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Original scientific paper

ERYTHROCYTE PROFILES OF *RANA DALMATINA* AND *PELOPHYLAX RIDIBUNDUS* (AMPHIBIA: RANIDAE)

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Abstract

The research on the erythrocyte profile was conducted on a total of twenty-eight frog individuals, out of which fourteen were *Pelophylax ridibundus* and also fourteen were *Rana dalmatina*. The aim of this study was to determine the erythrocyte parameters in the two frog species mentioned, in order to compare the obtained results. The individuals used in the study were captured in the area of Mrkonjić Grad municipality near Lake Balkana. After capture, all individuals were transported to laboratory conditions where they underwent a 15-day adaptation period. Following the adaptation period, blood samples were collected, and erythrocyte parameters were determined, including the number of erythrocytes, hemoglobin concentration, hematocrit and MCV, MCH and MCHC parameters. Based on the obtained results, the study showed that the *Pelophylax ridibundus* species had significantly higher values of erythrocyte count, hemoglobin concentration and MCHC compared to the values obtained in the *Rana dalmatina*.

Key words: frogs, erythrocytes, hemoglobin, hematocrit, hematological indices

INTRODUCTION

The Ranidae family includes frogs with smooth skin and long legs used for jumping. Their well-developed hind limbs have webbed feet for swimming. They lack parotid glands, and their upper jaw is toothed (Šorić, 1997). Species of the *Pelophylax* and *Rana* genera inhabit almost the entire continent of Europe, except for its far northern regions. They are also found in North Africa and parts of southwestern Asia, extending to Pakistan and Tajikistan (Nicolas, 2003; Prokić, 2016). *Pelophylax ridibundus* (marsh frog) inhabits mixed and deciduous forests, forest steppes, steppes, semi-arid and arid regions. It prefers open, well-sunlit habitats with abundant vegetation. As a semi-aquatic species, it also occupies a wide range of flowing and stagnant waters, from shallow ponds, puddles, and all kinds of artificial water ecosystems to large rivers and lakes (Kuzmin *et al.,* 2009). *Rana dalmatina* (agile frog) is widely distributed in Europe, primarily inhabiting thermophilic deciduous forests, and the population density within its range varies significantly. In the Balkan Peninsula, it can be found at altitudes of up to 1700 m (Kaya *et al.,* 2009).

Understanding that the hematological characteristics of animals represent an important, efficient, and sensitive tool that can be used to monitor physiological and pathological changes, as well as to indirectly monitor changes in the environment, is crucial. Amphibian populations worldwide are known to be decreasing since the 1960s (Houlahan *et al*., 2000). One of the main reasons for this is anthropogenic pollution (Whittaker *et al*., 2013). Anthropogenic pollution occurs as a result of human activities such as agricultural and industrial activities (Thammachoti *et al*., 2012). Frogs, due to their life characteristics (complex life cycle, semi-permeable skin, presence in both aquatic and terrestrial environments), are potentially good bioindicators (Garg and Hippargi, 2007; Prokić, 2016). It is known that hematological parameter values in amphibians show variations when comparing different species (Arikan *et al*., 2010; Baraquet *et al*., 2013), and these differences are significantly influenced by other factors such as environmental conditions, age, gender and more. Also, hematological dynamics may reflect early and late effects of environmental stressors to which individuals were exposed at early life stages (Ceccato *et al*., 2016).

The number of erythrocytes in the blood of amphibians varies significantly between different species and among individuals within the same species (Hutchinson and Szarski, 1965). These differences depend on body mass, age, and gender (Arvy, 1947; Goniakowska, 1973; Sinha, 1983; Choubey, 1986; Banerjee, 1988), environmental conditions (Ruiz, 1983) and season (Zhukova and Kubantsev, 1979; Sinha, 1983; Samantaray, 1985; Wojtaszek *et al*., 1997).

Hematological studies conducted on various species of the *Rana* genus have primarily aimed to determine the blood cell counts (Alder and Huber, 1923; Klieneberger, 1927; Arvy, 1947; Kaplan, 1951, 1952; Schermer, 1954; Stephan, 1954; Hutchinson and Szarski, 1965; Arıkan, 1989) and to determine erythrocyte morphometric parameters (Atatür *et al*., 1999; Arıkan *et al*., 2001).

In previous studies (Gül *et al*., 2011), it has been reported that among the five anuran species with different habitat choice, the number of erythrocytes is higher in terrestrial (*Bufotes variabilis*) and aquatic (*Pelophylax ridibundus*) species when compared to the semi-aquatic (*Rana dalmatina*) species.

The aim of this study was to determine the erythrocyte parameters in two frog species: *Rana dalmatina* and *Pelophylax ridibundus*.

MATERIALS AND METHODS

The research on the erythrocyte profile was conducted on a total of twenty-eight individuals from the two species of the Ranidae family. Out of the total, fourteen individuals were identified as the *Pelophylax ridibundus*, while the fourteen individuals were identified as *Rana dalmatina*.

Sampling. The individuals used in the study were captured in the vicinity of Mrkonjić Grad, in the area between the natural Balkana Lake and the Zelenkovac excursion site, during the period between April 28, 2010 and May 7, 2010. Frogs were captured using nets and the immediate identification of the individuals was performed in the field after capture. The captured frogs were then transported to the laboratory at the Faculty of Natural Sciences and Mathematics, where they underwent an adaptation period. The adaptation to laboratory

conditions lasted for 15 days, during which daily water changes were carried out in the containers housing individuals of both studied species. After the adaptation period, blood samples were collected, and erythrocyte parameters were determined.

Hematological analysis. Blood for hematological analyses was obtained by direct puncture of the heart using a sharp and wide sterile needle (1.0 to 1.2 mm) following sterile procedures. Native blood without the addition of anticoagulant was used for further analysis. The following parameters of the erythrocyte profile were determined in the collected blood samples: the number of erythrocytes (RBC), hemoglobin concentration (Hb), hematocrit (Hct) and hematological indices (MCV, MCH and MCHC).

The number of erythrocytes was determined by counting in a chamber (hemocytometer) using the Kekić and Ivanc method (1982), while the determination of hemoglobin concentration (Hb) was carried out using the Drabkin's hemoglobin cyanide method (Blaxhall and Daisly, 1973). Hematocrit (Hct) was determined by centrifugation using a microhematocrit centrifuge, and the hematological indices were calculated based on the values of hematocrit, the number of erythrocytes and hemoglobin concentration.

Mean corpuscular volume (MCV):

$$MCV = \frac{Hct}{\text{RBC/l}}$$

Mean corpuscular hemoglobin (MCH):

$$MCH = \frac{Hb/l}{\text{RBC/l}}$$

Mean corpuscular hemoglobin concentration (MCHC):

$$MCHC = \frac{Hb/l}{Hct}$$

Statistical analysis. In the analysis of research results, both descriptive and analytical statistics were used, and the data analysis and processing itself was conducted using the statistical software Microsoft Excel 2007 and GraphPad Prism 9. The following statistical parameters were determined: medium value, standard deviation, minimum value, maximum value and coefficient of variation. The data were interpreted considering the significance level as $p<0.05$.

RESULTS AND DISCUSSION

Erythrocyte parameters were studied in a total of fourteen individuals of *Pelophylax ridibundus* and fourteen individuals of *Rana dalmatina*. The results of the hematological analyses, along with statistical data processing, are presented in Table 1.

Table 1. Erythrocyte parameters of *Pelophylax ridibundus* and *Rana dalmatina*

	RBC (x 10^{12}/l)	**Hb (g/l)**	**Hct (l/l)**	**MCV (fl)**	**MCH (pg)**	**MCHC (g/l erythrocytes)**
Pelophylax ridibundus						

Medium value	0.929	56.98	0.362	455.865	67.451	169.695
Standard deviation	0.280	16.32	0.111	178.856	18.327	62.401
Minimal value	0.600	33.33	0.208	232.867	34.821	64.057
Maximum value	1.430	81.48	0.636	748.333	86.877	267.915
Coefficient of variation	30.14	28.64	30.663	39.234	27.171	36.772
Rana dalmatina						
Medium value	0.647	36.70	0.301	462.156	56.840	121.981
Standard deviation	0.165	10.66	0.083	126.497	16.702	29.655
Minimal value	0.370	22.22	0.214	304.878	30.869	77.868
Maximum value	0.870	62.96	0.471	672.857	89.943	162.960
Coefficient of variation	25.502	29.05	27.575	27.371	29.384	24.311

By comparing erythrocyte profile parameters in the examined frog species, significant differences in the values of individual erythrocyte parameters were demonstrated. Based on all the obtained research results, it was shown that all the erythrocytes parameters, except for the MCV index, exhibited higher values in individuals of the *Pelophylax ridibundus* species.

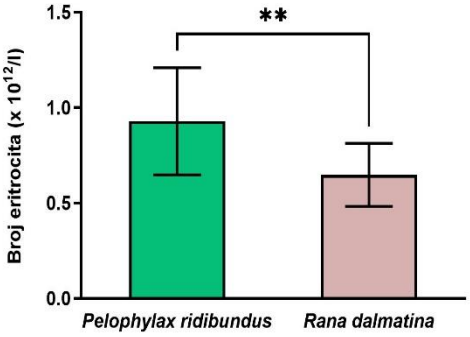


Figure 1. Number of erythrocytes

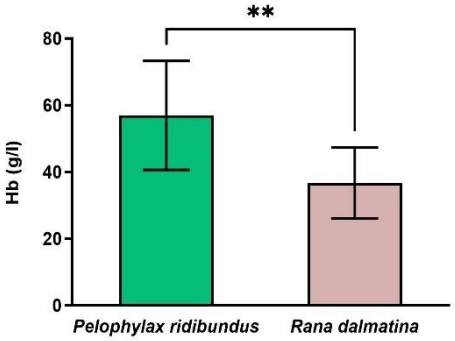
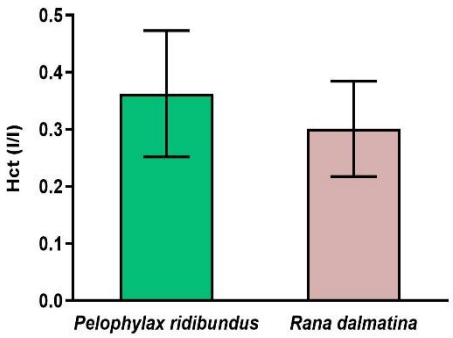


Figure 2. Hemoglobin concentration



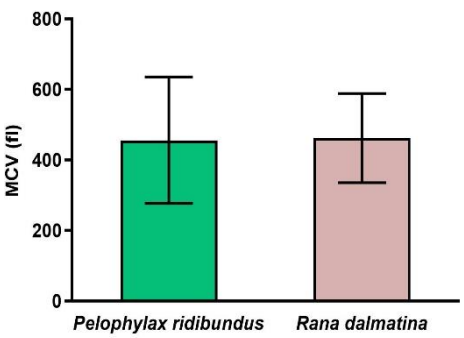
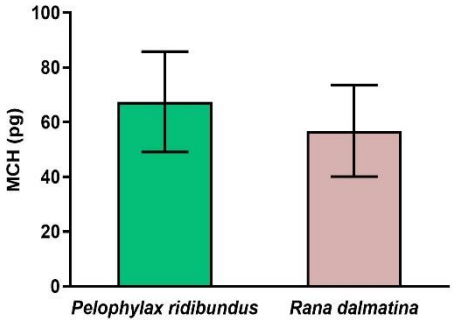


Figure 3. Hematocrit values

Figure 4. MCV values



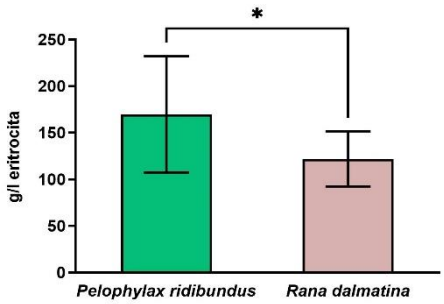


Figure 5. MCH values

Figure 6. MCHC values

Medium value of the erythrocyte count for *Pelophylax ridibundus* was 0.929 x 10^{12}/l, while the medium erythrocyte count for *Rana dalmatina* was 0.647 x 10^{12}/l (Figure 1). By means of statistical comparison, a significant statistical difference was shown between these two values, with a p-value of p=0.019. *Pelophylax ridibundus* also had significantly higher hemoglobin concentration values (p=0.002) (Figure 2) and MCHC parameter values (p=0.031) (Figure 6). Relevant statistical analyses of the remaining examined erythrocyte profile parameters in the two studied species did not reveal a statistically significant difference (Figures 3, 4 and 5).

Hematological parameter values in amphibians generally vary widely depending on the species, location, environmental conditions and so on. These data are in line with other similar studies, as Glomski *et al*. (1997) reported that the erythrocyte count in amphibian blood can range from 0.500 x 10^{12}/l to 1.500 x 10^{12}/l.

The highest values were recorded in *Rana cyanophlyctis* (2.060 x 10^{12}/l) and *Rana tigerina* (1.850 x 10^{12}/l) during the monsoon season (Samantaray, 1985).

Alder and Huber (1923) reported erythrocyte count values for *Rana temporaria* (0.408 x 10^{12}/l) and *Rana esculenta* (0.800 x 10^{12}/l), while in the population of *Pelophylax ridibundus*, it was found that erythrocyte count values ranged from 0.180 x 10^{12}/l to 0.590 x 10^{12}/l (Arikan, 1989).

Arserim and Mermer (2008) state that the medium erythrocyte count in *Rana macrocnemis* is 0.514 x 10^{12}/l, with variations ranging from 0.280 x 10^{12}/l to 0.940 x 10^{12}/l. Minor differences were also observed between genders, but they are negligible. The same authors (Arserim and Mermer, 2008) investigated claims that the erythrocyte count in *Rana macrocnemis* is higher in spring compared to summer and autumn. These authors demonstrated that variations exist between genders as well, with females having a higher erythrocyte count, also in the spring. The increased erythrocyte count in spring is likely associated with the elevated metabolism and activity after winter dormancy.

In amphibians, prior to the breeding season, the erythrocyte count significantly increases, simultaneously reflecting an increased demand for oxygen within the organism (Arikan *et al*., 2001). In addition to the mentioned variations, changes in erythrocyte counts can also be influenced by geographic variations (Hutchison and Szarski, 1965; Rouf, 1969). Arikan (1989) noted that in the case of *Pelophylax ridibundus*, the erythrocyte count in Anatolia is not season-dependent but rather changes with the geographical location of the

habitat. In some studies, it has been found that environmental conditions and habitat location can significantly impact the maintenance of a normal erythrocyte count and the preservation of their functionality. Gül *et al*. (2011), through a comparative analysis of hematological parameters in five frog species, discovered higher erythrocyte count, hemoglobin concentrations and hematocrit values in terrestrial species compared to semi-aquatic and aquatic species. Zhelev *et al*. (2020), based on the results of their research, found that the studied individuals of *P. ridibundus* inhabiting a polluted area in the southern part of Bulgaria were anemic and had a low body mass. In their study, individuals of both genders were exhausted, with suppressed hematopoiesis and weakened immunity. The authors attributed the deteriorated overall health of *P. ridibundus* individuals to high concentrations of anthropogenic pollutants.

Furthermore, in Delalić *et al*. (2022) research, erythrocyte morphology of the *Bufo bufo* species from certain sites in the Tuzla Canton was examined. They established that certain erythrocytes harbored intracellular parasites, potentially a result of environmental conditions. Based on all the aforementioned research results, it is confirmed that the environmental conditions in which various frog species live have an influence on the hematological parameters of individuals.

Regarding the values of hemoglobin concentration determined in the studied species in this research (56.98 g/l and 36.70 g/l), they are slightly lower compared to similar studies, since Arserim and Mermer (2008) reported hemoglobin values ranging from 56.00 g/L to 121.00 g/L, with an average value of 81.00 g/l for *Rana macrocnemis*.

In our study, the medium hematocrit value for *Pelophylax ridibundus* was 0.362 l/l with a range from 0.208 l/l to a maximum of 0.636 l/l, while for *Rana dalmatina*, the medium value of 0.301 l/l was observed. These values are higher compared to those found in *Rana pipiens* (Kaplan, 1989). Similarly, in the case of *Rana macrocnemis*, the hematocrit value was 0.340 l/l, with a range from 0.160 l/l to 0.460 l/l. This parameter also exhibited seasonal changes, with the lowest value in the autumn (0.240 l/l) and the highest in the spring (0.370 l/l) (Arserim and Mermer, 2008).

The MCV, MCH, and MCHC values in the studied species are slightly lower compared to the data reported for *Rana macrocnemis* (Arserim and Mermer, 2008). The same authors mention that hematological indices exhibit seasonal variations.

Numerous studies indicate the presence of variations in hematological parameters based on gender. For example, in the study by Glomski *et al*. (1997) on the species *Bombina bombina*, a difference in erythrocyte count between males and females was observed. Red blood cell counts were higher in males (0.340×10^{12}/l) compared to females (0.290×10^{12}/l), while other blood parameters were approximately the same.

Hematological parameter research was also conducted on individuals of the species *Bufo bufo* (Donmez *et al*., 2009). Simultaneously, differences between genders and changes that occur during different stages of their life cycle (pre-reproductive and post-reproductive periods) were monitored. When comparing values between males and females during the pre-reproductive phase, no significant differences were found. However, the number of red blood cells showed increased values in both males and females during the reproductive phase. The results from this study demonstrated that the erythrocyte count in this species is higher in males during the reproductive period (Donmez *et al*., 2009), and similar variations are seen in hemoglobin concentration and hematocrit. Hematological studies are very important, because

for example Özgül *et al.* (2020) in their study showed that hematological and genotoxicological parameters of the terrestrial and aquatic amphibian species showed changes when compared with the literature results. Within the hematological parameters of the *P. ridibundus* and *B. variabilis*, the statistical difference in their research was only in the hemoglobin value between the two mentioned species.

CONCLUSION

Based on all the obtained results in the research, it has been demonstrated that all the monitored erythrocyte parameters, except for the MCV parameter, showed higher values in individuals of the species *Pelophylax ridibundus*. The values of erythrocyte count, hemoglobin concentration, and MCHC were statistically significantly higher in *Pelophylax ridibundus*. The results obtained in our study showed values similar to those reported in research by other authors.

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ERITROCITNI PROFILI *RANA DALMATINA* I *PELOPHYLAX RIDIBUNDUS* (AMPHIBIA: RANIDAE)

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Sažetak

Istraživanje eritrocitnog profila provedeno je na ukupno dvadeset osam jedinki žaba, od kojih je četrnaest identifikovano kao *Pelophylax ridibundus*, a preostalih četrnaest kao *Rana dalmatina.* Cilj ovog istraživanja je bio određivanje parametara eritrocitne loze kod dvije navedene vrste u cilju poređenja dobijenih vrijednosti. Jedinke koje su korištene u istraživanju su ulovljene na području opštine Mrkonjić Grad u blizini jezera Balkana. Nakon ulova sve jedinke su transportovane u laboratorijske uslove u kojima su prošle period adaptacije u trajanju od 15 dana. Nakon perioda adaptacije sakupljeni su uzorci krvi i određeni su parametri eritrocitne loze, koji su obuhvatali: broj eritrocita, koncentraciju hemoglobina, hematokrit i parametre MCV, MCH i MCHC. Na osnovu dobijenih rezultata u istraživanju je pokazano da vrsta *Pelophylax ridibundus* ima značajno više vrijednosti broja eritrocita, koncentracije hemoglobina i MCHC parametra u odnosu na vrijednosti dobijene kod vrste *Rana dalmatina.*

Ključne riječi: žabe, eritrociti, hemoglobin, hematokrit, hematološki indeksi

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