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***Original scientific paper***

# THE BLOOD CELLS AND DIFFERENTIAL BLOOD COUNT OF *BUFO BUFO* FROM SEVERAL LOCALITIES OF TUZLA CANTON

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**Summary**

The hematology of the class Amphibia is insufficiently researched in Bosnia and Herzegovina. There is almost no data about the hematology of the lower taxa of this class, especially non-specific and "uninteresting" species such as *Bufo bufo* (common toad), which is not endangered or particularly attractive to scientists and the general public. The paper presents the morphology of blood cells as well as the differential blood count of two populations of the common toad from the area of the Tuzla Canton. All types of blood cells were determined, and the differential blood count showed that there are certain differences in the representation of certain forms of leukocytes in the observed populations, and the presence of certain parasites in the blood of the investigated species. This paper emphasizes the importance of having a comprehensive, accessible register of data on the biology of all species that surround us, including *Bufo bufo*, and provides an overview of the available information on the biology and specific hematology of the mentioned species.

**Key words**: *Bufo,* common toad, hematology, blood count

## INTRODUCTION

A complete registry of knowledge on the morphology, physiology, genetics, evolution, ecology, and other valuable data concerning the common toad, *Bufo bufo* (Linnaeus, 1758), still hasn't been created. According to The IUCN's Red List, *B. bufo* has the least concerning conservation status thanks to its notable distribution, resistance to variable conditions, and adaptability to its unprotected habitat (Vörös *et al*., 2016).

However, at population levels, we can identify that the most dangerous threats to this species - responsible for the extinction of several populations (Wilkinson *et al*., 2012) - are increasing and can be seen in the destruction of forests and meadows along with the artificial draining of wetlands. The common toad is a native species in forty-seven world countries, including Bosnia and Herzegovina (Clarke *et al*., 1974).

Amphibian red blood cells (RBCs) are larger than mammal RBCs, oval-shaped, nucleated, and the most frequent in amphibian blood. The nucleus is constructed of condensed chromatin and is an elongated serrated oval shape. Their function is the same as in other animals - to transport oxygen with the help of hemoglobin (Allender and Fry, 2008).

Toad platelets are a functional equivalent of those in mammals, but their morphology differs - an ellipsoid, sometimes very elongated, nucleated cell contrasts those present in more evolved life forms (Allender and Fry, 2008). Unlike them, leukocyte morphology and function follow the pattern seen in other animals.

Its presumed lymphocytes take up a bigger form than usual when a toad has been infected or hurt (Allender and Fry, 2008). They are the most dominant type of leukocyte in Amphibia, followed by neutrophils, basophils, and finally monocytes (Davis *et al*., 2009).

There are differences in the process of erythropoiesis in juvenile and adult forms of *B. bufo*. Erythropoiesis of the tadpole primarily occurs in the liver and kidneys as opposed to the liver, spleen, and bone marrow of a toad (Allender and Fry, 2008).

Knowledge of the hematological pattern may help improve the use of frogs as bioindicators of environmental degradation (Lilian *et al*., 2022).

## MATERIALS AND METHODS

In this paper, the blood smears of 30 adult common toads, of both sexes, were analyzed. From each locality, 15 individuals were analyzed, which were approximately equal in size and weight, and sexually mature. The sample was collected during the month of August 2021, and the individuals were taken from two different localities. The first site is a lake at the Dubrava open pit (44° 50' 55" N, 18° 71' 54" E), and the second site is the stream Mednica in Babice Donje, near Lake Modrac (44° 48' 79" N , 18°43' 07"E).

Catching individuals was carried out with an improvised net. The individuals were then transferred to the laboratory, and anesthetized with an ether solution, after which their blood was sampled. The frogs' blood was taken by heart puncture, and differential blood smears were made.

Well dried blood smears were kept in the concentrated May-Grünwald solution for seven minutes, washed with distilled water afterward, and finally placed in a 2:1 Giemsa solution for 12 minutes. After final washing and drying, the smears were investigated with the Motic RED223 microscope. We photographed the cells using the Optika Microscopes B Series camera and Optika Vision Pro software. Statistical analysis was conducted within Statistica.

## RESULTS AND DISCUSSION

### Blood cell morphology

Red blood cells were by far the most frequently represented cell type and had similar morphologic features in most cases - an ellipsoid form of the cell, a light to medium dark blue cytoplasm with a centrally positioned dark purple or blue nucleus of uneven, serrated edges. A scattering of RBCs were bottle, tear, heart, or tadpole-shaped (Figure 1).

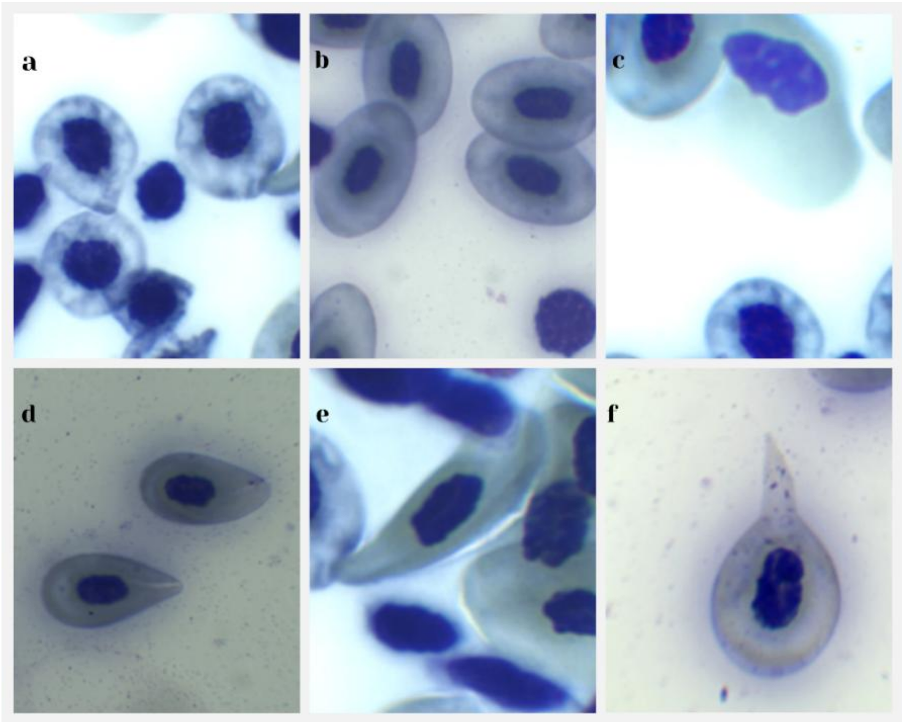


**Figure 1.** Microscopic view of erythrocytes found on blood smears (a – immature erythrocytes, b – mature erythrocytes, c – old erythrocytes, d – tear-shaped erythrocytes, e – spindle-shaped erythrocytes, f – tadpole-shaped erythrocytes)

Apart from characteristic ellipsoid shapes, amphibians are known to show wide variation in erythrocyte morphology among species (Wernberg, 1955; Kuramoto, 1981; Davis *et al*., 2009; Madhusmita and Pravati, 2014). The average recorded dimensions of erythrocytes are 13.40 - 20.70 µm, and there are reports of some erythrocytes whose diameter reaches up to 70.00 µm. Small, round to irregular, basophilic cytoplasmic inclusions, similar to those often incidentally found in reptiles, are found within the cytoplasm of amphibian erythrocytes and are considered a normal finding (Allender and Fry, 2008).

Immature and old RBCs were identified on the blood smears. Immature RBCs retained the oval centrally nucleated cell with a blue-colored cytoplasm, darker than the one in mature RBCs (Figure 1). Reticulocytes (immature erythrocytes) may occasionally be considered a normal finding in amphibians. Reticulocytes are round (less ellipsoidal) and smaller than erythrocytes (Allender and Fry, 2008).

In contrast, old RBCs had much lighter colored or transparent cytoplasm with unclear cell membrane edges. Their nucleus is of an irregular shape, with less serrated edges than in mature RBCs. Several erythrocytes had intracellular parasites in their cytoplasm (Figure 2).

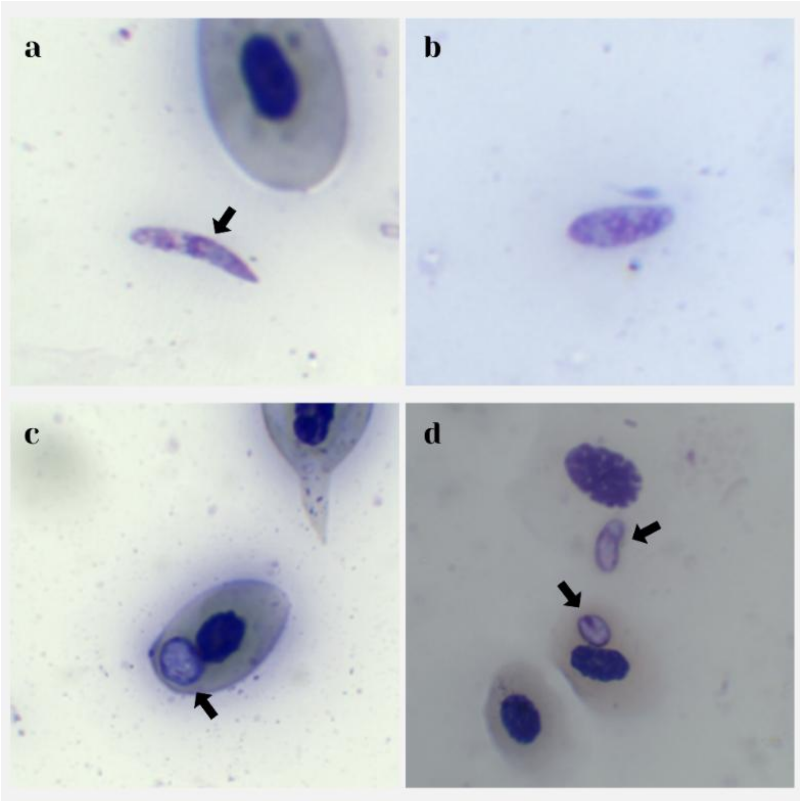


**Figure 2.** Microscopic view of parasites found during the research (a, b – extracellular parasites, c, d – intracellular parasites)

Platelets found on blood smears from both toad populations had a round cell shape with a round dark purple or blue nucleus and little cytoplasm surrounding it. They were mostly found grouped next to accumulations of erythrocytes (Figure 3). In similar studies, platelets were long and oval in shape with large and ovoid nuclei, arranged in clusters (Arserim and Mermer, 2008; Madhusmita and Pravati, 2014).

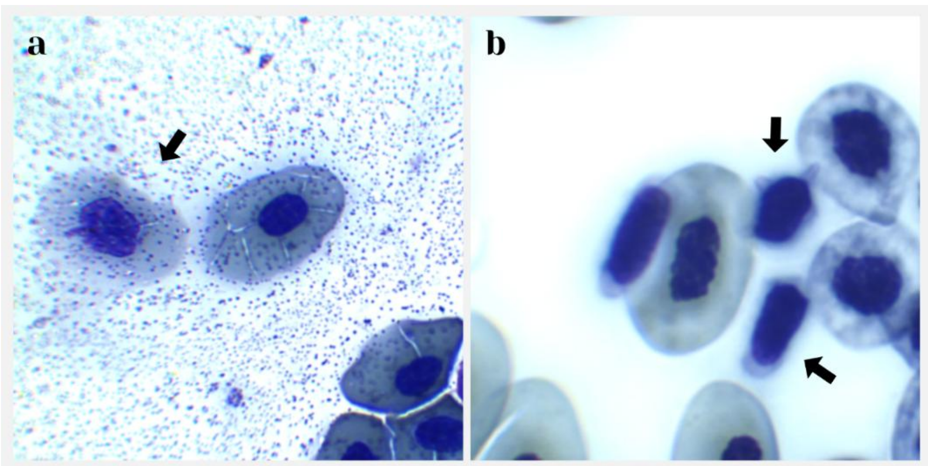


**Figure 2.** Microscopic view of platelets found on blood smears (a – immature platelets, b – mature platelets)

All forms of leukocytes were identified (Figure 4): agranulocytes (lymphocytes and monocytes) and granulocytes (eosinophilic, basophilic, and neutrophilic granulocytes).

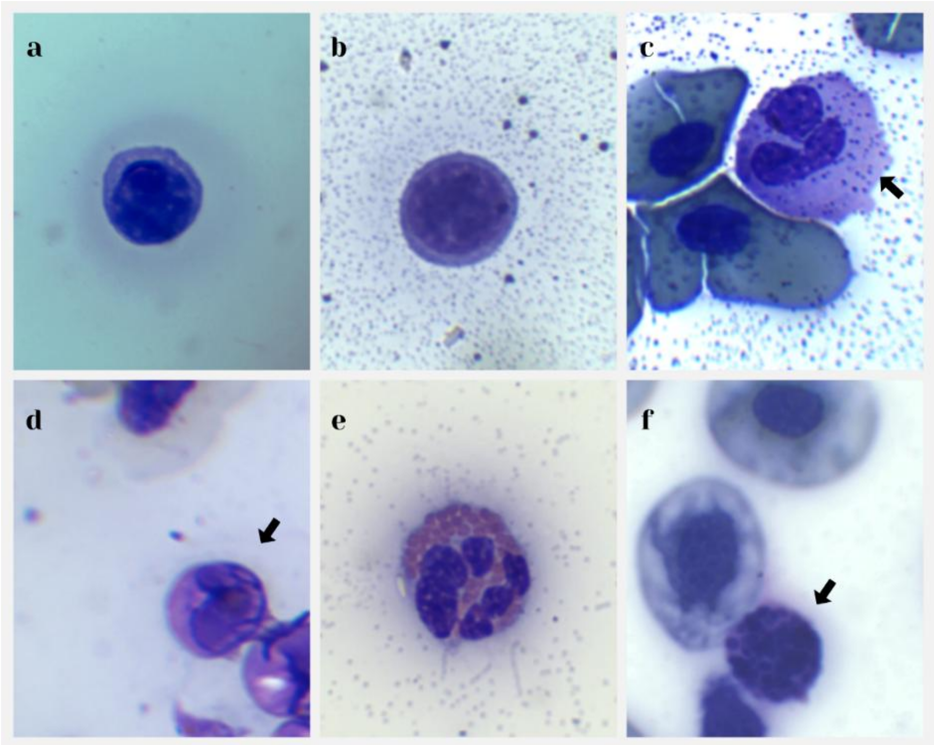


**Figure 4.** Microscopic view of leukocytes found on blood smears (a – small lymphocyte, b – large lymphocyte, c - neutrophil, d - monocyte, e - eosinophil, f - basophil)

Large and small lymphocytes could be distinguished. Lymphocyte morphology in *Bufo bufo* followed the general morphology of lymphocytes found in other species of frogs, amphibians, and vertebrates in general. They are round in shape with a large, round, unsegmented dark blue nucleus, which is surrounded by a very thin layer of lighter blue colored cytoplasm. The morphology of amphibian lymphocytes is similar to that of other vertebrates (Madhusmita and Pravati, 2014). They are the dominant type of leukocytes in most amphibians, including toads. Small lymphocytes can be misidentified as platelets and vice versa. Also, contamination of the blood sample with lymph in the field can lead to incorrect interpretation of lymphocytosis during work in the laboratory (Cathers *et al.*, 1997; Allender and Fry, 2008).

Monocytes were observed to have a round cell shape. Their cytoplasm occupies a large part of the cell, and its color is similar to that of lymphocytes. The nucleus has a kidney shape and a dark blue coloration, but it's not segmented. A similar morphology of monocytes was found in the species *Polypedates teraiensis* (Madhusmita and Pravati, 2014). Some researchers have described in their works the presence of azurophils in addition to or instead of monocytes in the blood of amphibians (Allender and Fry, 2008).

The most frequent granulocytes were neutrophils - large, round cells with a dark, segmented (usually up to three lobes) nucleus surrounded by cytoplasm of a lighter blue coloration. It is generally assumed that the function of amphibian granulocytes is similar to that of cells with similar morphology in other vertebrates (Allender and Fry, 2008).

Similar sized cells, eosinophils, present with a transparent to white cytoplasm filled with a large number of intensely pink colored granules. The nucleus of eosinophils is purple and segmented into multiple lobes - 2, 3, or 4, and often positioned eccentrically, close to the cell membrane. Segmented nuclei were also found in eosinophils of the species *Polypedates teraiensis* (Madhusmita and Pravati, 2014).

Basophils have an irregularly shaped, sometimes segmented into multiple lobes, dark purple or blue colored nucleus. The interior of the basophil cell, which is very difficult to see, contains numerous dark blue granules. Basophils in the species *Polypedates teraiensis* are round cells with large dark purple colored granules over irregular nuclei as well as whole cells (Madhusmita and Pravati, 2014).

Research of the morphology of blood cells was also carried out on the species *Rana rugulosa* (Xianxian *et al*., 2022), where no statistically significant difference in the size of erythrocytes between the sexes was found. However, the size of the nucleus of the erythrocytes of males is significantly larger than that of females. In the mentioned species, it was found that basophils were the smallest among granulocytes, while neutrophils were the largest.

Madhusmita and Pravanti (2012) investigated the shapes and forms of blood cells during the development of tadpoles of the species *Polypedates teraiensis*, where a certain correlation was determined in the morphometric changes during different stages of development of this species. The aforementioned studies represent the beginnings of tadpole hematology research.

**Frequency of certain forms of leukocytes**

The frequencies of certain forms of leukocytes found on blood smears from different localities are presented in Table 1 and Table 2. Blood smears from both localities contained all types of leukocytes.

**Table 1.** Leukocyte frequency on blood smears from the locality Babice Donje (in %)

| | Mean | Minimum | Maximum | SD |
|---|---|---|---|---|
| Lymphocytes | 54.867 | 50 | 61 | 3.270 |
| Neutrophils | 22.067 | 19 | 26 | 2.251 |
| Monocytes | 2.600 | 0 | 7 | 2.131 |
| Eosinophils | 19.600 | 10 | 28 | 4.611 |
| Basophils | 0.867 | 0 | 2 | 0.834 |

**Table 2.** Leukocyte frequency on blood smears from the locality Kop Dubrave (in %)

| | Mean | Minimum | Maximum | SD |
|---|---|---|---|---|
| Lymphocytes | 57.133 | 49 | 98 | 11.753 |
| Neutrophils | 20.133 | 1 | 26 | 5.743 |
| Monocytes | 11.267 | 0 | 17 | 5.405 |
| Eosinophils | 6.467 | 1 | 13 | 2.642 |
| Basophils | 5.000 | 0 | 19 | 6.990 |

Analysis of variance (ANOVA) did not show any significant differences in the frequency of lymphocytes ($F=0.51786$, $p=0.477722$) or neutrophils ($F=1.47359$, $p=0.23492$)

between the two populations. However, there was a significant variation in the frequency of basophils (F=5.17163, p=0.03082), monocytes (F=33.38036, p<0.00001), and eosinophils ( =91.62327, p<0.00001).

Additional literature regarding the differential blood count of the *Bufo bufo* species was not found. Instead, we compared our results to the previously reported differential blood counts from other *Bufo* species, as well as other genera (Table 3).

**Table 3.** Overview of multiple species' differential blood counts

| Species | N | L (%) | N (%) | M (%) | E (%) | B (%) | Source |
|---|---|---|---|---|---|---|---|
| *Bufo bufo* | 30 | 56.0 | 21.1 | 6.9 | 13.0 | 2.9 | Our results |
| *Bufo arenarum* | 12 | 60.9 | 27.3 | 1.7 | 3.7 | 3.8 | (Chiesa *et al.*, 2006.) |
| *Bufo arenarum* | 24 | 64.0 | 20.9 | 1.3 | 13.7 | 0.0 | (Cabagna *et al.*, 2005) |
| *Bufo americanus* | 27 | 20.0 | 68.0 | 1.5 | 3.3 | 7.4 | (Forbes *et al.*, 2006) |
| *Bufo alvarius* | * | 37.0 | 48.0 | 5.0 | 9.0 | 1.0 | (Cannon and Cannon, 1979) |
| *Bufo fowleri* | 6 | 72.3 | 8.5 | 0.8 | 8.5 | 9.8 | (Davis and Maerz, 2009) |
| *Bombina bombina* | 31 | 51.6 | 25.0 | 12.6 | 3.9 | 7.6 | (Wojtaszek and Adamowicz, 2003) |
| *Rana catesbeiana* | 14 | 62.9 | 22.0 | 0.6 | 8.9 | 2.5 | (Cathers *et al.*, 1997) |
| *Rana catesbeiana* | 302 | 26.8 | 60.9 | 2.9 | 5.8 | 3.5 | (Coppo *et al.*, 2005) |
| *Rana catesbeiana* ** | 40 | 73.0 | 23.8 | 0.3 | 3.6 | 2.3 | (Davis and Maerz, 2009) |
| *Rana pipiens* | 50 | 53.4 | 26.5 | 11.0 | 7.3 | 4.4 | (Rouf, 1969) |
| *Rana pipiens* | 18 | 55.4 | 11.3 | 4.1 | 10.1 | 19.2 | (Maniero and Carey, 1997) |
| *Rana pipiens* | 14 | 25.4 | 61.8 | 5.2 | 7.0 | 1.8 | (Bennett and Alspaugh, 1964) |
| *Rana esculenta* | 136 | 57.6 | 15.2 | 0.5 | 14.4 | 12.4 | (Romanova and Romanova, 2003) |
| *Rana clamitans* | 35 | 66.0 | 16.0 | 1.0 | 17.0 | 1.0 | (Shutler *et al.*, 2009) |
| *Acris c. Crepitans* | 79 | 68.3 | 22.4 | 2.7 | 1.6 | 5.0 | (Davis and Durso, 2009) |
| *Xaenopus laevis* | 10 | 30.1 | 26.5 | 1.6 | 1.2 | 40.5 | (Hadji – Azimi *et al.*, 1987) |
| *Glyphoglossus molossus* | 18 | 41.6 | 26.3 | 22.7 | 1.1 | 8.3 | (Ponsen *et al.*, 2008) |

N – number of individuals, L – lymphoctes, N – neutrophils, M – monocytes, E – eosinophils, B – basophils,
* number of individuals not specified, ** juvenile form

The mean leukocyte frequencies are notably susceptible to varying, the result of which can be observed in the data presented in Table 3. The reason for this increased variability can be attributed to multiple factors. One of them could be the great impact of the stress hormone (glucocortiocoids) on the leukocytes found in the blood of amphibians, meaning, if a toad is exposed to stress before or during the sampling of its blood, a disturbance in the frequencies of leukocyte forms can be expected. With the influx of glucocorticoids there is a rise in the number of neutrophils present in blood, which are pulled from the reserves in bone marrow. A decrease in the number of lymphocytes found on blood smears can also be expected as these white blood cells cling to the endothelial cells of blood vessels when a toad is stressed (Bennett and Alspaugh, 1964; Davis and Durso, 2009). However, additional literature shows that the lymphocyte and neutrophil ratio doesn't have to point to stress immediately before or during blood sampling, but can be an important sign of existing stressors or disrupted living conditions in the species' habitat (Davis *et al*., 2009).

The increased eosinophil and decreased basophil frequencies can further be connected to the presence of parasites in blood. A necessary way of moving forward is now to conduct detailed research of amphibian blood parasites and only then point to their significance in varying frequencies of leukocyte forms. Basophil frequency variants can not only be explained by the aforementioned elements but additionally with a potential viral infection (Forzan *et al*., 2017).

## CONCLUSION

This paper emphasizes the importance of creating an open access registry of all species, including *Bufo bufo*. Many internal and external elements that require extensive research, including hematology and precisely the hematological parameters of a taxon, can affect any aspect of a species' biology and lead to changes in its resistance, adaptability, abundance, and finally, its vulnerability. Hematological parameters are a clear indicator of changes in the environment, so this and similar research should be intensified, especially taking into account the fact that there is almost no data on the hematology of the researched species. We recommend that future research focus on the influence of potential stressors in the creation of various hematological parameters in frogs.

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