	1.69	161.42	409.84
NS (μm^2)	480	46.83	0.31	25.31	74.41
NCSh (µm)	480	0.16	0.00	0.10	0.33

The first principal component explained 74.84 of total variance whereas the second component was loaded with 17.61 of total variance. In total, two principal components described 92.46% of total variance. Most of the variables showed positive loadings for PC1 more relevant to shape parameters of blood cells (Table 2).

Table 2. Principal component loadings, eigenvalues, and associated variances described by the first two components (PC1 and PC2) based on blood cell parameters

Variables	**PC1**	**PC2**
EL	0.206	-0.628
EW	0.367	0.120
NL	0.332	-0.362
NW	0.381	0.074
ESh	-0.305	-0.441
NSh	-0.284	-0.482
ES	0.366	-0.116
NS	0.380	-0.095
NCSh	0.337	-0.045
Eigenvalue	6.73	1.58
Variance (%)	74.84	17.61
Cumulative Variance (%)	74.84	92.46

The highest loadings were in ES, NS, EW and NW variables. However, the highest load was observed for blood cell length (EL) in PC2 and most of variables were negatively loaded (Figure 2). The species were not clearly distinguished in the morphospace based on blood cell parameters (Figure 3).

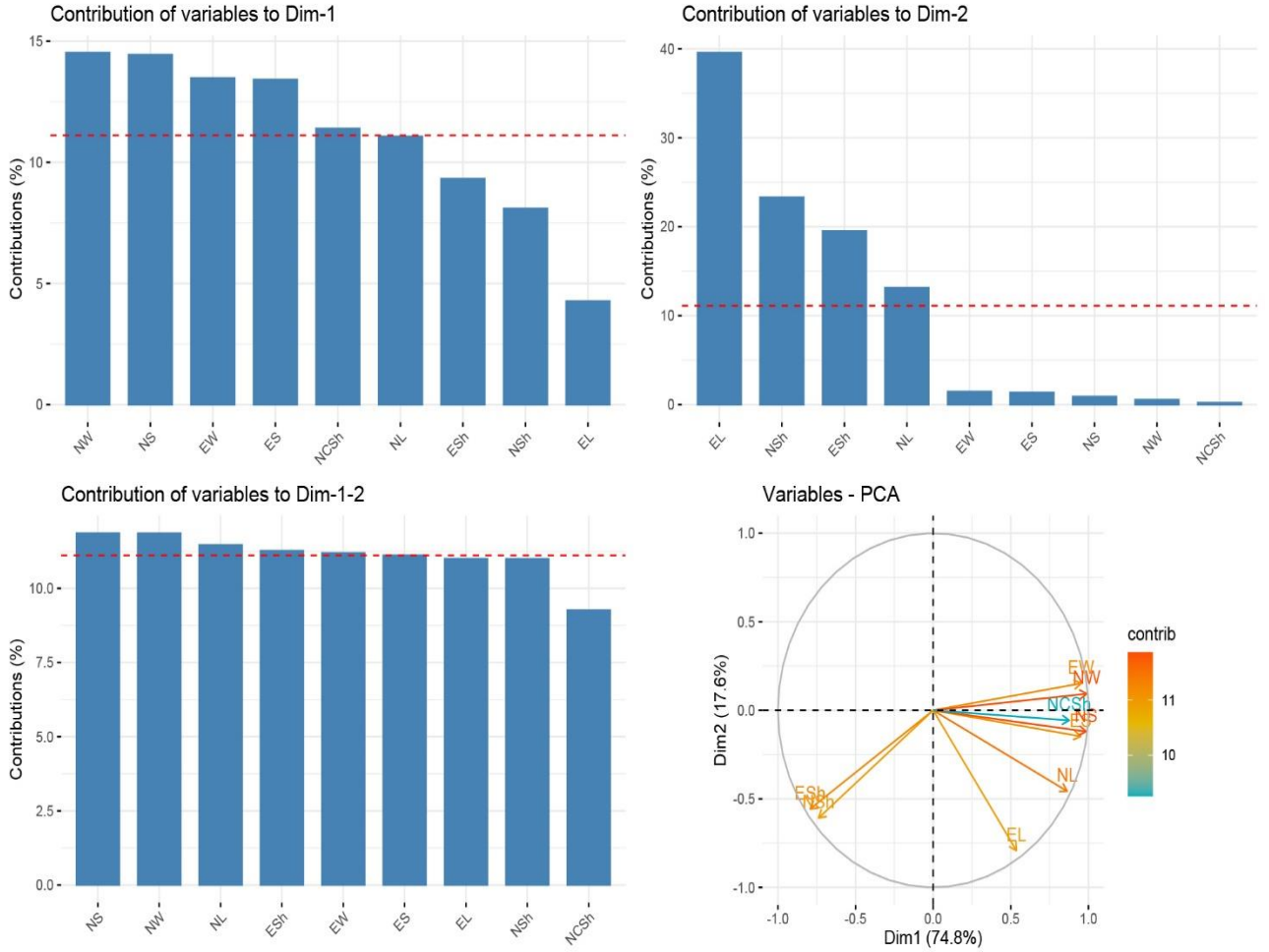

Figure 2. The contributions of each variable to the PC1, PC2 and cumulative PC1-PC2. Correlation circle demonstrates the relationship between blood cell parameters, and their relationship with the first two principal components

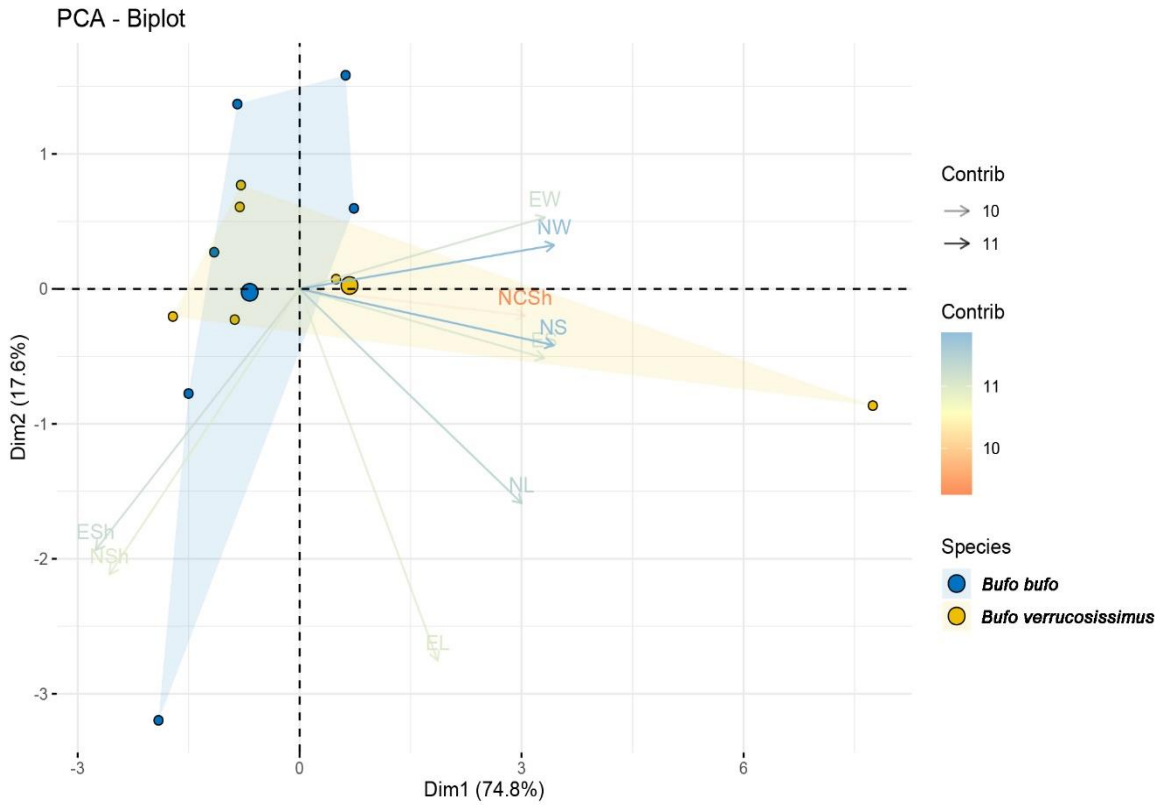

Figure 3. Principal component analysis of blood cell parameters based on species. Arrows indicate variable contributions

According to the correlation analysis of SVL and blood cell parameters, a significant negative correlation was found only between SVL and NSh (r = -0.61; p<0.05). Correlogram of all variables was demonstrated in Figure 4.

Figure 4. The matrix of scatter plots for visualizing the correlation between blood cell parameters and SVL (Bb: *Bufo bufo*; Bv: *Bufo verrucosissimus*)

4. Conclusions and discussion

In this study, the species were compared in terms of some blood parameters, and we contributed to the systematics of taxa regarding serological characters. Regardless of taxa, it was found that female individuals had larger values for measurement of blood cell parameters. The erythrocyte dimension was proportional to body size. Suljevic et al. [18] have investigated *Bufo bufo* in terms of general, seasonal, and sexual haematopoietic distribution and they reported that females had larger erythrocytes than males. Wei et al. [19] also handled erythrocyte morphology in some amphibians, and they indicated that the body size of organism is affecting erythrocyte dimension. From this aspect, we have found similar results in both taxa supported by literature data.

Our findings suggested mean erythrocyte and leucocyte numbers were higher in the blood of *B. bufo* than *B. verrucosissimus* but there was not a significant difference between species for EN and LeuN. The number of erythrocytes and leucocytes in amphibian blood demonstrates a wide range of interspecific variation regarding gender, age, season, and habitat conditions [20]. Considering the similarity of vegetation and altitude of Uzungöl and Karagöl [21, 22], sampling season as well as phylogenetic history of species [9, 10], we assume this observed situation is reasonable. On the other hand, it is asserted that terrestrial and aquatic anuran species have higher number of erythrocytes comparing to semi-aquatic species. For instance, Özgül et al. [23] indicated the mean erythrocyte number difference of *Pelophylax ridibundus* (mean: 119555.55) and *Bufotes variabilis* (mean: 186133.33) per 1 mm^3 blood samples. Similarly, Gül et al. [4] reported the number of erythrocytes in *Pseudepidalea viridis* (mean: 937666), *Pelobates syriacus* (mean: 765909) and *Hyla arborea* (mean: 733636) as terrestrial, *Pelophylax ridibundus* (mean: 886000) and *Rana dalmatina* (mean: 716660) as semi-aquatic. Although our results supported literature findings for *B. bufo*, the mean value of *B. verrucosissimus* was lower than referenced values. Therefore, this assertation is remained uncorrected with the recent data. However, Dönmez et al. [13] investigated haematological values in *B. bufo*, and they calculated number of erythrocyte 460000-920000 in females and 390000-900000 in males corresponding to range in our study. Moreover, Liu et al. [24] scored the number of erythrocytes and leucocytes between 443000-701000 and 23300-39400 for *B. gargarizans* in $1mm^3$ blood sample. In this study, number of erythrocytes and leucocytes were ranged between 640000-900000 and 18200-46800 for *B. bufo* (Uzungöl); between 410000-880000 and 20000-30000 for *B. verrucosissimus* (Karagöl). Regarding the comparison, it was observed that the average leukocyte and erythrocyte numbers of the three species were similar, but the number of erythrocytes of the *B. verrucosissimus* species was lower compared to the other two toads.

The measurement of erythrocyte cells became a subject of heamatological studies in *Bufo* taxa. Atatür et al. [13] handled erythrocyte sizes of some anurans from Türkiye, and they reported mean length, width, and size as 20,85 µm, 13,45 µm and 221,22 $µm^2$, respectively for *Bufo bufo* from Marmaris. In this study, we have found larger values in both taxa. This can be caused due to latitudinal and altitudinal differences because our sampling areas are located at the north and higher altitudes. Arıkan and Çiçek [15] also collected *B. bufo* samples from Marmaris (corresponding to *B. bufo*) and they recorded compatible values with Atatür et al. [12], but lower values than our study. As for other bufonid taxa, Xianguang et al. [25] studied on the blood cells of *Bufo gargarizans* in China, and they reported the mean erythrocyte length and width as 19.41 µm and 14.25 µm, respectively. Liu et al. [24] also assessed annual variation in peripheral blood cells in the same species and they reported erythrocyte length, width, and shape as 19.91-21.49 µm, 13.87- 15.47 µm and 1.38-1.46 µm; nucleus length, width, and shape as 8.29-10.69 µm, 5.43-6.5 µm, and 1.49-1.66 µm. These measurements were also lower than our samples. However, Wei et al. [19] handled evolution of erythrocyte morphology in amphibians, and they reported these mean length, width, and size as 28.17 µm, 20.18 µm and 447.56 $µm^2$. Accordingly, *B. gargarizans* species surpassing our measurements in both species.

In females, erythrocytes were larger and narrower in *B. verrucosissimus* than *B. bufo* species. Alike, nuclei were larger and narrower as observed in *B. verrucosissimus*, as observed in erythrocytes. However, *B. bufo* had larger and wider erythrocytes but larger and narrower nuclei in males. Given the differences between sexes, these characters thought as not diagnostic. Therefore, the other characters which are derived from these main measurements were also represented same situation. In addition, PCA analysis showed overlapped position of species in morphospace and supported the weak discrimination power of the characters.

To conclude, we presented serological comparison of two closely related taxa to literature for the first time. Our findings will contribute to the future studies as a reference source for basic blood parameters. New studies can deal with other peripheral blood cells such as granulocytes and their ratios between these species.

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