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Haematology of the Levant Green Frog, *Pelophylax bedriagae* (Amphibia: Ranidae) in southern Iran

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Abstract
In this study, 68 specimens of the Levant Green Frog, *Pelophylax bedriagae*, were collected from agricultural fields around Yasouj and Noor-Abad cities, from August 2018 to May 2019 in southern Iran to investigate the blood cell morphology. Blood specimens were taken from the heart, or abdominal and facial veins. Smears were dried in room temperature, fixed by methanol and stained using Giemsa method. Blood cells (erythrocytes, leucocytes, and thrombocytes) were counted and measured using light microscopy under an optical micrometer. Based on the findings of this study, erythrocytes from the Noor-Abad population has significantly greater values relative to Yasouj population in all of the six metric characters ($P < 0.05$). RBC's in male frogs are also greater than those in females in all six studied characters ($P < 0.05$). All studied metric characters in lymphocytes are also different between two localities and sexes ($P < 0.05$).

Key words: *RBC, Leucocyte, Yasouj, Noor-Abad, Haematology.*

INTRODUCTION

Amphibians comprise 8090 known species with a worldwide distribution except for Antarctica and some oceanic Islands (AmphibiaWeb, 2019). The Levant Green Frog *Pelophylax bedriagae* (Camerano, 1882) is one of the three known representatives of the family Ranidae in Iran (AmphibiaWeb, 2019). Phylogenetic relationships of water frogs in Iran have studied by Pesarakloo et al. (2017), demonstrating two distinct mtDNA clades. The first clade, *Pelophylax bedriagae*, is primarily found from northwestern to southwestern parts of Iran. The second clade, which is different from other water frogs in genetic composition, distributes in the northeastern part of Iran.

Blood analysis is a useful method to determine the health of animals as well as managing and treating diseases (Christopher *et al.*, 1999). Multicellular animals need a circulatory system to carry nutrients and oxygen to and/or excretions from cells. Regulating blood composition and volume at a constant level is necessary for the continuity of animal life (Arikan & Çiçek, 2014). There is an increasing tendency toward the hematology of anurans during the recent decade (Davis *et al.*, 2008; Shutler & Marcogliese, 2011; Baraquet *et al.*, 2013; Arikan & Çiçek, 2014). Amphibian blood cells comprise erythrocytes, leukocytes, and thrombocytes (Arikan & Çiçek, 2014). Frog's erythrocytes are oval, nucleated, and biconcave, while the leucocytes look like those found in human blood (Storer *et al.*, 1957). Amphibians show well-developed circulatory and immune systems (Manning & Horton, 1982). Erythrocyte durability and longevity in amphibians are longer than those in birds and mammals. *Bufo marinus* RBC's, for instance, remain in blood circulation for

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around 700-1400 days (Altland & Brace, 1962). Leucocyte quality and quantity are regarded as an important hematologic parameter and a determinant of immune response level against diseases (Tierney *et al.*, 2004), as when the number of eosinophils increases in blood parasite cases (Kiesecker, 2002). Leukocytes are divided into two groups based on the presence of granules in the cytoplasm and the shape of the nucleus: granulocytes (polymorphonuclear leukocytes) and agranulocytes (mononuclear leukocytes). Both of the two groups are spherical in blood plasma. Granulocytes comprise neutrophils, eosinophils, and basophils. The neutrophils are spherical cells with multiple nuclei connecting by thin threads of chromatin (Javanbakht *et al.*, 2013). The eosinophils are the same size as neutrophils, having a doublet nucleus with many large granules in cytoplasm stained well by eosin (Javanbakht *et al.*, 2013). The basophils contain a unilobed nucleus, cytoplasmic granules, but differ in size from species to species (Wright, 2001). The basophils are predominant leucocytes in some amphibian species (Thrall *et al.*, 2012). Agranulocytes lack specific granules, but azurophiles granules and round denticulate nucleus, comprising monocyte and lymphocytes. As predominant leukocytes, small and large lymphocytes present in the blood of many amphibian species (Thrall *et al.*, 2012). Small lymphocytes are almost filled with the nucleus. Confusing generally with each other, monocytes are similar morphologically to large lymphocytes. The former is recognized from the latter by having a bean-like nucleus (Arikan & Çiçek, 2014). All vertebrate classes, except for mammals, are characterized by nucleated erythrocytes. Unlike other vertebrates, the presence of a nucleus in mammalian erythrocytes is a pathologic symptom (Arserim & Mermer, 2008). Like all other blood cells in amphibians, thrombocytes are nucleated fusiform cells with the same function as mammalian platelets. Immature thrombocytes are spherical having a round nucleus and a more basophilic cytoplasm. These blood cells are generally found in clusters, making them more easily to be distinguished from small lymphocytes (Arıkan & Çiçek, 2014). Given the fact that the hematology of the Levant Green Frog (*Pelophyax bedriagae*) has not yet been investigated in the southern parts of Iran, we try in the current survey to study and report some primary morphological parameters of blood cells of this species.

MATERIAL AND METHODS

This study carried out in two different localities in southern parts of Iran: Noor-Abad Mamasani (51° 34′E, 30°13′N; 900 masl) and Yasouj city (51° 34′E, 30°40′N; 1810 masl) in Fars and Kohgilouyeh & Boyer-Ahmad Provinces, respectively (Fig. 1). We collected 68 *Pelophylax bedriagae* with uneven distribution between the two localities including 43 (22 males, 21 females) and 25 (nine males, 16 females) specimens from Yasouj and Noor-Abad, respectively. These frogs were measured from snout to vent (SVL) using a digital caliper with an accuracy of 0.01 mm. We used the presence of vocal sacs in males as a distinguishing character to determine the sex of each *Pelophylax bedriagae* specimen (Bamezar *et al.*, 2019). We took the blood from facial and abdominal veins as well as the heart in some specimens using insulin syringes. All the specimens were released immediately after blood taking. Two blood smears were prepared for each of the frogs, allowed to dry in ambient temperature for 10 minutes, fixed with absolute Methyl alcohol, and stained with the Giemsa method for 20 minutes. To study the morphology of blood cells, we analyzed ten different areas for each smear under 100X magnification using an Olympus CX31 microscope. Using an optical micrometer (BBT Krauss), four cellular characters measured in this study comprising cell length (CL), cell width (CW), nucleus length (NL), and nucleus width (NW) (Arıkan & Çiçek, 2014; Baraquet *et al*., 2013). To calculate the size of cells and nuclei, we used formulas $LW\pi/4$ for erythrocytes and $A = \pi r^2$ for leukocytes, respectively, in which: r = radius, L = length, and W = width (Arıkan & Çiçek, 2010; Javanbakht *et al*., 2013).

Statistical analyses carried out using Excel (2016) and SPSS 19. Normality tests were carried out to choose between the appropriate parametric (t-Test) or non-parametric (Mann-Whitney *U*) tests between two groups (both localities and sexes) at the significance level of 0.05.

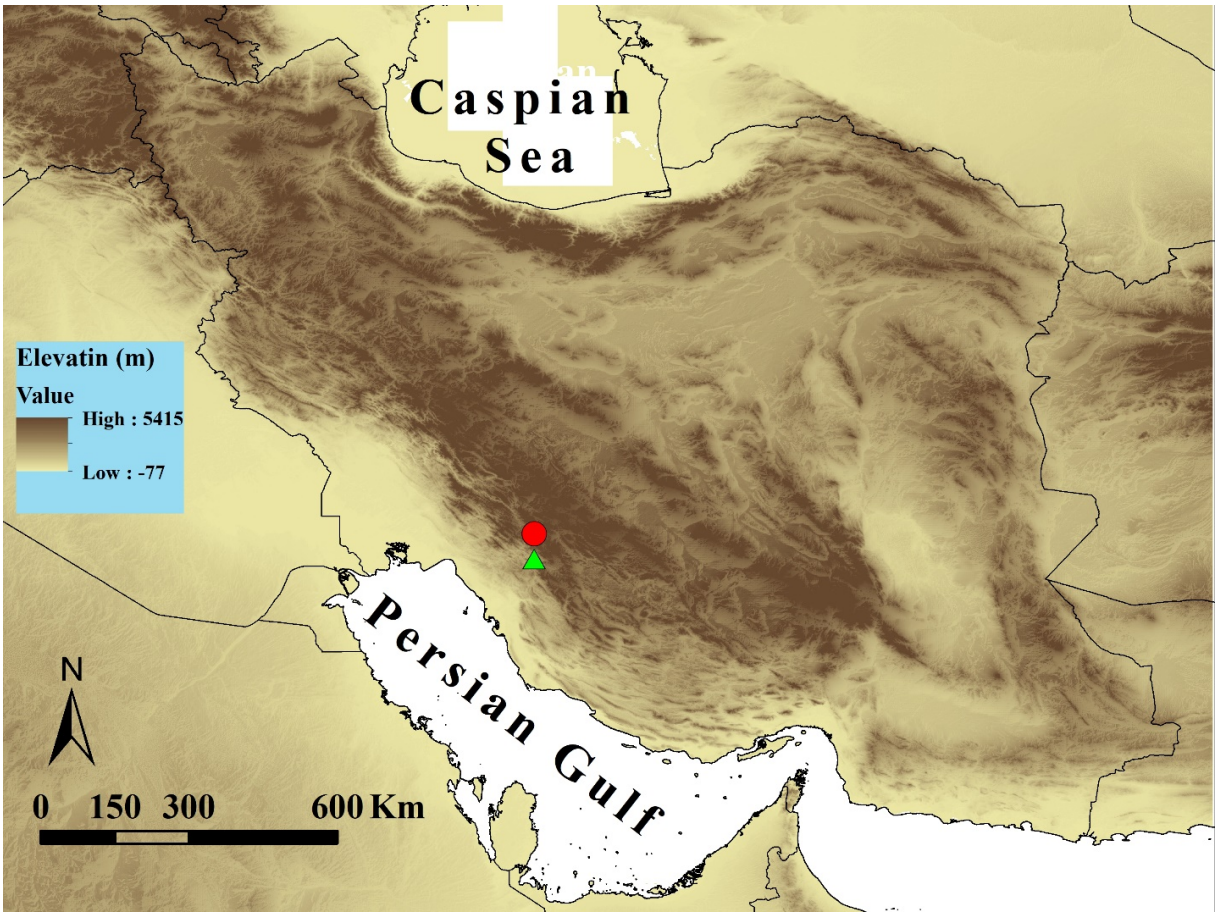

FIGURE 1. Position of collection sites of *Pelophylax bedriagae* in Noor-Abad Mamasani (green triangle) and Yasouj (red circle) in southern Iran.

RESULTS

Sex differences

The results of t-Test show that the mean value of SVL for females (71.51±11.53) is significantly larger than the males (62.02±7.24; P = 0.0001), while this value is not significantly different between two localities (i.e. Yasouj = 65.17±10.99 and Noor-Abad = 68.93±9.80) (P = 0.14).

Erythrocytes (RBCs, Fig. 2a)

A total number of 4032 RBCs were measured and analyzed in this study, of which 2533 cases were from Yasouj and 1499 from Noor-Abad regions, respectively. The values range from 17–28 µm (average = 22.37) for RBC length, 9–17 µm (average = 12.95) for RBC width, 6–12 µm (average = 8.96) for nucleus length, and 4–7 µm (average = 5.09) for nucleus width. All four characters showed non-normal distribution (Shapiro-Wilk test; P = 0.0001). The population from Noor-Ababd has significantly larger values than the Yasouj population for all the four characters (i.e., cell length, cell width, nucleus length, and nucleus width) (U-test; P = 0.0001; Table 1). Moreover, the Noor-Abad population has larger values in both cell and nucleus sizes than the Yasouj population (U-test; P = 0.0001; Table 1). Male frogs have greater values for the four RBS's characters as well as cell and nucleus sizes than female frogs (U-test; $P < 0.005$; Table 2).

Neutrophils (Fig. 2b)

Only two out of six characters (nucleus length and cell width) were normally distributed (Shapiro-Wilk test; $P > 0.05$). The smallest and largest size of neutrophils is 86.35 and 379.16 µm^2,

respectively with an average of 181.70±58.62 μm². There were no significant differences in all six characters between two sexes or localities (t-Test and U-test; P > 0.05; Tables 3-4).

Eosinophils (Fig. 2c)
Three out of six characters (i.e. nucleus length, cell length, and cell width) are normally distributed (Shapiro-Wilk test, P > 0.05). The average eosinophil size in both populations combined is 184.02±66.85 μm² ranging from 63.59 to 431.75 μm². Only one out of the six studied characters (i.e. cell size) are significantly different between Yasouj and Noor-Abad regions (*U*-test, *P* = 0.031; Table 5). Males and female frogs have the same values for all of the six studied characters (t-Test and U-test, *P* > 0.05; Table 6).

Basophils (Fig. 2d)
All of the three studied characters in basophils are normally distributed (Shapiro-Wilk test, P > 0.05). Basophil average size in both populations combined is 117.85±41.52 μm² ranging from 37.68 to 240.21 μm². There are no significant differences between the two groups (neither between two localities nor two genders) (t-Test, P > 0.05; Tables 7-8).

Lymphocytes (Fig. 2e)
All six relevant characters to lymphocytes show non-normal distribution (Shapiro-Wilk test, *P* = 0.0001). Lymphocyte average size in both populations combined is 126.17±56.84 μm² ranging from 38.47 to 373.66 μm². The lymphocyte nucleus average size is 75.19±30.54 ranging from 19.63 to 254.34 μm² (Table 9). All of the six studied characters are significantly different between to localities with larger values for Noor-Abad than Yasouj (U-test, P < 0.05; Table 9) as well as between two sexes with larger values for males than females (U-test, P < 0.05; Table 10).

Monocytes (Fig. 2f)
All six characters have a non-normal distribution (Shapiro-Wilk test; P < 0.05). Monocyte average size in both populations combined is 162.12±58.14 μm² ranging from 78.50 to 373.66 μm². The monocyte nucleus average size is 81.35±32.65 ranging from 28.26 to 200.96 μm² (Table 11). Only one out of the six studied characters (i.e. nucleus length) is significantly different between two localities with larger values for Noor-Abad than Yasouj (U-test, P = 0.033; Table 11) as well as between two sexes with larger values for males than females (U-test, P < 0.015; Table 12).

Thrombocytes (Fig. 2f)
All five characters studied in thrombocytes have a non-normal distribution (Shapiro-Wilk test; P < 0.05). Thrombocyte average size in both populations combined is 81.80±20.45 μm² ranging from 47.10 to 138.1 μm² (Table 13). Three out of the five studied characters (i.e. nucleus width, cell width, and cell size) are significantly different between two localities with larger values for Noor-Abad than Yasouj (U-test, P < 0.05; Table 13). Thrombocyte cell width and size in male *Pelophylax bedriagae* have significantly larger values than females (U-test, P < 0.05; Table 14).

TABLE 1. Descriptive statistics and significant analysis in erythrocytes of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min.	Max.	Range	Sig.	Test used
RBC N. Length	Yasouj	2533	8.69±0.96	5.00	13.00	8.00	0.0001	**U-Test**
	Noor-Abad	1499	9.40±1.01	5.00	13.00	8.00		
	Total	4032	8.90±1.14	5.00	13.00	8.00		
RBC N. Width	Yasouj	2533	4.96±0.74	4.00	8.00	4.00	0.0001	**U-Test**
	Noor-Abad	1499	5.33±0.70	4.00	8.00	4.00		
	Total	4032	5.10±0.75	4.00	8.00	4.00		
RBC Length	Yasouj	2533	21.70±1.91	15.00	29.00	14.00	0.0001	**U-Test**
	Noor-Abad	1499	23.50±2.14	17.00	33.00	16.00		
	Total	4032	22.38±2.20	15.00	33.00	18.00		
RBC Width	Yasouj	2533	12.55±1.31	8.00	19.00	11.00	0.0001	**U-Test**
	Noor-Abad	1499	13.63±1.54	9.00	19.00	10.00		
	Total	4032	12.95±1.49	8.00	19.00	11.00		
N. S.	Yasouj	2533	33.96±7.16	16.49	26.80	46.32	0.0001	**U-Test**
	Noor-Abad	1499	39.64±7.81	18.84	70.65	51.81		
	Total	4032	36.07±7.90	16.49	70.65	54.17		
RBC S.	Yasouj	2533	213.68±32.90	94.20	346.97	252.77	0.0001	**U-Test**
	Noor-Abad	1499	252.05±41.93	103.62	381.51	277.89		
	Total	4032	227.94±40.95	94.20	381.51	381.51		

TABLE 2. Descriptive statistics and significant analysis in erythrocytes of male and female *Pelophylax bedriagae* in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min.	Max.	Range	Sig.	Test used
RBC N. Length	Male	2213	9.05±1.09	5.00	13.00	8.00	0.006	**U-Test**
	Female	1819	8.84±0.97	5.00	12.00	7.00		
	Total	4032	8.96±1.04	5.00	13.00	8.00		
RBC N. Width	Male	2213	5.20±0.80	4.00	8.00	0.30	0.0001	**U-Test**
	Female	1819	4.97±0.65	4.00	8.00	0.30		
	Total	4032	5.10±0.75	4.00	8.00	0.30		
RBC Length	Male	2213	22.80±2.35	16.00	33.00	1.10	0.0001	**U-Test**
	Female	1819	21.87±1.88	15.00	29.00	1.10		
	Total	4032	22.38±2.20	15.00	33.00	1.10		
RBC Width	Male	2213	13.10±1.52	9.00	19.00	0.80	0.0001	**U-Test**
	Female	1819	12.78±1.44	8.00	19.00	0.80		
	Total	4032	12.95±1.49	8.00	19.00	11.00		
Nucleus Size	Male	2213	37.24±8.58	16.49	69.08	52.60	0.0001	**U-Test**
	Female	1819	34.66±6.71	16.49	70.65	54.17		
	Total	4032	36.07±7.90	16.49	70.65	54.17		
RBC Size	Male	2213	235.03±43.61	94.20	372.88	278.68	0.0001	**U-Test**
	Female	1819	219.32±35.63	103.62	381.51	227.89		
	Total	4032	227.94±40.95	94.20	381.51	287.31		

TABLE 3. Descriptive statistics and significant analysis in neutrophils of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Neutrophil N. Length	Yasouj	43	6.55±1.27	4.00	10.30	6.30	0.072	**T- test**
	Noor-Abad	19	7.13±1.32	4.50	9.70	5.20		
	Total	62	6.72±1.31	4.00	10.30	6.30		
Neutrophil N. Width	Yasouj	43	4.29±0.79	2.70	6.00	3.30	0.398	**U- test**
	Noor-Abad	19	4.47±0.87	2.30	6.30	4.00		
	Total	62	4.34±0.82	2.30	6.30	4.00		
Neutrophil Length	Yasouj	43	15.53±2.64	11.00	23.00	12.00	0.07	**U- test**
	Noor-Abad	19	16.89±2.49	14.00	22.00	8.00		
	Total	62	15.95±2.65	11.00	23.00	12.00		
Neutrophil Width	Yasouj	43	13.90±2.59	9.00	21.00	12.00	0.132	**T- test**
	Noor-Abad	19	14.94±2.14	11.00	19.00	8.00		
	Total	62	14.22±2.49	9.00	21.00	12.00		
Nucleus Size	Yasouj	43	24.44±10.26	9.62	68.38	58.76	0.11	**U- test**
	Noor-Abad	19	27.08±9.18	11.54	50.24	38.70		
	Total	62	25.25±9.94	9.62	68.38	58.76		
Neutrophil Size	Yasouj	43	174.59±59.11	86.55	379.94	293.39	0.051	**U- test**
	Noor-Abad	19	202.25±54.30	132.67	329.90	197.23		
	Total	62	183.07±58.66	86.55	379.94	293.39		

TABLE 4. Descriptive statistics and significant analysis in neutrophils of male and female *Pelophylax bedriagae* in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Neutrophil N. Length	Male	29	6.88±1.39	4.30	10.30	6.00	0.526	**T- test**
	Female	33	6.58±1.23	4.00	9.00	5.00		
	Total	62	6.72±1.31	4.00	10.30	6.30		
Neutrophil N. Width	Male	29	4.38±0.94	2.30	6.30	4.00	0.782	**U- test**
	Female	33	4.30±0.70	2.70	6.00	3.30		
	Total	62	4.34±0.82	2.30	6.30	4.00		
Neutrophil Length	Male	29	15.72±2.69	11.00	22.00	11.00	0.545	**U- test**
	Female	33	16.15±2.64	11.00	23.00	12.00		
	Total	62	15.95±2.65	11.00	22.00	12.00		
Neutrophil Width	Male	29	13.86±2.40	10.00	18.00	8.00	0.286	**T- test**
	Female	33	14.54±2.57	9.00	21.00	12.00		
	Total	62	14.22±2.49	9.00	21.00	12.00		
Nucleus Size	Male	29	25.27±9.19	11.54	50.24	38.70	0.918	**U- test**
	Female	33	25.23±10.70	9.62	68.38	58.76		
	Total	62	25.25±9.94	9.62	68.38	58.76		
Neutrophil Size	Male	29	175.70±53.00	86.55	283.39	196.84	0.456	**U- test**
	Female	33	189.54±63.32	86.55	379.94	293.39		
	Total	62	183.07±58.66	86.55	379.94	293.39		

TABLE 5. Descriptive statistics and significant analysis in eosinophils of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Eosinophil N. Length	Yasouj	38	9.21±1.78	5.50	13.00	7.50	0.748	**T- test**
	Noor-Abad	16	9.37±1.50	6.50	13.00	6.50		
	Total	54	9.26±1.70	5.50	13.00	7.50		
Eosinophil N. Width	Yasouj	38	5.41±1.14	3.50	9.00	5.50	0.946	**U- test**
	Noor-Abad	16	5.31±0.96	3.50	7.00	3.50		
	Total	54	5.37±1.08	3.50	9.00	5.50		
Eosinophil Length	Yasouj	38	15.47±3.28	9.00	25.00	16.00	0.107	**T- test**
	Noor-Abad	16	16.62±1.82	13.00	20.00	7.00		
	Total	54	15.81±2.95	9.00	25.00	16.00		
Eosinophil Width	Yasouj	38	14.02±2.75	9.00	22.00	13.00	0.09	**T- test**
	Noor-Abad	16	15.31±1.66	13.00	18.00	5.00		
	Total	54	14.40±2.53	9.00	22.00	13.00		
Nucleus Size	Yasouj	38	43.14±15.38	23.75	94.99	71.24	0.690	**U-test**
	Noor-Abad	16	42.89±10.07	23.75	67.17	43.42		
	Total	54	43.07±13.92	23.75	94.99	71.24		
Eosinophil Size	Yasouj	38	177.52.33±75.22	63.59	433.52	369.93	0.031	**U- test**
	Noor-Abad	16	201.92±39.06	132.67	268.67	136.00		
	Total	54	184.75±67.14	63.59	433.52	369.93		

TABLE 6. Descriptive statistics and significant analysis in eosinophils of male and female *Pelophylax bedriagae* in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min.	Max	Range	Sig.	Test used
Eosinophil N. Length	Male	20	8.75 ±1.62	6.50	12.00	5.50	0.091	**T- test**
	Female	34	9.55±1.68	5.50	13.00	7.50		
	Total	54	9.33±2.70	5.50	13.00	7.50		
Eosinophil N. Width	Male	20	5.42±1.06	3.50	7.00	3.50	0.536	**U- test**
	Female	34	5.32±1.11	3.50	9.00	5.50		
	Total	54	5.42±1.07	3.50	9.00	5.50		
Eosinophil Length	Male	20	15.25±2.75	9.00	19.00	10.00	0.286	**T- test**
	Female	34	16.14±3.06	12.00	25.00	13.00		
	Total	54	15.75±2.80	9.00	25.00	16.00		
Eosinophil Width	Male	20	14.00±2.42	9.00	18.00	9.00	0.370	**T- test**
	Female	34	14.64±2.60	10.00	22.00	12.00		
	Total	54	14.37±2.43	9.00	22.00	13.00		
Nucleus Size	Male	20	40.44±13.45	23.75	67.17	43.42	0.232	**U- test**
	Female	34	44.61±14.17	23.75	94.99	71.24		
	Total	54	43.07±13.92	23.75	94.99	71.24		
Eosinophil Size	Male	20	172.49±55.87	63.59	268.67	205.08	0.489	**U- test**
	Female	34	191.97±72.79	103.82	433.52	329.70		
	Total	54	184.75±67.14	63.59	433.52	369.93		

TABLE 7. Descriptive statistics and significant analysis in basophils of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in µm and size in µm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Basophil Length	Yasouj	41	13.41±2.94	8.00	21.00	13.00	0.414	**T- test**
	Noor-Abad	6	12.33±3.38	9.00	17.00	8.00		
	Total	47	13.27±2.99	8.00	21.00	13.00		
Basophil Width	Yasouj	41	11.09±2.25	6.00	17.00	11.00	0.945	**T- test**
	Noor-Abad	6	11.16±2.31	9.00	15.00	6.00		
	Total	47	11.10±2.23	6.00	17.00	11.00		
Basophil Size	Yasouj	41	121.5±41.95	38.47	240.41	201.94	0.714	**T- test**
	Noor-Abad	6	113.00±51.55	63.59	188.60	125.01		
	Total	47	120.4±42.75	38.47	240.41	201.94		

TABLE 8. Descriptive statistics and significant analysis in basophils of male and female *Pelophylax bedriagae* in southern Iran. Length and width in µm and size in µm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min.	Max.	Range	Sig.	Test used
Basophil Length	Male	14	12.28±2.75	8.00	17.00	9.00	0.141	**T- test**
	Female	33	13.69±3.02	8.00	21.00	13.00		
	Total	47	13.27±2.99	8.00	21.00	13.00		
Basophil Width	Male	14	10.57±2.24	8.00	15.00	7.00	0.291	**T- test**
	Female	33	11.33±2.23	6.00	17.00	11.00		
	Total	47	11.10±2.23	6.00	17.00	11.00		
Basophil Size	Male	14	106.31±42.34	50.24	188.60	138.36	0.149	**T- test**
	Female	33	126.38±42.13	38.47	240.41	201.94		
	Total	47	120.40±42.75	38.47	240.41	201.94		

TABLE 9. Descriptive statistics and significant analysis in lymphocytes of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in µm and size in µm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Lymphocyte N. Length	Yasouj	406	10.33±2.38	6.00	19.00	13.00	0.007	**U- test**
	Noor-Abad	298	10.75±2.19	5.00	19.00	14.00		
	Total	704	10.51±2.31	5.00	19.00	14.00		
Lymphocyte N. Width	Yasouj	406	8.51±1.92	4.00	17.00	13.00	0.014	**U- test**
	Noor-Abad	298	8.89±1.90	5.00	17.00	12.00		
	Total	704	8.67±1.92	4.00	17.00	13.00		
Lymphocyte Length	Yasouj	406	13.01±3.14	4.00	28.00	24.00	0.0001	**U-Test**
	Noor-Abad	298	14.36±3.82	7.00	27.00	20.00		
	Total	704	13.60±3.51	4.00	28.00	24.00		
Lymphocyte Width	Yasouj	406	11.02±2.38	6.00	21.00	15.00	0.0001	**U- test**
	Noor-Abad	298	11.78±2.62	4.00	19.00	15.00		
	Total	704	11.35±2.51	4.00	21.00	17.00		
Nucleus Size	Yasouj	406	72.74±30.78	8.55	226.87	218.32	0.007	**U- test**
	Noor-Abad	298	78.45±30.07	19.63	254.34	234.71		
	Total	704	75.16±30.59	8.55	254.34	254.79		
Lymphocyte Size	Yasouj	406	118.94±51.92	38.47	397.41	358.94	0.0001	**U- test**
	Noor-Abad	298	141.23±64.81	38.47	362.87	324.40		
	Total	704	128.38±58.73	38.47	397.41	358.94		

TABLE 10. Descriptive statistics and significant analysis in lymphocytes of male and female *Pelophylax bedriagae* in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Lymphocyte N. Length	Male	385	10.78±2.24	5.00	19.00	14.00	0.0001	**U-Test**
	Female	319	10.19±2.37	6.00	19.00	13.00		
	Total	704	10.51±2.32	5.00	19.00	14.00		
Lymphocyte N. Width	Male	385	8.80±1.92	4.00	17.00	13.00	0.041	**U-Test**
	Female	319	8.52±1.92	5.00	17.00	12.00		
	Total	704	8.67±1.92	4.00	17.00	13.00		
Lymphocyte Length	Male	385	14.26±3.70	7.00	28.00	21.00	0.0001	**U-Test**
	Female	319	12.84±3.05	7.00	23.00	16.00		
	Total	704	13.62±3.49	7.00	28.00	21.00		
Lymphocyte Width	Male	385	11.54±2.47	4.00	19.00	15.00	0.012	**U-Test**
	Female	319	11.13±2.56	6.00	21.00	15.00		
	Total	704	11.35±2.52	4.00	21.00	17.00		
Nucleus Size	Male	385	77.98±29.58	19.63	254.34	234.71	0.001	**U-Test**
	Female	319	71.82±31.38	23.75	226.87	203.12		
	Total	704	75.19±30.54	19.63	254.34	234.71		
Lymphocyte Size	Male	385	136.72±60.50	38.47	397.41	358.94	0.0001	**U-Test**
	Female	319	118.31±54.94	38.47	362.87	324.40		
	Total	704	128.38±58.73	38.47	397.41	358.94		

TABLE 11. Descriptive statistics and significant analysis in monocytes of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Monocyte N. Length	Yasouj	36	11.86±2.72	8.00	20.00	12.00	0.033	**U- test**
	Noor-Abad	18	13.50±3.24	9.00	24.00	15.00		
	Total	54	12.41±2.97	8.00	24.00	16.00		
Monocyte N. Width	Yasouj	36	7.86±2.26	3.00	12.00	9.00	0.282	**U- test**
	Noor-Abad	18	7.00±2.52	3.00	11.00	8.00		
	Total	54	7.57±2.36	3.00	12.00	9.00		
Monocyte Length	Yasouj	36	15.81±3.45	11.00	28.00	17.00	0.321	**U- test**
	Noor-Abad	18	14.44±2.18	10.00	18.00	8.00		
	Total	54	15.35±3.13	10.00	28.00	18.00		
Monocyte Width	Yasouj	36	13.31±2.27	10.00	19.00	9.00	0.486	**U- test**
	Noor-Abad	18	12.83±2.18	10.00	18.00	8.00		
	Total	54	13.15±2.23	10.00	19.00	9.00		
Nucleus Size	Yasouj	36	79.62±34.15	28.26	200.96	172.70	0.586	**U- test**
	Noor-Abad	18	84.82±30.05	38.47	165.05	126.58		
	Total	54	81.35±32.65	28.26	200.96	172.70		
Monocyte Size	Yasouj	36	171.53±66.07	94.99	397.41	302.42	0.314	**U- test**
	Noor-Abad	18	149.16±44.70	78.50	254.34	175.84		
	Total	54	164.07±60.31	78.50	397.41	318.91		

TABLE 12. Descriptive statistics and significant analysis in monocytes of male and female *Pelophylax bedriagae* in southern Iran. Length and width in µm and size in µm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Monocyte N. Length	Male	21	13.57±3.54	8.00	24.00	16.00	0.015	**U- test**
	Female	33	11.67±2.31	8.00	18.00	10.00		
	Total	54	12.41±2.97	8.00	24.00	16.00		
Monocyte N. Width	Male	21	7.24±2.19	4.00	12.00	8.00	0.324	**U- test**
	Female	33	7.79±2.47	3.00	12.00	9.00		
	Total	54	7.57±2.36	3.00	12.00	9.00		
Monocyte Length	Male	21	16.19±3.60	11.00	28.00	17.00	0.073	**U- test**
	Female	33	14.82±2.71	10.00	22.00	12.00		
	Total	54	15.35±3.13	10.00	28.00	18.00		
Monocyte Width	Male	21	13.78±2.27	10.00	18.00	8.00	0.327	**U- test**
	Female	33	12.94±2.21	10.00	19.00	9.00		
	Total	54	13.15±2.23	10.00	19.00	9.00		
Nucleus Size	Male	21	8.75±39.38	28.26	200.96	172.70	0.358	**U-test**
	Female	33	76.64±27.15	28.26	165.05	136.79		
	Total	54	81.35±32.65	28.26	200.96	172.70		
Monocyte Size	Male	21	178.02±68.23	94.99	397.41	302.42	0.141	**U- test**
	Female	33	155.20±53.90	78.50	298.50	220.00		
	Total	54	164.01±60.31	78.50	397.41	318.91		

TABLE 13. Descriptive statistics and significant analysis in thrombocytes of *Pelophylax bedriagae* between Yasouj and Noor-Abad regions in southern Iran. Length and width in µm and size in µm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Locality	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Thrombocyte N.Length	Yasouj	173	11.50±1.83	7.00	16.00	9.00	0.065	**U- test**
	Noor-Abad	42	12.09±1.60	10.00	15.00	5.00		
	Total	215	11.62±1.80	7.00	16.00	9.00		
Thrombocyte N.Width	Yasouj	173	5.25±0.92	4.00	7.00	3.00	0.0001	**U- test**
	Noor-Abad	42	6.09±0.84	5.00	8.00	3.00		
	Total	215	5.41±0.96	4.00	8.00	4.00		
Thrombocyte Length	Yasouj	173	15.77±2.89	10.00	22.00	12.00	0.615	**U- test**
	Noor-Abad	42	15.47±2.17	12.00	22.00	10.00		
	Total	215	15.71±2.76	10.00	22.00	12.00		
Thrombocyte Width	Yasouj	173	6.41±1.00	5.00	8.00	3.00	0.0001	**U- test**
	Noor-Abad	42	7.42±0.63	6.00	8.00	2.00		
	Total	215	6.61±1.02	5.00	8.00	3.00		
Thrombocyte Size	Yasouj	173	79.69±20.83	47.10	138.10	91.06	0.001	**U- test**
	Noor-Abad	42	90.46±16.37	65.94	138.10	72.22		
	Total	215	81.80±20.45	47.10	138.10	91.06		

TABLE 14. Descriptive statistics and significant analysis in thrombocytes of male and female *Pelophylax bedriagae* in southern Iran. Length and width in μm and size in μm². Abbreviations: N., nucleus; No., number; Max., maximum; Min., minimum; SD, standard deviation; Sig., significance.

Characters	Sex	No.	Mean±SD	Min	Max	Range	Sig.	Test used
Thrombocyte N. Length	Male	103	11.77±1.89	8.00	15.00	7.00	0.261	**U- test**
	Female	112	11.48±1.71	7.00	16.00	9.00		
	Total	215	11.62±1.80	7.00	16.00	9.00		
Thrombocyte N. Width	Male	103	5.52±0.95	4.00	8.00	4.00	0.184	**U- test**
	Female	112	5.32±0.96	4.00	7.00	3.00		
	Total	215	5.41±0.96	4.00	8.00	4.00		
Thrombocyte Length	Male	103	15.99±2.98	10.00	22.00	12.00	0.150	**U- test**
	Female	112	15.46±2.53	10.00	22.00	12.00		
	Total	215	15.71±2.76	10.00	22.00	12.00		
Thrombocyte Width	Male	103	6.82±1.00	5.00	8.00	3.00	0.004	**U- test**
	Female	112	6.41±1.01	5.00	8.00	3.00		
	Total	215	6.61±1.02	5.00	8.00	3.00		
Thrombocyte Size	Male	103	85.97±21.60	47.10	138.16	91.06	0.007	**U- test**
	Female	112	77.96±18.62	47.10	138.16	91.06		
	Total	215	81.80±20.45	47.10	138.16	91.06		

DISCUSSION

Stained with Giemsa, erythrocytes show a blue homogenous cytoplasm. Adult erythrocytes have a basophilic nucleus (Javanbakht *et al.*, 2013). Carrying O2 and CO_2 is one of the most important functions performing by RBCs. The ratio of erythrocyte surface area to size is regarded as a determining factor in tissues, with a higher rate of gas and nutrient exchange for smaller erythrocytes than larger ones. Erythrocyte size reflects the position of a species on an evolutionary scale. For instance, lower vertebrates and animal groups, which do not show a success evolutionary past (i.e. Cyclostomes, Elasmobranches, and Urodela), possess larger erythrocytes unlike smaller enucleated erythrocytes in the higher vertebrates such as mammals (Hartman & Lessler, 1964; Szarski & Czopek, 1966; Saint Girons, 1970). Noor-Abad population lies at a lower altitude than the Yasouj population (900 vs. 1810 masl). The results of this study show that the size of erythrocytes between two localities is significantly different, with larger average values in all six studied characters for the Noor-Abad than the Yasouj population. Some other studies have shown the blood cell size differences between different populations of the same species inhabiting different altitudes. For instance, the size of erythrocytes and their nuclei in *Hypsiboas cordobae* decreased significantly with increasing altitude (Baraquet *et al.*, 2013). The smaller but higher number of erythrocytes improve the uptake of oxygen; finally adapt the organism to the environments having low oxygen pressures (Hutchison *et al*., 1976). Due to higher ratio of surface area to volume and subsequently more efficiency in gas exchanges for smaller erythrocytes (Ruiz *et al*. 1983; Arıkan, 1989; Weber, 2007), possessing smaller erythrocytes might be regarded as an advantage for frogs inhabiting higher altitudes with lower oxygen pressure (like Yasouj population in this study) than lower altitudes with higher oxygen pressure (i.e. Noor-Abad population), explaining size differences between the two different localities. The same negative correlation between erythrocyte size and altitude has been reported in other amphibians (Ruiz *et al*. 1983; Arıkan, 1989; Weber, 2007; Baraquet *et al.*, 2013). Like other amphibians, frogs have oval erythrocytes encompassing an oval central nucleus. There is a negative correlation between the rate of metabolism and erythrocyte size, in that, more active species have smaller erythrocytes while the ones with less oxygen consumption have larger erythrocytes (Smith, 1925; Evans, 1939; Vernberg, 1955; Monnickendam & Balls, 1973).

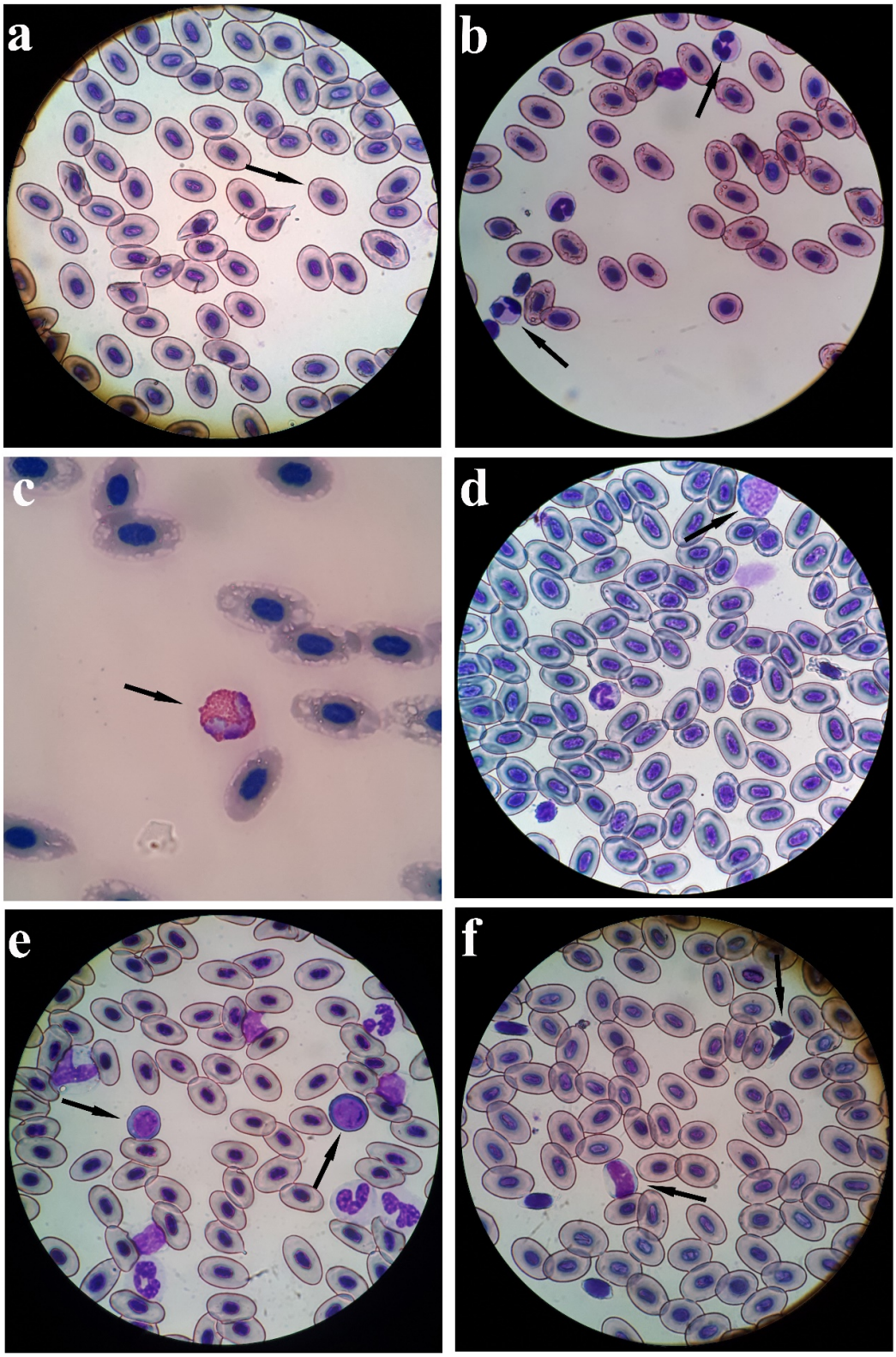

FIGURE 2. Blood cells in the peripheral circulatory system of *Pelophylax bedriagae*, including erythrocytes (a), neutrophils (b), eosinophil (c), basophil (d), lymphocytes (e), monocyte (f; lower arrow) and thrombocytes (f, upper arrow), under 100X magnification for (c) and 40X for the remaining visual fields.

Male-biased differences were also observed in all six characters studied in erythrocytes. The largest and most oval erythrocytes in anuran species have previously reported for aquatic *Pelophylax caralitanus*, while the smallest ones have reported in the terrestrial *Pelodytes caucasicus* (Atatür *et al.*, 1999; Arıkan *et al.*, 2001; Arikan *et al.*, 2003; Dönmez *et al.*, 2009; Erişmiş *et al.*, 2010; Arikan & Çiçek, 2014). The Farmed Bullfrog, *Lithobates catesbeianus*, have been shown to have larger erythrocyte morphometric parameters (i.e., surface area, the volume of RBCs and their nuclei) during active than hibernating period (Peng *et al*., 2016). The erythrocyte size influenced by numerous factors including size of the animals (Vernberg, 1955), environmental conditions like temperature and atmospheric pressure (Ruiz *et al.*, 1983, 1989), and other conditions such as health status, reproduction, hibernation, feeding, and daily activities (Wojtaszek *et al.*, 1997; Allender & Fry, 2008; Thrall *et al.*, 2012).

Leukocytes are specialized blood cells that act primarily in specific and unspecific immunological responses (Iwama and Nakanishi, 1996). Leukocyte numbers differ significantly between active and hibernating seasons in the Farmed Bullfrog, *Lithobates catesbeianus* (Peng *et al*., 2016). Several factors determine the levels of leukocyte in the whole blood including environmental quality (LeaMaster *et al*., 1990), nutritional state (Barros *et al*., 2002), the presence of infectious agents (Martins *et al*., 2008), and parasitism (Martins *et al*., 2004; Arıkan, 1989). Parasitic infections, for example, increase the number of monocytes and other leukocytes (Tavares-Dias *et al.*, 2007). Analyses of the leukocytes in this study indicated that six characters of lymphocytes are significantly different between two localities as well as sexes. Only one out of six characters (nucleus length) in monocytes is different between the two localities and sexes, while the remaining characters of monocytes and all the characters in other leukocytes are the same between localities and sexes. This study showed that lymphocytes are the most abundant leukocytes in the peripheral blood circulation of *Pelophylax bedriagae* which is consistent with the results of other studies conducted on anurans, say, Farmed Bullfrog, *Lithobates catesbeianus* (Peng *et al*., 2016), Dubio's Tree Frog, *Polypedates teraiensis* (Das & Mahapatra, 2014), American Bullfrog *Rana catesbeiana* (Cathers *et al*., 1997), and various species of frogs in Turkey (Arıkan & Çiçek, 2010), suggesting the primary role of immunological responses for lymphocytes among other leukocytes (Peng *et al*., 2016). On the other hand, basophils have the smallest number of leukocytes in *Pelophylax bedriagae*, which is accordant with Dubio's Tree Frog *Polypedates teraiensis* (Das & Mahapatra, 2014) and Farmed Bullfrog *Lithobates catesbeianus* (Peng *et al*., 2016). The basophil abundance in peripheral blood circulation depends on some factors such as species, season, geographic location, age of the animal, or probably blood parasites or viral infections (Vasaruchapong *et al*., 2014). Like other studies (Claver & Quaglia, 2009; Arıkan & Çiçek, 2010), a high concentration of granules in eosinophils and basophils, prevented us to observe and investigate nuclei of the two leukocytes.

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