ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 289-320

Use of Haematological Indicators in Anurans for Assessing Their Health Status When Inhabiting Conditions of Anthropogenic Stress. Pelophylax ridibundus (Amphibia: Ranidae) as an Example: A Review and Appraisal

***Zhivko M. Zhelev*[1*], *Georgi S. Popgeorgiev*[2]**

1 - University of Plovdiv "Paisii Hilendarski", Faculty of Biology, Department of Human
Anatomy and Physiology, 24 Tsar Asen Str., 4000, Plovdiv, BULGARIA
2 - National Museum of Natural History, Bulgarian Academy of Sciences,
1 Tsar Osvoboditel Blvd., Sofia 1000, BULGARIA
[*]Corresponding author: zhivko-m@mail.bg

Abstract. We review evidence for and against the use of haematological parameters as indicators for assessing the health status in free-living anuran populations that live in conditions of ecological stress. We focus on the use of direct measured blood parameters, such as erythrocyte and leukocyte counts, haemoglobin concentration, haematocrit value, leukogram and calculated haematological indexes: mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and mean corpuscular volume. In addition, we evaluate the possibilities for use as biomarkers of the metric parameters of erythrocytes. This paper summarizes the results from our long-year *in situ* analyses of the populations of Marsh frog *P. ridibundus* inhabiting freshwater ecosystems in Southern Bulgaria with different degrees of anthropogenic transformation. It presents the results from using different haematological indicators as markers for the assessment of changes in their organism when inhabiting areas affected by anthropogenic stressors. Our results strongly support using blood changes in anuran amphibians as objective indicators for assessing their health status. We believe they are a reliable biodiagnostics instrument in modern in field ecotoxicological studies.

Key words: Anurans, Marsh frog, haematological parameters, anthropogenic stress, biomarkers, health status.

Introduction

Why are the haematological parameters in anuran amphibians' suitable biomarkers for assessing the effects of various anthropogenic stressors?

Blood is a highly differentiated and reactive internal environment of the body. It is a type of specialized connective tissue of mesenchymal origin, consisting of blood plasma, which includes water, nutrients, waste, enzymes, antibodies, hormones and shaped elements (blood cells) – erythrocytes, leukocytes and platelets (Seiverd, 1972). The formation of plasma and its components is generally related to tissue metabolism, starting with the organs of the gastrointestinal tract. Differentiated blood cells have a relatively short life and are formed continuously by stem cells produced in haematopoietic organs (Glomski et al., 1997). In all vertebrates, the first blood cells appear during the embryonic period, but the haematopoietic

Union of Scientists in Bulgaria – Plovdiv
University of Plovdiv Publishing House

centers are different in different taxonomic groups and this largely depends on the age of the individuals (Jordan, 1933; Foxon, 1964; Glomski et al., 1997). Although in amphibians (including anurans) blood composition is similar to that in other vertebrates, such as heterothermic animals, they have developed a number of adaptive structures and mechanisms to deal with changes in the environment (Foxon, 1964; Wojtaszek & Adamowicz, 2003). Many of these adaptations are the result of their phylogenetic history, involving a transition from water to land life (Wei et al., 2015; Franco-Belussi et al., 2021). In all vertebrates, blood is responsible for important bodily functions such as immune response, transport of nutrients, transport of gases and metabolic waste (Wells & Baldwin, 1990; Allender & Fry, 2008; Álvarez-Mendoza et al., 2011).

All changes in environmental factors, including those related to anthropogenic stress, cause changes in functional state of the blood (Cajaraville et al., 2000; Davis et al., 2008; Gonçalves et al., 2019; Oliveira et al., 2019). This makes it very difficult and practically impossible to derive reliable reference values for different haematological parameters in different species of tailless amphibians, although in the past many studies have been directed at this. However, research from the end of the past century and the present century give clear evidence that setting reference values for haematological parameters in anurans without parallel tracing and analysis of environmental factors and the possible presence of such with anthropogenic origin in their habitats, does not give reliable results (Cabagna et al., 2005; Romanova & Egorikhina, 2006; Davis et al., 2008; Priyadarshani et al., 2015; Pollo et al., 2016, 2017; Salinas et al., 2017; Sures et al., 2017; Corduk et al., 2018; de Assis et al., 2018; Xiong et al., 2018; Davis & Golladay, 2019; Oliveira et al., 2019; Zamaletdinov et al., 2019; Brodeur et al., 2020). Most of the above tests with anuran amphibians include mainly analyses using as biomarkers quantitative and qualitative characteristics of the erythrogram and leukogram, since variations in their parameter values provide the main volume of information about the indication of changes in main physiological processes such as breathing and immune defense of the organism when inhabiting anthropogenically transformed environment (Venturino et al., 2004; Davis et al., 2008). In addition, this type of research can be performed immediately after the capture of the animals and does not require expensive laboratory equipment and consumables.

Changes in the erythrogram of anurans inhabiting under conditions of anthropogenic stress.

The erythrogram is that part of the blood picture that quantitatively and qualitatively evaluates erythrocytes. This is done by counting the total number of these cells (RBCs), measuring haemoglobin concentration (Hb) and calculating haematocrit value (Hct) or packed cell volume (PCV). In addition, haematological indices (MCH: mean corpuscular haemoglobin: MCHC: mean corpuscular haemoglobin concentration in erythrocytes and MCV: mean corpuscular volume) are also part of the erythrogram. Here we explicitly clarify that research on the values of haematological indices in "wild populations" of amphibians is very scarce (Johnstone et al., 2017).

The number of erythrocytes in amphibians is the lowest compared to all other groups of vertebrates (Hutchison & Szarski, 1965). This is due to the morphological features of their erythrocytes: relatively large nuclear cells, and it is known that the number of erythrocytes is inversely correlated with their volume (Wintrobe, 1933; Banerjee, 1979). Researchers believe that animals in better physical condition maintain higher levels of Hb and Hct, so these indicators should be positively correlated with body condition indexes, at least when mobilization of metabolic energy sources is beneficial for the body (Artacho et al., 2007; Norte et al., 2008). The benefit of higher Hb or Hct is associated with a greater ability to transport oxygen in the blood and its faster delivery to metabolizing tissues and therefore meeting high energy needs. This assumption supposes that animals in better overall physical condition have the ability to better allocate metabolic resources to maintain a higher "healthy" level of Hb and Hct. This suggests that each species should have an upper limit of Hct ("optimal haematocrit"), beyond which increased blood viscosity would lead to deterioration in blood flow velocity (de Boer et al., 2006).

When *in situ* analyzes examine changes in the erythrogram of anurans, in order to obtain reliable and objective information on the condition of individuals from populations living under stress, it is necessary to compare the data with those obtained from a reference population (such living in optimal environmental conditions, without the presence of anthropogenic stressors) (Pollo et al., 2016, 2017; Salinas et al., 2017; Sures et al., 2017; Corduk et al., 2018; Xiong et al., 2018; Zamaletdinov et al., 2019; Şişman et al., 2021). The most objective information is obtained when complex changes in the erythrogram are monitored and analyzed, and not its individual components (Johnstone et al., 2017; Brodeur et al., 2020). According to literature data, short-term stress-induced changes in the erythrogram affecting Hb and Hct values are usually due to changes in plasma volume rather than erythrocytes. Thus, morphological variables in red blood cells, such as mean corpuscular volume (MCV) or MCHC, should not be altered by short-term stress (Johnstone et al., 2017). Haemoglobin and haematocrit values may decrease in anemia, or increase (more pronounced Hct) in general dehydration (Coppo et al., 2005). In the absence of food, or in the case of unbalanced food, as well as in the absence of minerals such as Fe, Cu, Co and Se, the values of haemoglobin and haematocrit may also decrease (Jain, 1993). Johnstone et al. (2017) generally classify anemias into two types: regenerative and nonregenerative. According to Lewis et al. (2006) anemia occurs as a result of either a reduced number of erythrocytes or a reduced concentration of haemoglobin in the erythrocytes [mean cell concentration, or corpuscular concentration of haemoglobin - MCHC (g/dl)]. Regenerative anemia occurs when an individual responds to this condition by releasing immature erythrocytes (reticulocytes) from the haematopoietic centers and / or increasing erythropoiesis. Typically, the stimulus that triggers the release of immature erythrocytes is mediated by hypothalamic-pituitary-adrenal stress, such as a hemorrhage injury or parasitic infection (McGrath, 1993; Tyler & Cowell, 1996). Non-regenerative anemia occurs when an individual is unable to respond and / or compensate for the loss of red blood cells and this condition may be the result of food stress, certain chronic diseases or toxic substances (McGrath, 1993; Tyler & Cowell, 1996). Regenerative anemias, as well as those of hypoxemic type, caused by toxicants present in the habitats of anurans (leading to a decrease in tissue oxygen levels) are an expression of long-term toxic (or other type) negative effects on their organism (Johnstone et al., 2017). However, in wild populations, the differences between the two types of anemia may not be clear, but combined synergistic effects are possible as well, as a result of physiological reactions caused by short-term and long-term stress (Dhabhar et al., 1996; Romero & Romero, 2002, Romero & Reed, 2005). The presence of a high number of immature erythrocytes in the peripheral blood of tailless amphibians is considered as an indicator of a compensatory regenerative response caused by anemia or loss of circulating erythrocytes (Valenzuela et al., 2006; Prieto et al., 2008; Allender & Fry, 2008; Briggs & Bain, 2012). This thesis is supported by specific results obtained in *in situ* studies in populations of anurans inhabiting conditions of anthropogenic stress in habitats heavily polluted with different types of xenobiotics (heavy metals, fertilizers and pesticides) (Peskova & Zhukova, 2005; Barni et al., 2007; Marques et al., 2008; Spirina, 2009; Mineeva & Mineev, 2010; Pollo et al., 2017; 2019).

One of the important but very poorly studied morphological characteristic of the erythrocyte cell is the changes in its size and shape (as well as those in the nucleus) in populations of anuran amphibians inhabiting conditions of anthropogenic stress. The ability of erythrocytes to change the size and shape of their cell depends on the ratio of surface to volume, the mechanical properties of their cell membrane and their internal viscosity (Chien, 1987; Baskurt & Meiselman, 2003). According to Baskurt & Meiselman (2003), changes in cell shape depend on the critical ratio between cell size and volume (S/V).

When moving from an ellipse to a sphere, due to the smaller volume of the latter, its area increases. It should be noted that under conditions of environmental and physiological stress, changes in the shape of erythrocytes are accompanied by changes in the properties of blood flow such as osmotic balance, viscosity, flow rate, etc. (Baskurt & Meiselman, 2003; Allender & Fry, 2008; Kim et al., 2015). Rounding of erythrocyte cells (oval or spherical erythrocytes) can be considered an adaptive response in conditions of hypoxia, as it increases the contact surface of the cell (Wells & Baldwin, 1990) and ensures the diffusion of more oxygen to the tissues (Hartman & Lessler, 1964; Allender & Fry, 2008).

The opposite effects have also been reported in literature – increase of cell size and reducing cell contact surface leads to difficulties in gas metabolism (Nicol et al., 1988; Lay & Baldwin, 1999; Bondarieva et al., 2012). However, we must clarify that positive biological effects in tissue hypoxia can be expected not only from the presence of small cells with a larger relative surface, but also from accelerated haematopoiesis, accompanied by normal haemoglobin synthesis. The size of erythrocyte nuclei in tailless amphibians (and in other groups of vertebrates with erythrocytes with nuclei) depends on the size of the genome and the degree of chromatin condensation. (Macadangdang et al., 2014; Levy & Heald, 2016), but these are not the only factors determining their size. According to Kim et al. (2015) changes in the size of the nuclei may be due to changes in the osmotic pressure of the cytoplasm and the activity of the motor structural elements of their cytoskeleton. In addition, changes in nuclear shape may be due to changes in nuclear plate (Webster et al., 2009). It is known that in amphibians the rate of metabolism is inversely proportional to the size of their genome (Gregory, 2001; Vinogradov & Anatskaya, 2006), but in general the causes and mechanisms leading to changes in the size of the nuclei remain unclear and very poorly studied (Edens & Levy, 2014; Mueller, 2015). Very little work has been devoted to studies on the morphology of erythrocytes in populations of anuran amphibians inhabiting conditions of anthropogenic pollution (Zhelev et al., 2006; Omelykovets & Berezyuk, 2009; Mineeva & Mineev, 2010; Salinas et al., 2017; Dönmez & Şişman, 2021).

Although the authors of these works report changes in the size and shape of erythrocytes in the blood of anurans inhabiting anthropogenically polluted habitats, compared to populations of frogs inhabiting background areas, there are no definite opinions about the causes of the observed changes and their biological role. In such *in situ* studies, it is even more difficult to find causal relationships between changes in erythrocyte size and specific toxicants. One of the reasons is that toxic agents in wastewater are usually in the composition of complex mixtures and enter into synergistic interactions both with each other and with various abiotic environmental factors such as altitude, temperature, hydrogen index, electrical conductivity of water, dissolved oxygen, etc.

(Haden, 1940; Hartman & Lessler, 1964; Ruiz et al., 1989; Addy et al., 2004).

Changes in the leukogram of anurans inhabiting conditions of anthropogenic stress.

The leukogram is a study that determines the total number of leukocytes (WBCs) and in particular in the differential count – the number of individual types of leukocytes with their percentages.

Leukocytes in anurans are identical to those in other groups of vertebrates: agranulocyte leukocytes – lymphocytes, monocytes and granulocyte leukocytes – neutrophils, eosinophils and basophils (Davis et al., 2008; Arikan & Çiçek, 2014; Zhelev et al., 2017a). Lymphocytes are involved in immune responses and the production of haematopoietic growth factors. They are morphologically divided into small and large lymphocytes, and immunologically and functionally into B and T cells (Campbell, 2004). Monocytes (mononuclear phagocytes) are relatively large cells, of different origin from lymphocytes (myeloid stem cell line) and are an important part of the monocyte-macrophage system, providing non-specific immune protection of the organism. Together, lymphocytes and monocytes make up 80% of the leukocytes of anuran amphibians (Arikan & Çiçek, 2014). The functions of granulocytes in amphibians and anurans in particular are similar to these cells in other vertebrate species and have been identified in terms of their morphological characteristics (Wright, 2001). Neutrophils (rod-nuclear and segment-nuclear) are the largest group of granulocytes. They are associated with chemotaxis and are involved in the inflammatory process (recognizing and binding cytokines and leukotrienes released during inflammation) and the killing of bacteria by phagocytosis. Neutrophils carry out the so-called "oxidative burst" (secretion in the phagosome of toxic to pathogens oxygen compounds, such as hydrogen peroxide and hypochlorous acid as well as lysozyme and interferon), which makes them major participants in the fight against bacterial and fungal infections.

In birds and reptiles, neutrophil is replaced by heterophile that performs the same immunological function (Hawkey & Dennett, 1989; Jain, 1993). Eusinophils are also involved in phagocytosis, especially in allergic-type reactions (they absorb biologically active substances such as histamine). They are major participants in the disposal of tumor cells and parasites (Thrall et al., 2006). In most vertebrates, basophils are the smallest group of granulocytes and their functions are still unclear. However, in some species of anurans, there is evidence of a high percentage of basophils in peripheral blood (Campbell, 2004). They are thought to be involved in "delayed-type" immunobiological reactions (releasing proteolytic enzymes, heparin, but also histamine, which causes blood flow to the inflamed areas). They participate in the management of asthma attacks and other allergic conditions. In amphibians and anurans in particular, the number of leukocytes in 1 mm^3 of blood varies. Variations in leukocyte count are due to sex, age, season, diet, pregnancy in females, environmental and pathological factors (Thrall et al., 2006; Davis et al., 2008; Liu et al., 2013; Arikan & Çiçek, 2014; Bunjerdluk et al., 2021; de Gregorio et al., 2021; Franco-Belussi et al., 2021). All this makes it very difficult to define reference ranges, both for the total number and for the percentage ratio of different types of leukocytes in different types of anurans. An additional problem in this regard is the heterothermic metabolism and its strong dependence on abiotic environmental factors, among which temperature is in the first place.

Low temperatures are a major problem for amphibians, and one strategy they use to deal with low temperatures is thermal acclimatization: tuning cellular and physiological processes in response to environmental conditions (Feder, 1992;

Greenspan et al., 2017). However, thermal acclimatization requires time, which leads to suboptimal levels of immunity after temperature changes, while the immune system undergoes adjustments (Raffel et al., 2006, 2015). Therefore, it is always necessary in *in situ* analyzes, when examining the leukogram parameters in anurans inhabiting conditions of anthropogenic stress, to analyze and compare them in parallel with data from a reference population living as close as possible to the optimal environmental conditions.

The WBC profile (the number of circulating immune cells in the bloodstream) can be used to assess haematopoietic productivity and the level of activation of the immune system (Davis et al., 2008). In recent years, this type of analysis has been increasingly used in various in situ studies in "wild" populations, including populations of amphibians inhabiting conditions of anthropogenic stress (Rohr et al., 2008; Shutler & Marcogliese, 2011; Peterson et al., 2013; Das & Mahapatra, 2014; Vallejo et al., 2015). Lymphocytes and neutrophils usually make up the majority of leukocytes (Davis & Durso, 2009) and the ratio of these cells is often used alone as a reliable indicator of the state of the immune system (Davis et al., 2008; Silva et al., 2021). High relative proportions of circulating neutrophils and low relative proportions of circulating lymphocytes (high N:L ratio) are characteristic of a haematopoietic stress response or an activated immune system in amphibians (Bennett & Daigle, 1983; Cooper et al., 1992; Maniero & Carey, 1997; Davis & Maerz, 2008a; Vallejo et al., 2015; Greenspan et al., 2017; de Gregorio et al., 2021) and other vertebrates (Davis et al., 2008). The functional explanation for the high N:L ratios is that they can increase immune readiness under stressful conditions when animals are likely to face an immune challenge (Dhabhar et al., 1994, 1995; Franchimont, 2004). The increase in neutrophils in blood may intensify the inflammatory response, while the trafficking of lymphocytes out of circulation and into tissues may improve immune control or protect these cells from the inhibitory effects of stress hormones (Dhabhar et al., 1994; 1995; Rich & Romero, 2005). Interpretation of changes in leukocyte profiles (ie, attribution of stress against infection) can be problematic, especially if the state of the infection is unknown (Davis et al., 2004). This can be a problem in field analysis, although the two factors (stress and infections) are closely related and stress usually leads to infections and diseases, weakening the body's overall immunity. One of the approaches to overcome this problem is the parallel analysis of other haematological parameters (erythrogram).

In literature there are many studies, though unsystematized, reporting changes in the leukogram of different groups of vertebrates living under stress (Dhabhar et al., 1994; 1995; Davis et al., 2008; Davis & Maerz, 2008a; b) but research in populations of anurans from anthropogenically polluted habitats is generally very limited. There are studies reporting high leukocyte count in severe neutrophilia and monocytosis in the blood of *P. ridibundus* inhabiting the area of a chemical plant in Ukraine (Tarasenko, 1981), a system of rice fields in Southern Russia (Peskova & Zhukova, 2005) and of *Rhinella arenarum* (Hensel, 1867) from rice fields in Argentina (Pollo et al., 2017). The reverse changes – general leukopenia in combination with reduction of leykocyte and monocytes count is reported in populations of *P. ridibundus* inhabiting urbanized environment – in a residential area in Ekaterinburg densely built-up with residential buildings (Sils & Vershinin, 2005; Sils, 2006, 2008) and Novgorod (Romanova & Romanova, 2003; Romanova, 2005; Romanova & Egorikhina, 2006) in the Russian Federation. Peskova (2001) proposes changes in the differential formula of blood in populations of anurans inhabiting conditions of anthropogenic stress to be generally referred to two types: In the "first type of changes" (under conditions of relatively low anthropogenic load on the environment) the leukogram is characterized by leukocytosis and neutrophilia (increases of the total number of neutrophils or only that of rod-nuclear cells accompanied by a decrease in segment-nuclear neutrophils). In addition, monocytosis is almost always observed compared to controls. This general picture of changes in the leukogram of amphibians may show some variation with diverse changes in the number of lymphocytes, basophils and eosinophils.

The "second type of changes" in the leukogram in anurans is characterized by leukocytosis and neutropenia, which may be accompanied by monocytosis and eosinophilia, or monocytosis and eosinopenia. In the opinion of the author (Peskova, 2001), when toxicant concentration does not exceed any critical value, characteristic for the given polluting agent, the leukogram of the amphibians reacts according to the first type. Significant neutrophilia is an expression of a complete protective reaction of the body. In combination with monocytosis, it provides adaptation of amphibians to inhabiting conditions of pollution and elimination of toxic and / or associated bacterial agents that have entered the body. The second type of leukogram reaction is evidence not only of the body's attempts to cope with high doses of toxicants, but also a sign of depletion of its resistance in an environment with chronic presence of stressors (anthropogenic pollutants). However, severe neutropenia leaves little hope for amphibians to survive in such severe environmental conditions, despite the appearance of immature granulocyte forms, which is evidence of compensatory stimulation of neutrophilic granulocytopoiesis. The second type of changes in the leukogram of anuran amphibians is an indication for transition from adaptations to pathological changes that probably lead to the subsequent death of the animals – third phase (exhaustion of the organism), according to the theory of general adaptation syndrome (GAS) (Selye, 1956).

This paper summarizes and presents the results from our long-term *in situ* analyzes conducted in the populations of Marsh frog *Pelophylax ridibundus* (Pallas, 1771) inhabiting freshwater ecosystems in Southern Bulgaria with varying degrees of anthropogenic pollution. It covers the period from 2001 to 2017 and includes studies on the application of blood markers complex for the purpose of assessment of general health status of frogs that inhabit areas, polluted with toxicants of different types and in different concentrations. To achieve the goals of research, the values of tested blood parameters of frogs from polluted habitats were compared with those obtained from frogs inhabiting less disrupted habitats (references sites). In the course of research different and specific working hypotheses related to the objectives of the respective analyses were tested, but what they had in common was the assumption of expected differences in the values of the tested blood markers in the populations of anurans inhabiting conditions of anthropogenic stress when compared with the corresponding values obtained for the individuals from the reference populations. This makes it possible to assess how exposure to toxic agents (usually over prolonged time) in anthropogenically contaminated habitats affects marsh frogs *P. ridibundus* and what changes are caused by the toxicants in the body of these anurans (indication of stress and exhaustion of the body, or adaptation to life in a hostile environment).

Materials and Methods

The material for the analyzes was collected during the spring-autumn seasons of 2001-2017 from rivers and dams (natural and artificial water bodies) in Southern Bulgaria with test subject – Marsh frog *P. ridibundus*. This species of water frog has a large range in Eurasia (Amphibia Web, 2021) and in Bulgaria it is distributed throughout the country (Beschkov & Nanev, 2002; Stojanov et al., 2011). The species is under protection: BDA (IV), 92/43 (V), BERN (III), IUCN (LC) (Biserkov et al., 2007). According to Article 42, Article 41 and Appendix II of Article 41 of BDA, permits for catching *P. ridibundus* are not required when collecting them for scientific purposes. Frogs were caught in the evening by the light of strong lamps placed on the shore of the waterbody, or with the help of an electric lantern. In the second case, areas with length of about 1-2 km and width of 4 m along the river or along the shoreline of dams were scanned, following Sutherland's (2000) methodology. Adult individuals of both sexes (SVL > 60.0 mm, according to Bannikov et al., 1977) were used for the analyses. They were separated by sex, using secondary sexual characteristics: the presence of "marital corns" on the first finger and resonator bubbles in the corners of the mouth of the males (detailed physiographic maps with exact locations where frogs were caught are presented in Zhelev et al. (2013; 2015; 2016a; b; 2017b; 2018; 2020; 2021).

The studies in the period 2001-2017 are compliant and performed with basic data on the ecological status of each of the water bodies where frogs were caught; such data were obtained on the basis of physio-chemical analyzes of the water carried out on behalf of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, (Newsletters on the state of water in each studied waterbody–rivers and dams, in the period 2001-2017). Physio-chemical analyzes measure the values of priority substances for each of the water bodies; such values are determined according to the monitoring plans of the East Aegean River Basin Directorate / Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, in compliance with Water Framework Directive WED 2000/60/EO (EC, 2000), Ordinance No H-4/14.09.2012 (Ordinance, 2012) and No 256/1.11.2010 (Ordinance, 2010). Detailed data (physicochemical analysis of water) on the condition of each tested water body in the period 2001-2017 are presented in Zhelev et al. (2013; 2015; 2016a; b, 2017b; 2018; 2020; 2021).

After the catches in each of the studied habitats, in order to reduce stress, the frogs were transported to the laboratory in containers with water. The frogs were treated and the experiments were performed within 24 h of capture. The manipulations were carried out in compliance with ethical standards for working with animals (Fellers et al., 1994; Steven et al., 2004). The frogs were anesthetized with ether according to the procedure proposed by Stetter (2001). In a living condition, a 20 mm long heparinized needle was used to pierce the heart and draw up to 0.20 ml of blood according to the procedure proposed by Wright (2001). All haematological analyzes were performed according to standard clinical methods (Pavlov et al., 1980). Erythrtocyte count (RBC) and leucocyte count (WBC) were manually quantified using Hayem and Turck's solution and a Burker haemocytometer chamber. The haemoglobin concentration (Hb) was determined by the cyanmethaemoglobin spectrophotometric method and the packed cell volume (PCV), or (Hct value) by the microhaematocrit method. Leukograms (differential leukocyte counts) were performed in blood smear with Giemsa–Romanowsky staining. The derivative haematological parameters (MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, and MCV: mean corpuscular volume) were calculated by the Brown's formulas (1980). MCV was calculated by dividing haematocrit per liter of blood by total RBC count. The erythrocyte sizes were determined with an Olympus stereo microscope (SZX16, resolution 900 line pair/mm, Germany). We used the methodology proposed by Atatür et al. (1999) and Arserim & Mermer (2008) for nuclear erythrocytes: 40 randomly selected erythrocytes from each individual were measured with an ocular micrometer (MOB-1–15x). Four basic erythrocyte sizes were evaluated: cells length (EL) and width (EW) and nuclear length (NL) and width (NW). Cell and nuclear shapes were assessed with EL/EW and NL/NW ratios, and nucleus/cytoplasm with a NS/ES ratio. We used the formula: [$ES = EL \times EW \times \pi / 4$ and $NS = NL \times NW \times \pi / 4$] to calculate the erythrocyte sizes (ES μm^2) and their nuclear sizes (NS μm^2).

Statistical processing of the data was performed, using the STATISTICA 7.0 Software (Statistica, 2004) or R language (version 3.1.2, R Development Core Team, 2015). A Shapiro-Wilk test was used to evaluate the normal distribution of data, which proved to be statistically significant. For differences between individuals of *P. ridibundus* inhabiting habitats with different degrees and different types of pollution and those inhabiting relatively clean habitats (control groups) parametric Student's t-test for normally distributed independent samples and analysis of variance (one-way ANOVA) were applied, with subsequent verification of the statistical significance of the differences (depending on the homogeneity of the variances) by LSD, Tukey or Games-Howell *post-hoc* tests. Results with $p < 0.05$ [$\alpha = 5\%$] were considered significant. Data was given as Mean ± SEM and their confidence interval – 95% (or Minimum and Maximum values). Multivariate statistical analyses (a principal component analysis PCA and a standard discriminant analysis DA) were applied as an integral assessment of the health status of *P. ridibundus* from the investigated sites in Southern Bulgaria (for details see Zhelev et al., 2013; 2015; 2016a; b; 2017b; 2018; 2020; 2021).

Results and Discussion

The descriptive statistics and results from the comparisons by a one-way ANOVA analysis of the erythrogram and leukogram parameters in *P. ridibundus* individuals from investigated sites in Southern Bulgaria are presented in Table 1 and Table 2.

Table 1. The erythrogram parameters (Mean±SEM, Minimum-Maximum, or confidence interval – 95%) used for evaluation of health status of *P. ridibundus* individuals from freshwater ecosystems with different degree of anthropogenic pollution in southern Bulgaria. *Legend:* Sites: Site 1 (Sazliyka River near the village Rakitnitsa), site 2 (Sazliyka River near the town of Radnevo), site 3 (Topolnitsa River near the village Poibrene), site 4 (Studen Kladenets Dam Lake near the LZP "Kardzhali"), site 5 (Vacha River), site 6 (Tsalapitsa Rice Fields), site 7 (Vacha River) and site 8 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen (NH^+_4-N), total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), erythrocyte count (RBC), haemoglobin concentration (Hb), packed cell volume, or haematocrit value (PCV), mean corpuscular haemoglobin (MCH),: mean corpuscular haemoglobin concentration (MCHC),: mean corpuscular volume (MCV). Significance codes: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns $p > 0.05$.

Sites	**Parameters**						**Publications**
	RBC (x 10^6/µl)	**Hb (g/dl)**	**PCV (L/l)**	**MCH (pg)**	**MCHC (g/l)**	**MCV (fl)**	
Site 1(RS) (n=30, ♀+♂)	366.670±10.40 (270.000–490.000)	5.53±1.38 (4.57–7.10)	0.25±0.005 (0.21–0.31)	153.32±5.02 (105.17–220.17)	225.05±5.28 (149.77–298.45)	682.57±17.82 (517.44–937.50)	Zhelev et al. (2013; 2015; 2016a)
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=30, ♀+♂)	482.670±12.12 (380.000–590.000	6.88±2.04 (4.87–8.64)	0.34±0.005 (0.30–0.40)	160.30±2.77 (129.02–186.25)	203.98±4.27 (152.41–245.12)	774.44±9.83 (670.00–931.25)	
Site 3 (PS:	629.670±26.84	8.28±1.02	0.37±0.006	146.08±5.09	221.18±3.74	650.91±20.82	

Cu, Fe, Pb, Mn, As) (n=30, ♀+♂)	(400.000–920.000)	(7.18–9.25)	(0.31–0.43)	(98.55–191.05)	(141.72–265.19)	(452.78–912.50)	
Site 4 (PS: Cd, Zn, Pb, Cu) (n=30, ♀+♂)	571.633±10.72 (480.000–690.000)	7.16±1.83 (5.21–8.97)	0.34±0.005 (0.30–0.41)	134.59±2.92 (110.04–172.85)	211.65±4.13 (157.97–254.53)	652.82±12.59 (540.50–806.25)	
Statistics (one way ANOVA, F, LSD tests)	F=8450.612; 1/4***; 2/4***; 3/4*	F=1.917; 1/4***; 2/4ns; 3/4***	F=3.107; 1/2***; 1/3***; 2/3***; 1/4***; 2/4ns; 3/4***	F=2.667; 1/2ns; 1/3ns; 2/3*; 1/4***; 2/4***; 3/4ns	F=2.525; 1/2***; 1/3ns; 2/3***; 1/4*; 2/4ns; 3/4ns	F=7.072; 1/2***; 1/3ns; 2/3***; 1/4ns; 2/4***; 3/4ns	
Site 5 (RS) Female (1) n=25 Male (2) n=25	♀: 610.720±25.89 (450.000–920.000) ♂: 616.400±22.13 (450.000–910.000)	♀: 7.82±1.98 (4.64–9.25) ♂: 7.61±1.99 (5.21–9.10)	♀: 0.35±0.01 (0.30–0.42) ♂: 0.35±0.01 (0.30–0.42)	♀: 144.37±5.93 (98.55–196.48) ♂: 138.03±6.36 (105.68–228.49)	♀: 218.62±4.83 (157.97–254.33) ♂: 208.05±4.57 (141.72–246.52)	♀: 644.42±20.87 (452.78–781.25) ♂: 656.33±16.43 (478.39–818.75)	Zhelev et al. (2018)
Site 6 (PS: fertilizers, pesticides) Female (3) n=25 Male (4) n=25	♀: 395.200±14.83 (270.000–600.000) ♂: 357.600±12.10 (270.000–510.000)	♀: 5.65±1.77 (4.43–7.16) ♂: 5.37±1.34 (4.49–7.24)	♀: 0.25±0.01 (0.22–0.31) ♂: 0.24±0.01 (0.21–0.30)	♀: 146.76±5.85 (107.41–198.97) ♂: 154.53±5.79 (105.17–220.70)	♀: 226.21±5.50 (156.49–298.45) ♂: 221.56±5.13 (149.77–261.12)	♀: 635.59±17.817 (517.44–810.34) ♂: 689.05±19.48 (511.31–937.50)	
Statistics (two way ANOVA, Tukey HSD)	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1/3ns; 1/4ns; 2/3ns; 2/4ns	1/2ns; 3/4ns; 1/3ns; 1/4ns; 2/3ns; 2/4ns	1/2ns; 3/4ns; 1/3ns; 1/4ns, 2/3ns; 2/4ns	Zhelev et al. (2020)
Site 7 (RS) Female (1) n=10 Male (2) n=10	♀: 543.900±23.21 (497.391–596.407 ♂: 659.000±43.47 (560.657–757.342)	♀: 8.02±1.59 (7.64–8.36) ♂: 7.92±2.73 (7.31–8.53)	♀: 0.35±0.01 (0.33–0.37) ♂: 0.36±0.01 (0.34–0.38)	♀: 160.28±7.99 (142.21–178.37) ♂: 142.86±12.56 (114.42–171.27)	♀: 219.14±9.38 (197.91–240.38) ♂: 216.25±4.92 (205.13–227.37)	♀: 712.46±23.34 (659.65–765.25) ♂: 647.66±31.13 (577.22–718.09)	
Site 8 (PS: NH^+_4-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu,As) Female (3) n=10 Male (4) n=10	♀: 360.100±16.79 (321.977–398.003) ♂: 355.200±14.24 (322.786–387.213)	♀: 5.57±2.63 (4.97–6.17) ♂: 5.31±2.03 (4.84–5.76)	♀: 0.23±0.01 (0.21–0.24) ♂: 0.23±0.01 (0.22–0.25)	♀: 154.08±11.49 (128.08–180.09) ♂: 149.69±6.89 (134.12–165.29)	♀: 238.58±8.89 (218.46–258.71) ♂: 227.79±7.68 (210.41–245.18)	♀: 612.65±27.62 (550.17–675.14) ♂: 651.11±29.42 (584.53–717.67)	
Statistics (one way ANOVA, F, LSD tests)	F=30.27, $1<2^{***}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=40.69, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=81.47, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=0.54, $1/2_{ns}$, $1/3_{ns}$, $1/4_{ns}$, $2/3_{ns}$, $2/4_{ns}$, $3/4_{ns}$	F=1.61, $1/2_{ns}$, $1/3_{ns}$, $1/4_{ns}$, $2/3_{ns}$, $2/4_{ns}$, $3/4_{ns}$	F=2.19, $1/2_{ns}$, $1>3^{**}$, $1/4_{ns}$, $2/3_{ns}$, $2/4_{ns}$, $3/4_{ns}$	

Table 2. The leukogram parameters (Mean±SEM, Minimum-Maximum, or confidence interval – 95%) used for evaluation of health status of *P. ridibundus* individuals from freshwater ecosystems with different degree of anthropogenic pollution in southern Bulgaria. *Legend:* Sites: Site 1 (Sazliyka River near the village Rakitnitsa), site 2 (Sazliyka River near the town Radnevo), site 3 (Topolnitsa River near the village Poibrene), site 4 (Dam Lake Studen Kladenets near the LZP "Kardzhali"), site 5 (Vacha River), site 6 (Tsalapitsa Rice Fields), site 7 (Vacha River) and site 8 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen NH^+_4-N, total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), leukocyte count (WBC), stab neutrophils (St), segmented nuclei neutrophils, total neutrophils (Ne), basophils (Ba), eosinophils (Eo), monocytes (Mo), lymphocytes (Ly). Significance codes: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns $p > 0.05$.

Sites	**Parameters**							**Publications**
	WBC (x 10^6/µl)	**St**	**Sg**	**Ba**	**Eo**	**Mo**	**Ly**	
Site 1(RS) (n=30,	2.396±0.08 (1.500–3.400)	2.40±0.23 (1–6)	7.20±0.29 (4–10)	2.13±0.18 (1–4)	1.83±0.14 (1–3)	3.40±0.35 (1–9)	83.04±0.80 (72–90)	Zhelev et al. (2013; 2015;

♀+♂)								2016a)
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=30, ♀+♂)	3.400±0.09 (2.600–4.400)	3.03±0.30 (1–8)	11.10±0.38 (5–15)	5.60±0.52 (2–10)	4.70±0.53 (1–11)	7.17±0.49 (3–13)	68.40±1.03 (57–78)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=30, ♀+♂)	4.123±0.09 (3.100–4.850)	4.80±0.26 (3–8)	3.46±0.29 (1–7)	0.77±0.08 (0–3)	0.37±0.10 (0–2)	12.30±0.62 (6–19)	78.30±0.74 (72–87)	
Site 4 (PS: Cd, Zn, Pb, Cu) (n=30, ♀+♂)	3.647±0.06 (3.200–4.300)	3.43±0.21 (2–6)	13.47±0.32 (10–17)	2.57±0.17 (2–6)	0.87±0.16 (0–3)	9.67±0.44 (5–16)	70.00±0.60 (65–75)	
Statistics (one way ANOVA, F, LSD tests)	F=48.9989, 1/4***; 2/4ns; 3/4***	F=73.3599, 1/4**; 2/4ns; 3/4***	F=92.3850, 1/4***; 2/4***; 3/4***	F=7.1650, 1/4ns; 2/4***; 3/4***	F=4.6820, 1/4*; 2/4***; 3/4ns	F=13.3772, 1/4***; 2/4***; 3/4***	F=78.2500, 1/4***; 2/4ns; 3/4***	
Site 5 (RS) Female (1) n = 25 Male (2) n=25	♀: 4.028±0.91 (3.200–4.800) ♂: 3.972±0.96 (3.200–4.900)	♀: 2.60±0.21 (1–6) ♂:2.20±0.15 (1–4)	♀: 7.68±0.23 (6–10) ♂:6.48±0.23 (5–9)	♀: 4.24±0.38 (1–6) ♂:4.20±0.26 (1–6)	♀:1.16±0.20 (0–3) ♂: 1.08±0.1 (0–3)	♀: 3.84±0.31 (1–9) ♂: 3.92±0.18 (2–5)	♀: 80.48±0.69 (71–88) ♂: 80.48±0.69 (71–88)	Zhelev et al. (2018)
Site 6 (PS: fertilizers, pesticides) Female (3) n = 25 Male (4) n = 25	♀: 2.332±0.81 (1.600–3.400) ♂: 2.340±0.10 (1.600–3.300)	♀: 5.16±0.19 (4–8) ♂:4.96±0.24 (3–8)	♀: 3.12±0.10 (2–4) ♂:3.44±0.23 (2–6)	♀: 1.04±0.18 (0–3) ♂:1.32±0.19 (0–3)	♀: 4.72±0.32 (0–6) ♂:4.72±0.32 (0–6)	♀: 14.80±0.36 (12–19) ♂: 12.80±0.44 (9–16)	♀: 71.16±0.49 (67–76) ♂: 72.28±0.49 (68–77)	
Statistics (two way ANOVA, Tukey HSD)	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1<3**, 1<4***; 2<3***; 2<4***	1>2***; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	1/2ns; 3/4ns; 1<3***; 1<4***; 2<3***; 2<4***	1/2ns; 3>4***; 1<3***; 1<4***; 2<3***; 2<4***	1/2ns; 3/4ns; 1>3***; 1>4***; 2>3***; 2>4***	
Site 7 (RS) Female (1) n = 10 Male (2) n = 10	♀: 4.680±1.23 (4.400–4.959) ♂: 4.290±1.25 (4.006–4.573)	Ne ♀: 9.10±0.31 (8.39–9.81) ♂: 9.41±0.34 (8.63–10.17)		♀: 4.50±0.56 (3.23–5.77) ♂: 3.20±0.29 (2.54–3.85)	♀: 1.0±0.33 (0.24–1.75) ♂: 0.70±0.26 (0.11–1.29)	♀: 3.60±0.41 (2.69–4.51) ♂: 4.50±0.22 (3.99–5.01)	♀: 81.80±0.80 (79.99–83.61) ♂: 82.20±0.51 (81.04–83.34)	Zhelev et al. (2020)
Site 8 (PS: NH^+_4-N, NO^-_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n = 10 Male (4) n = 10	♀: 2.130±0.63 (1.986–2.273) ♂: 2.128±1.29 (1.837–2.422)	Ne ♀: 19.60±0.42 (18.57–20.62) ♂: 20.21±0.51 (19.04–21.35)		♀: 0.70±0.21 (0.22–1.18) ♂: .50±0.17 (0.12–0.89)	♀: 4.40±0.39 (3.63–5.17) ♂: .50±0.27 (4.89–6.11)	♀: 11.80±0.42 (10.86–12.75) ♂: 11.30±0.36 (10.47–12.13)	♀: 63.50±0.65 (62.02–64.97) ♂: 62.50±0.73 (60.84–64.16)	
Statistics (one way ANOVA, F, LSD tests)	F=30.27, $1<2^{***}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=145.18, $1>2^{**}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, 3/4 ns		F=32.11, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=63.49, $1/2_{ns}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3/4_{ns}$	F=146.21, $1/2_{ns}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3/4_{ns}$	F=257.96, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	

In the study performed in the Sazliyka and Topolnitsa Rivers as well as of Studen Kladenets Dam Lake, the analyses were made with pooled samples – 30 individuals of both sexes from each population. In this study, the data obtained found the following statistically significant changes in the blood of frogs from anthropogenically contaminated sites compared to these inhabiting less disrupted habitats (site 1: reference group).

- in population 2 (site 2: domestic sewage pollution and nutrients) - erythrocytosis, hypohromia, leukocytosis, neutrophilia (with increasing Sg cells), basophilia, eosinophilia, monocytosis, lymphopenia.
- in population 3 (site 3: heavy metal pollution) - erythrocytosis, hyperchromia, leukocytosis, neutropenia, monocytosis, basopenia, eosinopenia, lymphopenia.
- in population 4 (site 4: heavy metal pollution) - erythrocytosis, hyperchromia, leukocytosis, neutrophilia (increasing St and Sg cells), monocytosis, eozinopeniya and lymphopenia.

Analyzing PCV (Hct) and the three derivative parameters: MCH, MCHC and MCV, we found increasing PCV values in each population that inhabit conditions of anthropogenic pollution, decreasing MCH values (in population 4) and MCHC (in populations 2 and 4) and an increase of MCV in population 2.

Since establishing reference values for haematological parameters in heterothermic animals is a difficult task, comparing the literature data is the only way to assess the fluctuations in their values and their dependence on environmental factors and anthropogenic pollutants. The most common cause of erythrocytosis in conditions of prolonged living in anthropogenic pollution is tissue hypoxia (Thrall et al., 2004; Allender & Fry, 2008). The increase in erythrocyte count leads to an increase in the oxygen capacity of blood and is considered a compensatory reaction to the general decrease of oxygen – hypoxemia (Randall, 1982; Randall & Perry, 1992; Lai et al., 2006). There is evidence in literature that a short stay of *P. ridibundus* in polluted industrial wastewater reduces the number of erythrocytes and the amount of haemoglobin (Romanova & Egorikhina, 2006; Vafis & Peskova, 2009). However, in long-term habitation of the species of *Rana (Pelophylax)* genus (*P. ridibundus* in particular) in an environment with persistent toxicants, the opposite effects are observed – increase of RBC counts and Hb values (Tarasenko & Tarasenko, 1988; Peskova & Zhukova, 2005; Sedalishtev, 2005; Toktamysova, 2005). In some anuran amphibians there is suppression of erythropoiesis as a result of the accumulation of heavy metals in the liver (Egorov et al., 2002; Akulenko, 2005; Chiesa et al., 2006). The results in our study for the population of *P. ridibundus* inhabiting Topolnitsa River (site 3), where pollutants are mainly heavy metals, however, show the opposite direction of change – erythrocytosis in combination with hyperchromia, a reaction that is adaptive rather than pathological. There is scarce and unsystematized data in literature on the status of the indicators PCV (Ht), MCH, MCHC and MCV in Anura, and most of the works is aimed at establishing reference values for different species (Arserim & Mermer, 2008; Gül et al., 2011; Mahapatra et al., 2012). It should be noted, however, that no environmental factor values were monitored in these works. Data on changes in PCV and the three derivative indicators in amphibian populations inhabiting conditions of anthropogenic pollution are scarce. In our previous paper (Zhelev et al., 2005) we found a statistically significant increase of PCV in populations of *P. ridibundus* inhabiting an area with well developed energy industry – TPP "Maritsa Iztok - 1" near Galabovo Town and we did not report differences in the values of the indicators MCH, MCHC and MCV in comparison with the reference group from the area of Harmanli Town (relatively clean biotope). At the same time, in the populations of *P. ridibundus* inhabiting another anthropogenically polluted region – the town of Dimitrovgrad, in the area of the chemical plant "Neochim Inc." we found a statistically significant decrease in the values of all four indicators (PCV, MCH, MCHC and MCV) in comparison with the animals from the control group. The analysis of differential leukocyte counts in *P. ridibundus* populations from four sites in Southern Bulgaria, shows that regardless of the living environment parameters in the four habitats the blood of frogs has a lymphoid character, but the quantity of the largest cell group is with highest statistical significance in the relatively non-polluted habitat (site 1) and lowest in the population from site 2 (Table 2). The found significant lymphopenia in the populations of anthropogenically polluted habitats is present on the background of statistically significant changes in the numerical ratio of all other mature white blood cells in the peripheral blood of the animals. This shows the general mobilization of the body's defenses. The changes in the differential leukocyte count of animal blood from population 2 (site 2) were referred to the so-called first type of reaction (adaptive), according to the classification proposed by Peskova (2001). Similar changes in the differential leukocyte count in Anura, in the presence of toxins of anthropogenic origin, have been found by other authors as well (Romanova & Romanova, 2003; Cabagna et al., 2005; Romanova & Egorikhina, 2006; Peskova & Sharpan, 2007; Peskova & Vafis, 2007; Zhelev, 2007; Mineeva & Mineev, 2011; Shutler & Marcogliese, 2011). The changes in the differential leukocyte count in population 3 (site 3) were referred to the second type of reaction by the terminology of Peskova (2001), according to which these changes are a result of serious violations in the physiological functions of the body (even these of pathological nature) under the influence of toxins. Similar changes in the differential formula in Anura, inhabiting conditions of toxicosis have also been reported by (Isaeva & Vyazov, 1997; Zhykova & Peskova, 1999; Cabagna et al., 2005; Davis et al. 2008). The changes in the leukogram in *P. ridibundus* individuals that inhabit in site 4 (Studen Kladenets Dam Lake) rather, they could be attributed to the so-called first type of reaction according to Pescova's (2001) classification. The results from the discriminant analysis (see Fig. 1) group together populations 2 and 4 and at the same time clearly separate them not only from population 1 but also from population 3. This is undoubtedly an interesting fact considering the nature of the toxicants present in Studen Kladenets Dam Lake – heavy metals (for details see Zhelev et al., 2015). The same type of pollutants is present in the habitat inhabited by population 3 (for details see Zhelev et al., 2013). The conclusion to be drawn is that with the same nature of toxicants, the disturbances in the physiological condition of the animals from the respective populations of *P. ridibundus* are different – in the Topolnitsa River they are more serious. The reason for the reported differences in the state of *P. ridibundus* populations can be traced to the type of water body and the concentration of toxicants. In closed water bodies (such as Studen Kladenets Dam Lake) the lack of currents and larger sizes create conditions for the accumulation of heavy metals in the bottom sediments, while in shallower and faster rivers this is not always the case. It is quite possible that the changes in the blood of *P. ridibundus* from the population inhabiting the Topolnitsa River (population 3) were preceded by changes similar to those found in the populations of the anthropogenically transformed habitats of the Sazliyka River (population 2) and Studen Kladenets Dam Lake (population 4). This makes it extremely important to establish a thin borderline between the changes characterizing the unique "battle phase" in the blood of amphibians, expressed in the mobilization of the body's defenses, and the threshold of toxicants leading to the predominance of degenerative changes of pathological nature. Of course, this task is complicated by the fact that in natural reservoirs of different types, along with the action of the anthropogenic factor, the effect of the combination of a large number of environmental factors must be taken into account. Such a direction outlines prospects for further research not only of fundamental, but also of strictly applied nature for the needs of bioindication and biomonitoring.

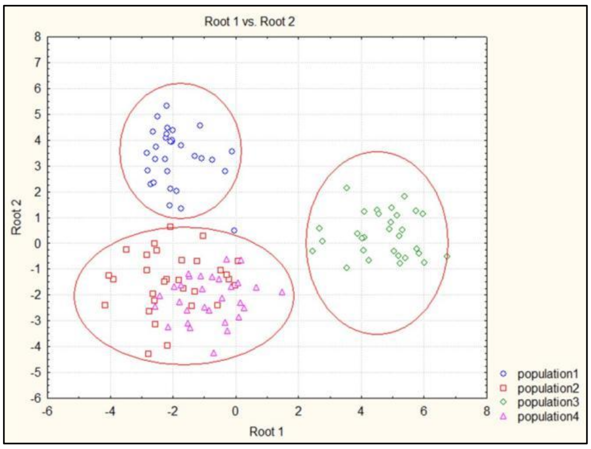

Fig. 1. Discriminatory coordinates by six most informative parameters (RBC, PCV, WBC, Sg, Ba, and Eo) of individuals from the four compared *Pelophylax ridibundus* populations from the sites with different levels of anthropogenic pollution in Southern Bulgaria. This diagram is published in Zhelev et al. (2015).

Studies conducted in anthropogenically polluted habitats of Tsalapitsa Rice Fields (pesticides and fertilizers) and the Chaya River (heavy metals), were carried out taking into account the sex of the frogs. The values of the studied haematological markers were compared with those obtained for the respective number of individuals of both sexes inhabiting the reference site (the Vacha River). Significant differences in the values of tested haematological markers were established in the blood of the frogs (in individuals of both sexes) inhabiting the Tsalapitsa Rice Fields compared to such values in the blood of frogs from the reference site. In our view, the erythropenia, leucopenia, hypochromia, lower values of PCV, St-neutrophilia, Sg-neutropenia, basopenia, eosinophilia, monocytosis and lymphopenia that were found in *Pelophylax ridibundus* individuals inhabiting the Tsalapitsa Rice Fields were probably caused by the pesticides and fertilizers that enter the paddy cages during the rice production process. These results are an indication of severely deteriorated health status of frogs inhabiting in the Tsalapitsa Rice Fields. This conclusion is also confirmed by the results of the discriminant analysis (Fig. 2). The spatial distribution of factor loadings separates in two clearly differentiated "clouds": the individuals of *P. ridibundus* from Tsalapitsa Rice Fields and also these from the Vacha River (for details see Zhelev et al., 2018).

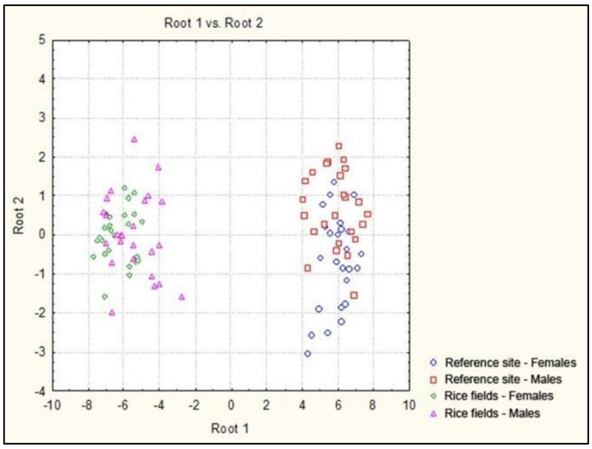

Fig. 2. Plot (discriminatory coordinates for haematological parameters of the importance of canonical discriminant function of the *Pelophylax ridibundus* individuals from the investigated sites (Tsalapitsa Rice Fields – polluted site and the Vacha River – reference site). Root 1 – the first canonical function Root 2 – the second canonical function. This diagram is published in Zhelev et al. (2018).

The studies of the populations' of *P. ridibundus*, inhabiting the industrial zone of the city of Plovdiv City and the Chaya River which flows through it, were conducted with a smaller number of test animals (10 individuals of both sexes). This number was consistent with analyzes for the bioaccumulation of heavy metals in the tissues of these frogs (muscle and liver). The values of the tested haematological markers were compared to these obtained for the same number of individuals inhabiting in the reference site: the Vacha River (see Tables 1 and 2). In addition to haematological parameters, a set of other morphophysiological markers were used in this study, namely: body condition index and organ indexes: hepatosomatic index, spleenosomatic index and renosomatic index.

The general adaptation syndrome theory (Selye, 1956), considers the physiological response of each organism to stress as a process involving three stages: During the first stage, the body's defenses are mobilized (alarm stage). The second stage is characterized by general adaptation of the body’s defenses and developing mechanisms in response to stressors (resistance stage). In the third stage, as a result of the chronic action of toxicants (or an increase in their concentration over time), the adaptive potential of the organism is depleted, its energy resources are reduced and death occurs (exhaustion stage). The results of this research showed that the exhaustion stage was reached for the frogs inhabiting an area of industrial pollution in Southern Bulgaria, and an impossibility to cope with the force and enduring action of the toxicants present in the environment was observed as well. Low body weight combined with values of the body condition index is observed in the frogs of both sexes inhabiting the Chaya River and they are anemic (erythropenia and hypochromia), their immunity is weakened (leucopenia, neutrophilia, monocytosis, lymphopenia and lower values of the spleenosomatic index) and the functions of their livers and kidneys are deteriorated (for details see Zhelev et al., 2020). The results of the discriminant analysis clearly differentiate and separate the *P. ridibundus* individuals in the Chaya River from these inhabiting the reference site (see Fig. 3).

Variations in the size and shape of erythrocytes (and their nuclei) in anurans have been very poorly studied. Single studies have focused on changes in erythrocyte morphology in anurans populations inhabiting conditions of anthropogenic stress (Zhelev et al., 2006; Omelykovets & Berezyuk, 2009; Bondarieva et al., 2012; Salinas et al., 2017; Dönmez & Şişman, 2021).

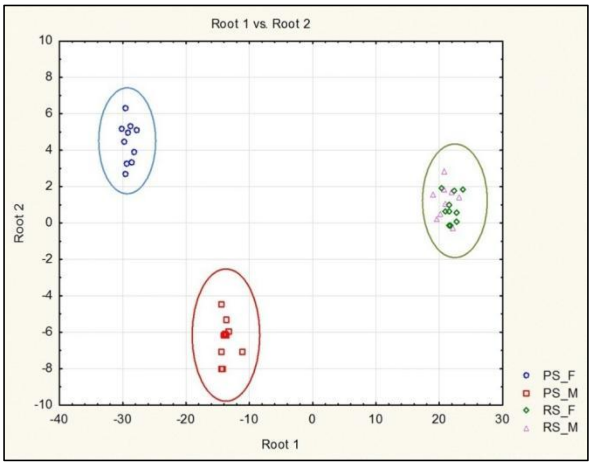

Fig. 3. Canonical discriminant functions scatter plot of tested biological parameters of *Pelophylax ridibundus* specimens from the sites studied. This diagram is published in Zhelev *et al.* (2020). *Legend:* Polluted siete (PS): the Chaya River, Reference site (RS): the Vacha River, F: females, M: males.

Although the authors of those works report changes in the size and shape of erythrocytes in anurans from polluted ecosystems, when comparing them to populations of frogs inhabiting background territories, there are no categorical opinions regarding the causes of the observed changes or their biological role.

Tables 3 and 4 present the results of our research, conducted in *P. ridibundus* populations that inhabit five water bodies in Southern Bulgaria (2 rivers and 3 reservoirs) with different degrees and types of anthropogenic pollution (less disrupted water bodies, domestic sewage pollution and heavy metal pollution).

Table 3. Erythrocyte cells measurements (Mean ± SEM values and their confidence interval – 95%) in *Pelophylax ridibundus* individuals from investigated sites in Southern Bulgaria and results from their comparisons. The signs > and <are used to compare the mean values of the parameters. *Legend*: Sites: Site 1 (Sazliyka River below the village of Rakitnitsa), site 2 (Sazliyka River below the town of Radnevo), site 3 (Topolnitsa River below the village of Chavdar), site 4 (Topolnitsa River below the village of Poibrene), site 5 (Vacha dam lake), site 6 (Rozov Kladenets dam lake), site 7 (Studen Kladenets dam lake), site 8 (Vacha River), site 9 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD$_5$), nitrite nitrogen (NO^{-}_{2}-N), nitrate nitrogen (NO^{-}_{3}-N), ammonium nitrogen (NH^{+}_{4}-N), total nitrogen (TN), orthophosphates (PO^{3-}_{4}), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), erythrocyte length (EL), erythrocyte width (EW), erythrocyte size (ES). Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05.

Sites	Parameters				Publications
	EL (μm)	EW (μm)	EL/EW ratio	ES (μm^2)	
Site 1 (RS) (n=15, ♀+♂)	24.01±0.07 (23.87–24.15)	13.90±0.05 (13.79–14.00)	1.73±0.01 (1.72–1.74)	263.21±1.75 (259.78–266.63)	Zhelev et al. (2017b)
Site 2 (PS: BOD$_5$, NO_2-N, NO_3-N, TN, PO^{3}_{4}) (n=15, ♀+♂)	24.98±0.08 (24.81–25.14)	14.50±0.07 (14.37–14.63)	1.73±0.01 (1.72–1.74)	286.57±2.32 (282.00–291.13)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	22.61±0.05 (22.51–22.71)	13.76±0.03 (13.69–13.82	1.64±0.01 (1.63–1.65)	244.63±1.02 (242.63–246.63)	
Site 4 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	22.28±0.06 (22.15–22.41)	13.65±0.05 (13.55–13.74)	1.63±0.01 (1.63–1.64)	239.79±1.49 (236.87–242.72)	
Site 5 (RS) (n=15, ♀+♂)	24.45±0.09 (24.27–24.62)	14.15±0.06 (14.03–14.27)	1.73±0.01 (1.72–1.74)	273.71±2.24 (269.32–278.11)	
Site 6 (PS: BOD$_5$, NO^{-}_{2}-N, NO^{-}_{3}-N, TN, PO^{3-}_{4}) (n=15, ♀+♂)	24.79±0.08 (24.63–24.95)	14.32±0.06 (14.20–14.44)	1.74±0.01 (1.73–1.75)	280.81±2.19 (276.51–285.12)	
Site 7 (PS: Cd, Zn, Pb, Cu) (n=15, ♀+♂)	22.50±0.06 (22.39–22.61)	13.59±0.05 (13.49–13.67)	1.66±0.01 (1.65–1.67)	240.98±1.35 (238.33–243.63)	
Statistics (one way ANOVA, F, LSD tests)	F=254.322, (1.2 = 4.1) > 1.1 > (2.1 = 5.1) > 2.2, (1.2 = 4.1) > 3.1 > (2.1 = 5.1) > 2.2, 1.2 > (2.1 = 5.1) > 2.2, 4.1 > (2.1 = 5.1) > 2.2	$F_{6,\ 4193}$ =42.262, 1.2 > 4.1 > 1.1 > (2.1 = 2.2) > 5.1, 1.2 > 4.1 > 3.1 > (2.1 = 2.2) > 5.1, 1.2 > (2.1 = 2.2) > 5.1, 4.1 > (2.1 = 2.2) > 5.1	F=136.312, (1.1 = 1.2 = 4.1) > 5.1 > (2.1 = 2.2), (3.1 = 1.2 = 4.1) > 5.1 > (2.1 = 2.2), 1.2 > 5.1 > (2.1 = 2.2), 4.1 > 5.1 > (2.1 = 2.2)	F=116.513, 1.2 > 4.1 > 1.1 > (2.1 = 2.2 = 5.1), 1.2 > 4.1 > 3.1 > (2.1 = 2.2 = 5.1), 1.2 > (2.1 = 2.2 = 5.1), 4.1 > (2.1 = 2.2 = 5.1)	
Site 8 (RS) Female (1) n = 10 Male (2) n = 10	♀: 24.35±0.09 (24.15–24.55) ♂: 24.23±0.10 (24.03–24.42)	♀: 14.15±0.07 (14.01–14.29) ♂: 14.14±0.07 (13.99–14.29)	♀: 1.73±0.01 (1.71–1.74) ♂: 1.72±0.01 (1.71–1.73)	♀: 272.22±2.46 (267.39–277.05) ♂: 270.89±2.59 (265.78–275.99)	Zhelev et al. (2021)
Site 9 (PS: NH^{+}_{4}-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n = 10 Male (4) n = 10	♀: 22.47±0.06 (22.35–22.59) ♂: 22.54±0.07 (22.40–22.67)	♀: 13.52±0.04 (13.52–13.69) ♂: 13.65±0.05 (13.55–13.75)	♀: 1.65±0.01 (1.64–1.66) ♂: 1.65±0.01 (1.64–1.66)	♀: 240.56±1.29 (238.02–243.09) ♂: 242.31±1.49 (239.37–245.25)	
Statistics (one way ANOVA, F, LSD tests)	F=148.091, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=24.074, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=65.079, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	F=72.719, 1/2$_{ns}$, 1>3***, 1>4***, 2>3***, 2>4***, 3/4$_{ns}$	

Table 4. Erythrocyte nuclei measurements (Means±SEM and their Confidence – 95%) in *Pelophylax ridibundus* individuals from investigated sites in Southern Bulgaria and results from their comparisons. The signs > and <are used to compare the mean values of the parameters. *Legend:* Sites: Site 1 (Sazliyka River below the village of Rakitnitsa), site 2 (Sazliyka River below the town of Radnevo), site 3 (Topolnitsa River below the village of Chavdar), site 4 (Topolnitsa River below the village of Poibrene), site 5 (Vacha dam lake), site 6 (Rozov Kladenets Dam Lake), site 7 (Studen Kladenets Dam Lake), site 8 (Vacha River), site 9 (Chaya River). Abbreviations: Reference site (RS), Polluted site (PS), number of individuals (n), biological oxygen demand five days (BOD_5), nitrite nitrogen (NO^-_2-N), nitrate nitrogen (NO^-_3-N), ammonium nitrogen (NH^+_4-N), total nitrogen (TN), orthophosphates (PO^{3-}_4), copper (Cu), iron dissolved in water (Fe), lead (Pb), manganese (Mn), arsenic (As), cadmium (Cd), zinc (Zn), nucleus length (NL), nucleus width (NW), nucleus size (NS). Significance codes: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05.

Sites	Parameters					Publications
	NL (µm)	NW (µm)	NL/NW ratio	NS (µm^2)	NS/ES ratio (µm^2)	
Site 1 (RS) (n=15, ♀+♂)	9.67±0.05 (9.56–9.77)	5.65±0.03 (5.58–5.71)	1.73±0.01 (1.71–1.74)	43.39±0.44 (42.53–44.24)	0.17±0.002 (0.16–0.17)	Zhelev et al. (2017b)
Site 2 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	9.78±0.05 (9.68–9.89)	5.64±0.03 (5.59–5.70)	1.74±0.01 (1.73–1.76)	43.73±0.40 (42.94–44.51)	0.16±0.002 (0.15–0.16)	
Site 3 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	8.08±0.05 (7.99–8.17)	5.14±0.03 (5.09–5.19)	1.58±0.01 (1.56–1.59)	33.01±0.34 (32.35–33.67)	0.13±0.001 (0.13–0.14)	
Site 4 (PS: Cu, Fe, Pb, Mn, As) (n=15, ♀+♂)	8.28±0.05 (8.18–8.37)	5.20±0.03 (5.15–5.27)	1.60±0.01 (1.58–1.61)	34.22±0.34 (33.54–34.90)	0.14±0.001 (0.14–0.15)	
Site 5 (RS) (n=15, ♀+♂)	9.62±0.05 (9.53–9.72)	5.39±0.03 (5.33–5.44)	1.80±0.01 (1.78–1.82)	41.07±0.37 (40.35–41.79)	0.15±0.001 (0.15–0.16)	
Site 6 (PS: BOD_5, NO^-_2-N, NO^-_3-N, TN, PO^{3-}_4) (n=15, ♀+♂)	9.79±0.05 (9.70–9.88)	5.53±0.03 (5.47–5.58)	1.79±0.01 (1.77–1.81)	42.71±0.34 (42.04–43.39)	0.16±0.001 (0.15–0.16)	
Site 7 (PS: Cd, Zn, Pb, Cu) (n=15, ♀+♂)	8.43±0.05 (8.34–8.53)	5.31±0.03 (5.26–5.36)	1.59±0.01 (1.58–1.61)	35.58±0.35 (34.89–36.27)	0.14±0.001 (0.14–0.15)	
Statistics (one way ANOVA, F, LSD tests)	F=252.757, (1.1 = 1.2 = 4.1) > 5.1 > 2.2 > 2.1, (1.2 = 4.1) > 3.1 > 5.1 > 2.2 > 2.1, 1.2 > 5.1 > 2.2 > 2.1, 4.1 > 5.1 > 2.2 > 2.1	F=54.115, (1.1 = 1.2) > 4.1 > 5. 1 > (2.1 = 2.2), 1.2 > 4.1 > 3.1 > 5. 1 > (2.1 = 2.2), 1.2 > 5. 1 > (2.1 = 2.2), 4.1 > 5.1 > (2.1 = 2.2)	F=145.099, 4.1 > (1.1 = 1.2) > (2.1 = 2.2 = 5.1), (3.1 = 4.1) > 1.2 > (2.1 = 2.2 = 5.1), 1.2 > (2.1 = 2.2 = 5.1), 4.1 > (2.1 = 2.2 = 5.1)	F=158.168, (1.1 = 1.2 = 4.1) > 5.1 > 2.2 > 2.1, (1.2 = 4.1) > 3.1 > 5.1 > 2.2 > 2.1, 1.2 > 5.1 > 2.2 > 2.1, 4.1 > 5.1 > 2.2 > 2.1	F=38.608, 1.1 > (1.2 = 1.4) > (2.2 = 5.1) > 2.1, (1.2 = 4.1) > 3.1 > (2.2 = 5.1) > 2.1, 1.2 > (2.2 = 5.1) > 2.1, 4.1 > (2 = 5.1) > 2.1	
Site 8 (RS) Female (1) n=10 Male (2) n = 10	♀: 9.58±0.06 (9.44–9.71) ♂: 9.65±0.06 (9.53–9.78)	♀: 5.49±0.04 (5.41–5.57) ♂: 5.54±0.04 (5.47–5.62)	♀: 1.76±0.01 (1.73–1.78) ♂: 1.75±0.01 (1.73–1.77)	♀: 41.76±0.51 (40.75–42.78) ♂: 42.51±0.51 (41.51–43.52)	♀: 0.16±0.002 (0.15–0.17) ♂: 0.16±0.002 (0.15–0.17)	Zhelev et al. (2021)
Site 9 (PS: NH^+_4-N, NO_3-N, NO_2-N, Pb, Cd, Zn, Cu, As) Female (3) n=10 Male (4) n=10	♀: 8.14±0.05 (8.02–8.25) ♂: 8.48±0.06 (8.35–8.61)	♀: 5.20±0.03 (5.14–5.26) ♂: 5.35±0.03 (5.28–5.41)	♀: 1.57±0.01 (1.55–1.59) ♂: 1.59±0.01 (1.57–1.60)	♀: 33.64±0.41 (32.82–34.45) ♂: 36.13±0.45 (35.23–37.02)	♀: 0.14±0.001 (0.13–0.15) ♂: 0.15±0.002 (0.14–0.16)	
Statistics (one way ANOVA, F, LSD tests)	F=148.181, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{***}$	F=18.807, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{**}$	F=114.870, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$	F=82.438, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{***}$	F=18.219, $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3<4^{**}$	

The analysis of the data showed that the changes in metric parameters of erythrocytes depend on the concentration of toxic substances and their type, and to a lesser extent show dependence on the type of water body. We found a statistically significant increase in the parameters: erythrocyte length (EL), erythrocyte width (EW) and erythrocyte size (ES) in populations of *P. ridibundus* inhabiting water bodies with domestic sewage pollution, compared to those of control groups from reference sites. This leads to a change in the shape of erythrocyte cells in these frogs which takes an elongated-elliptical shape (see Fig. 4). At the same time, in these populations of *P. ridibundus* the basic nuclear parameters (NL: nucleus length; NW: nucleus width and NS: nucleus size) are unchanged compared to the control groups. The nuclei retain their typical elliptical shape. In *P. ridibundus* populations inhabiting water bodies contaminated with heavy metals, the parameters EL, EW, ES, NL, NW and NS decrease compared to the control groups. The cells and nuclei were rounded (oval or spherical shape). In the populations of anthropogenically polluted water bodies the NS / ES decreases in comparison with the control groups, regardless of the type of toxicants (for details see Zhelev et al., 2018).

Our studies in the populations of *P. ridibundus* inhabiting habitats (sites) 1, 2 and 3 (see Tables 3 and 4) were continued in a seasonal aspect: analyses were performed in these habitats not only in the spring but also in the summer and autumn. The results of these studies are published in Zhelev et al. (2016b). Although some fluctuations in the metric parameters of erythrocyte cells and their nuclei were observed in the populations of *P. ridibundus* from the two anthropogenically contaminated habitats (sites 2 and 3), the changes observed in the spring persisted during the other two seasons. In the population from the habitat with domestic sewage pollution (site 2) the cells had a typically elliptical shape, but the ellipse became a little more elongated in summer and autumn.

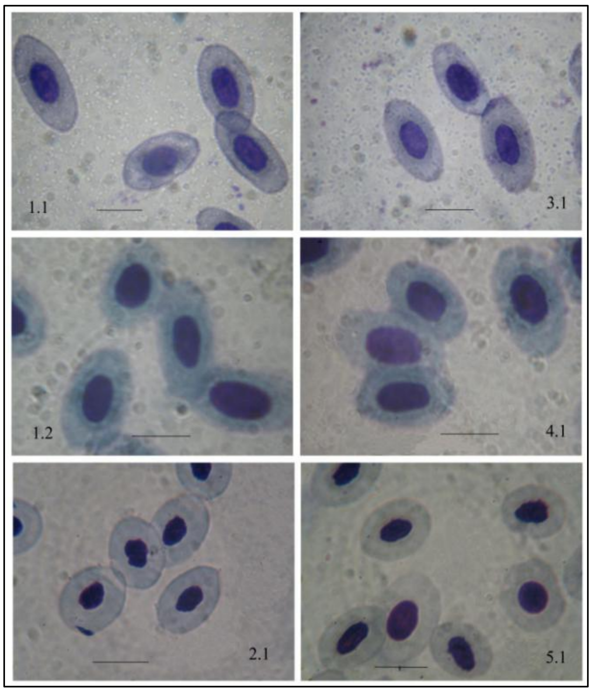

Fig. 4. Photomicrographs of erythrocytes of *Pelophylax ridibundus* populations from the investigated water bodies (*rivers and **reservoirs) in Southern Bulgaria: (1.1*, 3.1**) - less disrupted, (1.2*, 4.1**) - domestic sewage polluted and (2.1*, 5.1**) - heavy metal polluted water basins. Scale lines = 10 μm. Legend: 1.1 – the river Sazliyka below the village of Rakitnitsa, 1.2 – the river Sazliyka below the town of Radnevo, 2.1 – the river Topolnitsa below the village of Chavdar, 2.2 – the river Topolnitsa below the village of Poibrene, 3.1 – the Vacha reservoir, 4.1 – the Rozov Kladenets reservoir and 5.1 – the Studen Kladenets reservoir. This photos is published in Zhelev et al. (2018).

The nuclei had a typical oval shape, but some elongation of the ellipse also occurred (NW values were close to those from the less disrupted one, NL/NW became significantly higher in the population during summer and autumn). Throughout spring to autumn, NS/ES decreased in comparison with the less disrupted group (site 1). In the population from the habitat with heavy metal pollution (site 2), the values of all 9 cellular and nuclear parameters were the lowest throughout the investigation. EL, EW, NL, NW and ES experienced a progressive and statistically significant decrease during the seasonal transitions. EL/EW significantly increased throughout summer and showed a statistically significant decrease again in autumn. NL/NW experienced no changes in spring and declined in autumn. NS/ES reached its peak in spring, and significantly decreased during the course of the other two seasons. The shape of cells and nuclei became slightly more rounded; this was most pronounced during spring (for details see Zhelev et al., 2016b). The changes in the sizes of erythrocyte cells and their nuclei, found in the populations of *P. ridibundus* inhabiting the anthropogenically polluted sites (2 and 3), were confirmed in the study conducted in the populations of *P. ridibundus,* inhabiting the industrial zone of Plovdiv City (see Tables 3 and 4). In the blood of frogs from the anthropogenically polluted habitat of Chaya River (heavy metals, nitrates, nitrites and ammonium), oval and spherical erythrocytes circulate. Also, a significant reduction was found in the nucleus / cytoplasm ratio of the blood of these frogs (for details see Zhelev et al., 2021). The results from the PCA analysis confirmed that the differences between frogs from the polluted site (the Chaya River) and the reference site (the Vacha River) are mainly due to changes affecting the two main parameters of erythrocyte cells and their nuclei (length and width), and also to changes in nucleus sizes and nucleocytoplasmic ratio (Fig. 5).

We believe that the changes in the morphology of erythrocyte cells and their nuclei, found in the populations of *P. ridibundus* inhabiting anthropogenically polluted habitats in Southern Bulgaria (sites, 3, 4, 7 and 9 – see tables 3 and 4), can be considered adaptations increasing the contact surface and oxygen capacity of erythrocytes in conditions of hypoxia. Although these changes are essentially aimed at adaptation and a better life in an environment with deteriorating parameters, we cannot say for sure that this is actually taking place in these populations.

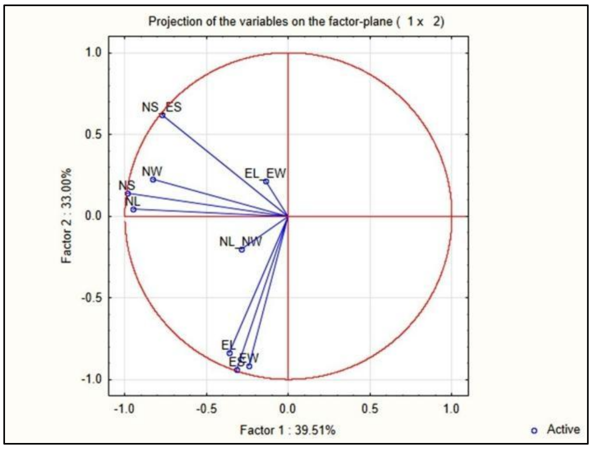

Fig. 5. Ordination on the two canonical variables (Factor 1) and (Factor 2) for tested erythrocyte-metric parameters in *Pelophylax ridibundus* specimens. The distinguishing force of the parameter is indicated by the arrow length; a large importance is shown by the long arrow and it is strongly correlated with the ordination axes. This diagram is published in Zhelev et al. (2021).

We believe that in order to be able to adequately assess the effects of morphological changes in the erythrocytes of these animals, they must be combined with analyzes of other physiological (in particular haematological) parameters. This thesis is supported by the results obtained in the analysis of a complex of morphophysiological parameters in the study conducted in the populations of *P. ridibundus* from the Chaya River (site 9). In these frogs, we found anemic changes (erythropenia and hypochromia) and weakened immunity (neutrophilia, eosinophilia, monocytosis, basopenia, and lymphopenia) compared to the frogs from the control group (site 8). The changes indicate a severe deterioration of the health status of these frogs (for details see Zhelev et al., 2020). On the other hand, it is very difficult to unambiguously assess the effects of changes in erythrocyte sizes in the polluted sites 2 and 5 (large erythrocytes with an elongated elliptical shape of the cell). There are interesting studies on birds in literature (Nadolski et al., 2006; Banbura et al., 2007; Janiga et al., 2017) and deep-sea marine mammals (Hedrick & Duffield, 1991; Promislow, 1991; Debey & Pyenson, 2013) where it is reported that large erythrocytes, combined with high haemoglobin and haematocrit values, may also be adaptive. In birds, they give a better chance of survival to young chicks, and in marine mammals, they supply cells slower and longer with oxygen when diving to great depths. Despite these examples, we believe that the effects of changes in erythrocyte size and shape found in homothermic animals should not be unambiguously interpreted and extrapolated to heterothermic animals (such as anurans). This would not be correct, at least because of the differences in the mechanisms of metabolism and the levels of its regulation in these different groups of animals (Kozłowski et al., 2010; Adrian et al., 2016).

The results of our studies provide conclusive evidence that erythrocyte measurements can be successfully used as biomarkers of physiological stress. They support the view of Davis & Maerz (2008a; 2008b), that erythrocyte sizes combined with other haematological parameters may be an objective marker for assessing environmental stress levels in amphibian populations living in anthropogenically contaminated habitats. However, we believe that the specific lifestyle of different groups of amphibians, as well as the characteristics of their habitat, should be taken into account when the morphology of blood cells (in combination with other blood parameters) is used to diagnose their health (physical fitness). The results of our studies once again emphasize the importance of haematological studies, including the morphology of blood cells as reliable biomarkers in the field of ecotoxicological studies. This necessitates rethinking of studies aimed at presenting "reference values" for quantitative (RBC and WBC counts), qualitative blood parameters (haemoglobin concentration haematocrit value and derivative parameters such as haematological indexes: MCH, MCHC and MCV), and for erythrocyte sizes. In our opinion, these studies would not be correct without providing data on the state of physio-chemical characteristics of the environment as well as tracing the possible presence of anthropogenic stressors in the specific habitat. Based on our results from studies with *P. ridibundus* test-subjects, we believe that the analysis of environmental factors is particularly necessary for analyses performed with water frogs, which spend a significant portion of their lives in aquatic habitats.

Conclusions

As a result of our long-term *in situ* studies in populations of marsh frog *P. ridibundus* inhabiting anthropogenically polluted water ecosystems in Southern Bulgaria, we find that the combination of erythrogram and leucogram parameters provides a powerful and objective tool for diagnosing their health status (physical fitness). Changes in the size of erythrocyte cells and their nuclei is also a promising biomarker for assessing the effects of environmental stress, but for a better understanding of the physiological changes occurring in the body of amphibians inhabiting conditions of chronic anthropogenic pollution, in our opinion these analyses should be combined with studies of other blood parameters, such as RBC, WBC count, Hb concentration, Hct value and differential leukocyte count. Of course, this type of analysis cannot completely replace the routine physio-chemical analyses used in the monitoring of ecological quality of water ecosystems, but they can provide sufficiently reliable supplementary information. In some cases, especially when looking for the long-term effects of toxic agents on biota, this information may be not only useful but also much more plausible than the one obtained from physio-chemical monitoring. Physio-chemical analysis gives a "snapshot" of the state of the waterbody – at the time of sampling, and haematological analysis assesses the general health status of frogs which is a total result of the long-term effects of contaminants on their body.

Acknowledgments

This research was supported by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science.

References

Addy, K., Green, L., & Herron, E. (2004). *pH and alkalinity*. Kingston, Australia: University of Rhode Island.

Adrian, G.J., Czarnoleski, M., & Angilletta, M.J. (2016). Flies evolved small bodies and cells at high or fluctuating temperatures. *Ecology and Evolution, 6*(22), 7991-7996. doi: 10.1002/ece3.2534.

Akulenko, N.M. (2005). Histological changes in the liver of green frogs *Rana esculenta* complex (Amphibia) anthropogenic landscapes. *Scientific statements, 21*(3), 76-78. (In Russian).

Allender, M.C., & Fry, M.M. (2008). Amphibian hematology. *Veterinary Clinics of North America, 11*(3), 463-480. doi: 10.1016/j.cvex.2008.03.006.

Álvarez-Mendoza, F.J., Tamez-Cantú, E.M., Lazcano, D., Setser, K.W., & Mociño-Deloya, E. (2011). Morfología de las células sanguíneas y perfil leucocitario de *Crotalus polystictus* (Cope 1865). *Ciencia UANL, 14*(1), 53-59.

Amphibia Web. (2021). Information on amphibian biology and conservation. Retrieved from amphibiaweb.org.

Arikan, H, & Çiçek, K. (2014). Haematology of amphibians and reptiles: a review. *North Western Journal of Zoology, 10*(1), 190-209.

Arserim, S., & Mermer, A. (2008). Hematology of the Uludağ frog *Rana macrocnemis* Boulenger, 1885 in Uludağ National Park (Bursa, Turkey). *Turkish Journal of Fisheries & Aquatic Sciences, 25*(1), 39-46.

Artacho, P., Soto-Gamboa, M., Verdugo, C. & Nespolo, R.F. (2007). Using haematological parameters to infer the health and nutritional status of an endangered black-necked swan population. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 147*(4), 1060-1066. doi: 10.1016/j.cbpa.2007.03.017.

Atatür, M.K., Arikan, H., & Çevik, İ.E. (1999). Erythrocyte sizes of some anurans from Turkey. *Turkish Journal of Zoology, 23*(2), 111-114.

Banbura, J., Banbura, M., Kalinski, A., Skwarska, J., Slomczynski, R., Wawrzyniak, J., & Zielinski, P. (2007). Habitat and year-to-year variation in haemoglobin concentration in nestling blue tits *Cyanistes caeruleus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 148*(3), 572-577. doi: 10.16/j.cdpa.2007.07.008.

Banerjee, V. (1979). Some aspects of haematology of *Bufo melanostictus* with relation to body weight. *Journal of the Zoological Society of India, 31*(1/2), 25-28.

Bannikov, A.G., Darevskii, I.S., Ishtenko, V.G., Rustamov, A.K., & Shterbak, N.N. (1977). *A Guide to the Amphibians and Reptiles of the USSR*. Moscow, Russian Federation: Prosveshtenie. (In Russian).

Barni, S., Boncompagni, E., Grosso, A., Bertone, V., Freitas, I., Fasola M., & Fenoglio, C. (2007). Evaluation of *Rana* snk *esculenta* blood cell response to chemical stressors in the environment during the larval and adult phases. *Aquatic Toxicology, 81*(1), 45-54. doi: 10.1016/j.aquatox.2006.10.012.

Baskurt, O.K., & Meiselman, H.J. (2003). Blood rheology and hemodynamics. *Seminars in Thrombosis and Hemostasis 29*(5), 435-450. doi: 10.1055/s-2003-44551.

Bennett, M.F., & Daigle, K.R. (1983). Temperature, stress and the distribution of leukocytes in red-spotted newts *Notophthalmus viridescens*. *Journal of Comparative Physiology Part A, 153*, 81-83.

Bern Convention. (1979). Convention on the Conservation of European Wildlife and Natural Habitats, Bern, 19.IX.1979. Retrieved from conventions.coe.int.

Beschkov, V., & Nanev, K. (2002). *Amphibians and reptiles in Bulgaria.* Sofia & Moscow, Bulgaria: Pensoft (In Bulgarian).

Biological Diversity Act. (2002). *State Gazette, 77*, 09.08.2002. (In Bulgarian).

Biserkov, V., Naumov, B., Tchankov, N., Stoyanov, A., Petrov, B., Dobrev, D., & Stoev, P. (2007). *A field guide to Amphibians and Reptiles of Bulgaria.* Sofia, Bulgaria: Green Balkans. (In Bulgarian).

Bondarieva, A.A., Bibik, Yu.S., Samilo, S.M., Shabanov, D.A. (2012). Erythrocytes cytogenetic characteristics of green frogs from Siversky Donets centre of *Pelophylax esculentus* complex diversity. *The Journal of Karazin Kharkiv Natural University, 15*(1008), 116-123. (In Russian).

Briggs, C., & Bain, B.J. (2012). Basic haematological techniques. In Bain, B.J., Bates, I., Laffan, M.A., & Lewis, S.M. (Eds.). *Dacie and Lewis Practical Haematology* (11th Edition, 23-56). London, England: Churchill Livingstone.

Brodeur, J.C., Bahl, M.F., Natale, G.S. & Poliserpi, M.B. (2020). Biomarker and hematological fieldwork with amphibians: is it necessary to sample all night? *Environmental Science and Pollution Research, 27*, 17152-17161. doi: 10.1007/s11356-020-08313-2.

Brown, B. (1980). *Hematology: Principles and procedures.* Philadelphia, USA: Lea and Febiger Publishing House.

Bunjerdluk, K., Kitana, J., Pomchote, P. (2021). Blood cell morphology and leukocyte profile of the Himalayan Newt *Tylototriton verrucosus* Anderson, 1871 (Urodela: Salamandridae) in Thailand. *Tropical Natural History, 21*(2), 274-284.

Cabagna, M.C., Lajmanovich, R.C., Stringhini, G.A., Sanchez-Hermandes J.C., & Peltzer, P.M. (2005). Hematological parameters of health status in the common toad *Bufo arenarum* in the agroecosystems of Santa Fe province, Argentina. *Applied Herpetology, 2*(4), 373-380. doi: 10.1163/157075405774483085.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., & Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment, 247*(2-3), 295-311. doi: 10.1016/s0048-9697(99)00499-4.

Campbell, T.W. (2004). Hematology of amphibians. In: Thrall, M.A. (Ed.). *Veterinary hematology and clinical chemistry* (2nd Edition, 291-297). Philadelphia, USA: Lippincott Williams & Wilkins.

Chien, S. (1987). Red cell deformability and its relevance to blood flow. *Annual Review of Physiology, 49*, 177-192. doi: 10.1146/annurev.ph.49.030187.001141.

Chiesa, M.E., Rosenberg, C.E., Fink, N.E., & Salibian, A. (2006). Serum protein profile and blood cell counts in adult toads *Bufo arenarum* (Amphibia: Anura: Bufonidae): effects of sublethal lead acetate. *Arhives of Environmental Contamination and Toxicology, 50*(3), 384-391.

Cooper, E.L., Wright, R.K., Klempau, A.E., & Smith, C.T. (1992). Hibernation alters the frog's immune system.

Cryobiology, 29(5), 616-631. doi: 10.1016/0011-2240(*92*)90066-b.

Coppo, J.A., Mussart, N.B., Fioranelli, S.A., & Zeinsteger, P.A. (2005). Blood and urine physiological values in captive bullfrog *Rana catesbeiana* (Anura: Ranidae). *Analecta Veterinaria, 25*(1), 15-17.

Corduk, N., Hacioglu-Dogru, N., Gul, C., & Tosunoglu, M. (2018). Monitoring of micronuclei and nuclear abnormalities in *Pelophylax ridibundus* erythrocytes from the Biga Stream (Canakkale, Turkey). *Fresenius Environmental Bulletin, 27*(1), 147-153. doi: 10.1007/s10646-010-0531-y.

Das, M., & Mahapatra, P.K. (2014). Hematology of wild caught Dubois's Tree Frog *Polypedates teraiensis*, Dubois, 1986 (Anura: Rhacophoridae). *Scientific World Journal, Article ID 491415*. doi: 10.1155/2014/491415.

Davis, A.K., Cook, K.C., & Altizer, S. (2004). Leukocyte profiles of House Finches with and without mycoplasmal conjunctivitis, a recently emerged bacterial disease. *EcoHealth, 1*, 362-373.

Davis, A.K., & Maerz, J.C. (2008a). Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. *Journal of Herpetology, 42*(1), 197-201.

Davis, A.K., & Maerz, J.C. (2008b). Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. *Copeia, 3*, 613-617.

Davis, A.K., Maney, D.L., & Maerz, J.C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology, 22*(5), 760-772. doi: 10.1111/j.1365-2435.2008.01467.x.

Davis, A.K., & Durso, A.M. (2009). Leukocyte differentials of Northern Cricket Frogs (*Acris c. crepitans*) with a compilation of published values from other amphibians. *Herpetologica, 65*(3), 260-267.

Davis, A.K., & Golladay, C., 2019. A survey of leukocyte profiles of red-backed salamanders from mountain like, Virginia, and associations with host parasite types. *Comparative Clinical Pathology, 28*, 1743-1750. doi: 10.1007/s00580-019-03015-9.

de Assis, V.R., Titon, S.C.M., & Gomes, F.R. (2018). Acute stress, steroid plasma levels, and innate immunity in Brazilian toads. *General and Comparative Endocrinology, 273*, 86-97. doi: 10.1016/j.ygcen. 2018.05.008.

Debey, L.B., & Pyenson, N.D. (2013). Osteological correlates and phylogenetic analysis of deep diving in living and extinct pinnipeds: what good are big eyes? *Marine Mammal Science, 29*(1), 48-83 doi: 10.1111/j.1748-7692.2011.00545.x.

de Boer, D., Ring, C. & Carroll, D. (2006). Time course and mechanisms of hemoconcentration in response to mental stress. *Biological Psychology, 72*(3), 318-324. doi: 10.1016/j.biopsycho.2005.12.004.

de Gregorio, L.S., Franco-Belussi, L., & de Oliveira, C. (2021). Leukocyte profile of tadpoles and juveniles of *Lithobates catesbeanus* Shaw, 1802 (Anura) and the effects of nonylphenol and cyproterone acetate. *South American Journal of Herpetology, 20*(1), 75-83. doi: 10.2994/SAJH-D-19-00111.1.

Dhabhar, F.S., Miller, A.H., Stein, M., McEwen, B.S., & Spencer, R.L. (1994). Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. *Brain, Behavior, and Immunity, 8*(1), 66-79. doi: 10.1006/brbi.1994.1006.

Dhabhar, F.S., Miller, A.H., McEwen, B.S., Spencer, R.L. (1995). Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms.

The Journal of Immunology, 154(10), 5511-5527.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. & Spencer, R.L. (1996). Stress-induced changes in blood leukocyte distribution. *Role of Adrenal Steroid Hormones Journal of Immunology, 157*(4), 1638-1644.

Dönmez, M., & Şişman, T. (2021). The morphometric and erythrometric analyses of *Pelophylax ridibundus* living in anthropogenic pollution resources. *Turkish Journal of Zoology, 45*, 314-328. doi: 10.3906/zoo-2104-16.

EC. (2000). Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for Community action in the field of water policy. *Official Journal of the European Union, L, 327*, 1-73. Retrieved from: eurlex.europa.eu.

Edens, L.J., & Levy, D.L. (2014). Size scaling of sub cellular organelles and structures in *Xenopus laevis* and *Xenopus tropicalis*. In Kloc, M., & Kubiak, J.Z. (Eds.). *Xenopus development* (2nd Edition, 325-345). Hoboken, NJ, USA: Wiley.

Egorov, M.A., Altufiev, Yu.V., & Altufieva, N.S. (2002). Developing an understanding of morphological and functional changes in the liver of the marsh frog (*Rana ridibunda*) at ekotoksikoze. In Altufieva, N.S (Ed.). *Ecological and Biological Problems of the Volga region and the Northern Caspian* (5th Edition, 90-91). Astrakhan, Russian Federation: ASPU Astrakhan, Publishing House. (In Russian).

Feder, M. (1992). *Environmental physiology of the amphibians.* Chicago, Illinois, USA: University of Chicago Press.

Fellers, G.M., Drost, C.A., &. Heyer, W.R. (1994). Handling live Amphibians. In Heyer, W.R., Donnelly, M.A, Mc Diarmid, L.C., & Foster, M.S. (Eds.). *Measuring and Monitoring Biological Diversity, Standard Methods for Amphibians,* (1st Edition, 275-276). Washington, USA: Smithsonian Institution Press.

Foxon, G.E.H. (1964). Blood and respiration. In Moore, J.A. (Ed.). *Physiology of the Amphibia*. (Vol. I., 151-209). London, England: Academic Press.

Franchimont, D. (2004). Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. *Annals of the New York Academy of Sciences, 1024*, 124-137. doi: 10.1196/annals.1321.009.

Franco-Belussi, L., Provete, D.B., Leão,T.R.F., Siqueira, M.S., Valverde,B.S.L., Martins, B.O., de Oliveira,C., Fernandes, C.E.S. (2021). Hematological parameters of a Neotropical wild frog population, with a phylogenetic perspective on blood cell composition in Anura. *Current Zoology, zoab059*. doi: 10.1093/cz/zoab059.

Glomski, C.A., Tamburlin, J., Hard, R., & Chainani, M. (1997). The phylogenetic odyssey of the erythrocyte. IV. The amphibians. *Histology and Histopathology, 12*(1), 147-170. doi: 10.14670/HH-12.147.

Gonçalves, M.V., de Campos, C.B.M., Godoy, F.R., Gambale, P.G., Nunes, H.F., Nomura, F., Bastos, R.P., da Cruz, A.D., de Melo, E., & Silva, D. (2019). Assessing genotoxicity and mutagenicity of three common Amphibian species inhabiting agroecosysten. *Archives of Environmental Contamination and Toxicology, 77*(3), 409-420. doi: 10.1007/s00244-019-00647-4.

Gregory, T.R. (2001). The bigger the C-value, the larger the cell: Genome size and red blood cell size in vertebrates. *Blood Cell, Molecules, and Diseases, 27*(5), 830-843. doi: 10.1006/bcmd.2001.0457.

Greenspan, S.E., Bower, D.S., Webb, R.J., Berger, L., Rudd, D., Schwarzkopf, L., & Alford, R.A. (2017). White blood cell profiles in amphibians help to explain disease susceptibility following temperature shifts, *Developmental and Comparative Immunology, 77,* 280-286. doi: 10.1016/j.dci.2017.08.018.

Gül, Ç., Tosunoğlu, M., Erdoğan, D., & Özdamar, D. (2011). Changes in the blood composition of some anurans. *Acta Herpetologica, 6*(2), 137-147.

Haden, R. (1940). Factors affecting the size and shape of the red cell. In: Moulton, F.R. (Ed.) *Blood, heart and circulation.* (1st Edition, 27-33). Lancaster, England: Science Press.

Hartman, F., & Lessler, M. (1964) Erythrocyte measurements in Fishes, Amphibians and Reptiles. *Biological Bulletin. 126*(1), 83-88.

Hawkey, C.M., & Dennett, T.B. (1989). *Color Atlas of Comparative Veterinary Hematology.* Ames, Iowa, USA: Iowa State University Press

Hedrick, M.S., & Duffield, D.A. (1991). Haematological and rheological characteristics of blood in seven marine mammal species: physiological implications for diving behavior. *Journal of Zoology, 225*(2), 273-283. doi: 10.1111/j.1469-7998.1991.tb03816.x.

Hutchison, V.H. & Szarski, H. (1965). Number of erythrocytes in some amphibians and reptiles. *Copeia, 3,* 373-375.

Isaeva, E.I., & Viazov, S.O. (1997). Overall assessment of immune status. In: Zinchenko, T.D., & Rosenberg, G.S. (Eds.). *Ecological status basin Chapaevka River in terms of human impact.* (2nd Edition, 292-296). Togliatti, Russian Federation: IEVB Science. (In Russian).

"IUCN". (2018). IUCN Red List of Threatened Species. Version 2018-2. Retrieved from iucnredlist.org.

Jain, N.C. (1993). *Essentials of Veterinary Hematology.* Philadelphia, USA: Blackwell.

Janiga, M., Haas, M., & Kufelová, M. (2017). Age, sex and seasonal variation in the shape and size of erythrocytes of the alpine accentor, *Prunella collaris* (Passeriformes: Prunellidae). *The European Zoological Journal, 84*(1), 583-590. doi: 10.1080/24750263.2017.1403656.

Johnstone, C.P., Lill, A., & Reina, R.D. (2017). Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal. *Biological Reviews, 92*(1), 150-168. 150 doi: 10.1111/brv.12219.

Jordan, H.E. (1933). The evolution of the blood-forming tissues. *Quarterly Review of Biology, 8*(1), 58-76.

Kim, D-H., Li, B., Si, F., Jude, M., Phillip, J.M., Wirtz, D., & Sun, S.X. (2015). Volume regulation and shape bifurcation in the cell nucleus. *Journal of Cell Science, 128*(18), 3375-3385. doi: 10.1242/jcs.166330.

Kozłowski, J., Czarnołeski, M., Francois-Krassowska, A., Maciak, S., & Pis, T. (2010). Cell size is positively correlated between different tissues in passerine birds and amphibians, but not necessarily in mammals. *Biology Letters, 6*(6), 792-796. doi: 10.1098/rsbl.2010.0288.

Lay, P.A., & Baldwin, J. (1999). What determines the size of teleost erythrocytes? correlations with oxygen transport and nuclear volume. *Fish Physiology and Biochemistry, 20*(1):31–35 doi: 10.1023/A:1007785202280.

Lai, J.C.C., Kakuta, I., Mok, H.O.L., Rummer, J.L., & Randall, D. (2006). Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. *Journal of Experimental Biology, 209*(14), 2734-2738. doi: 10.1242/jeb.02279.

Levy, D.L., & Heald, R. (2016). Biological scaling problems and solutions in Amphibians. *Cold Spring Harbor Perspectives in Biology, 8*(1), a019166. doi: 10.1101/cshperspect.a019166.

Lewis, S.M., Bain, B.J., Bates, I., & Dacie, J.V. (2006). *Dacie and Lewis Practical Haematology.* London, England: Churchill Livingstone.

Liu, C., Xia, C., Xie, Z., Jiao, Y., & She, Q. (2013). A research of peripheral blood cells annually in *Bufo bufo gargarizans*. *International Journal of Morphology, 31*(4), 1282–1288. doi: 10.4067/S0717-95022013000400022.

Macadangdang, B.R., Oberai, A., Spektor, T., Campos, O.A., Sheng, F., Carey, M.F., Vogelauer, M., & Kurdistani, S.K. (2014). Evolution of histone 2A for chromatin compaction in eukaryotes. *eLife, 3*, e02792. doi: 10.7554/eLife.02792.

Mahapatra, B.B., Das, M., Dutta, S.K., & Mahapatra, P.K. (2012). Hematology of Indian tree frog *Polypedates maculates* Gray, 1833 (Anura: Rhacophoridae). *Comparative Clinical Pathology, 21*, 453-460. doi: 10.1007/s00580-010-1118-y.

Maniero, G.D., & Carey, C. (1997). Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *Journal of Comparative Physiology B, 167*(4), 256-263. doi: 10.1007/s003600050072.

Marques, S.M., Goncalves, F., & Pereira, R. (2008). Efects of a uranium mine effluent in the early-life stades of *Rana parezi* Seoane. *Sciense of The Total Environmenrt, 402*(1): 29-35. doi: 10.1016/j.scitotenv.2008.04.005.

McGrath, J.P. (1993). Assessment of hemolytic and hemorrhagic anemias in preclinical safety assessment studies. *Toxicologic Pathology, 21*(2), 158-163. doi: 10.1177/019262339302100207.

Mineeva, O.V. & Mineev, A.K. (2010). Morphology defects in peripheral blood erythrocytes of lake frog *Rana ridibunda* Pallas, 1771. *Bulletin of the University of Nizhny Novgorod, 2*(2), 664-667. (In Russian).

Mineeva, O.V., & Mineev, A.K. (2011). Disorders of blood leukocyte formula in the lake frog of the Saratov reservoir. *Bulletin of the University of Nizhny Novgorod, 2*(2), 94-97. (In Russian).

Mueller, R.L. (2015). Genome biology and the evolution of cell size diversity. *Cold Spring Harbor Perspectives in Biology, 7*(11), a019125. doi: 10.1101/cshperspect.a019125.

Nadolski, J., Skwarska, J., Kalinski, A., Banbura, M., Sniegula, R., & Banbura, J. (2006). Blood parameters as consistent predictors of nestling performance in great tits (*Parus major*) in the wild. *Comparative Biochemistry and Physiology Part A: Physiology, 143*(1), 50-54. doi: 10.1016/j.cbpa.2005.10.021.

Nicol, S.C., Melrose, W., & Stahel, C.D. (1988). Haematology and metabolism of the blood of the little penguin, *Eudyptula minor*. *Comparative Biochemistry and Physiology Part A: Physiology, 89*(3):383-386 doi: 10.1016/0300-9629(88)91044-4.

Norte, A.C., Ramos, J.A., Ara´ujo, P.M., Sousa, J.P. & Sheldon, B.C. (2008). Health-state variables and enzymatic biomarkers as survival predictors in nestling Great Tits (*Parus major*): effects of environmental conditions. *The Auk, 125*(4), 943-952. doi: 10.1525/auk.2008.07188.

Oliveira, C.R., Garcia, T.D., Franco-Belussi, L., Salla, R.F., Souza, B.F.S., DeMelo, N.F.S., Irazusta, S.P., Jones-Costa, M., Silva-Zacarin, E.C.M., & Fraceto, L.F. (2019). *Pyrethrum* extract encapsulated in nanoparticles: Toxicity studies based on genotoxic and hematological effects in bullfrog tadpoles. *Environmental Pollution, 253*, 1009-1020. doi: 10.1016/j.envpol.2019.07.037.

Omelykovets, Ya.A., & Berezyuk, M.V. (2009). Research of change of motfo-metrical index of red corpuscles tales Amphibious in different periods of ontogenesis. *Zoology, 9*, 83-88.

Ordinance No H-4/14.09.2012 on the characterization of surface waters (2012). *State Gazette, 22*, 5.03.2013. (In Bulgarian).

Ordinance No 256 of 1.11.2010 for Standards on environmental quality for priority substances and for certain other pollutants, *State Gazette, 88*, 9.11.2010. (in Bulgarian).

Pavlov, D.N., Romanov, M.G., Vasilev, M.K. & Popov, I.C. (1980). *Chemical laboratory methods*. Sofia, Bulgaria: Medicine and Physical Culture. (In Bulgarian).

Peskova T.Yu. (2001). *Influence of anthropogenically polluted environment of amphibians*. Volgograd, Russia: Publishing House of the Volgograd State Pedagogical Institute. (In Russian).

Peskova, T.Yu., & Zhukova, T.I. (2005). Hematological indexes of *Rana ridibunda* inhabiting in clean and contaminated ponds. *Russian Journal of Herpetology, 12* (Suppl.1), 296-297.

Peskova, T.Yu., & Sharpan, Yu.V. (2007). Effect of oil on the haematological parameters of the marsh frog. *Bulletin of science of the university of Uzhgorod, 21*, 96-99. (In Russian).

Peskova, T.Yu., & Vafis, A.A. (2007). Effect of gasoline on hematological indices of the marsh frog. *Bulletin of science of the University of Uzhgorod, 21*, 100-104. (In Russian).

Peterson, J.D., Steffen, J.E., Reinert, L.K., Cobine, P.A., Appel, A., Rollins-Smith, L., & Mendonca, M.T. (2013). Host stress response is important for the pathogenesis of the deadly amphibian disease, chytridiomycosis, in *Litoria caerulea*. *PLoS ONE, 8*(4), e62146. doi: 10.1371/journal.pone.0062146.

Pollo, F., Grenat, P., Otero, M., Salas, N., & Martino, A. (2016). Health status of tadpoles and adults of frog *Hypsiboas cordobae* (Barrio 1965) that inhabit aquatic ecosystems associated to fluorite mine. *Ecotoxicology and Environmental Safety, 133*, 466-474. doi: 10.1016/j.ecoenv.2016.08.003.

Pollo, F.E., Grenat, P.R., Salinas, Z.A., Otero, M.A., Salas, N.E., & Martino, A.E. (2017). Evaluation *in situ* of genotoxicity and stress in South American common toad *Rhinella arenarum* in environments related to fluorite mine. *Environmental Science and Pollution Research, 24*(22), 18179-18187. doi: 10.1007/s11356-017-9479-2.

Pollo, F., Bionda, C., Otero, M., Grenat, P., Babini, S., Flores, P., Grisolia, M., Salas, N., Martino, A. (2019). Morphological abnormalities in natural populations of the common South American toad *Rhinella arenarum* inhabiting fluoride-rich environments. *Ecotoxicology and Environmental Safety, 177*(15), 32-38. doi: 10.1016/j.ecoenv.2019.03.098.

Prieto, Z., Incio, J.L., Jara, Q.C., Fernández, R., Polo. B.E., VallejoRodríguez, R., & Villegas Sanchez, L. (2008). Efecto genotóxico deldicromato de potasio en eritrocitos de sangre periférica de *Oreochromis niloticus* (Tilapia). *Revista Peruana Medicina Experimantal y Salud Publica, 25*(1), 51-58.

Priyadarshani, S., Madhushani, W.A.N., Jayawardena, U.A., Wickramasinghe, D.D. & Udagama, P.V. (2015). Heavy metal mediated immunomodulation of the Indian green frog, *Euphlyctis hexadactylus* (Anura: Ranidae) in urban wetlands. *Ecotoxicology and Environmental Safety, 116*, 40-49. doi: 10.1016/j.ecoenv.2015.02.037.

Promislow, D.E.L. (1991). The evolution of mammalian blood parameters: patterns and their interpretation. *Physiological Zoology, 64*(2), 393-431. doi: 10.1086/physzool.64.2.30158183.

R: The R Project for Statistical Computing. Retrieved from R-project.org.

Raffel, T.R., Rohr, J.R., Kiesecker, J.M., & Hudson, P.J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology, 20*(5), 819-828. doi: 10.1111/j.1365-2435.2006.01159.x.

Raffel, T.R., Halstead, N.T., Mcmahon, T.A., Davis, A.K., & Rohr, J.R. (2015).

Temperature variability and moisture synergistically interact to exacerbate an epizootic disease. *Proceedings of the Royal Society of London Part B: Biological Sciences, 282*(1801), 20142039. doi: 10.1098/rspb.2014.2039.

Randall, D.J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *Journal of Experimental Biology, 100*, 275-288.

Randall, D.J., & Perry, S.F. (1992). Catecholamines, In (Hoar, W.S., & Randall, D.J. (Eds.). *Fish Physiology.* (Vol. XII: The Cardiovascular System, 255-300). Orlando, USA: Academic Press.

Rich, E.L. & Romero, L.M. (2005). Exposure to chronic stress downregulates corticosterone responses to acute stressors. *American Journal of Physiology, 288*(6), R1628-R1636. doi: 10.1152/ajpregu.00484.2004.

Rohr, J.R., Schotthoefer, A.M., Raffel, T.R., Carrick, H.J., Halstead, N., Hoverman, J.T., Johnson, C.M., Johnson, L.B., Lieske, C., Piwoni, M.D., Schoff, P.K., & Beasley, V.R. (2008). Agrochemicals increase trematode infections in a declining amphibian species. *Nature, 455*(7217), 1235-1239. doi: 10.1038/nature07281.

Romanova, E.B., & Romanova, O.Yu. (2003). Peculiarities of leukocytic formula of peripheral blood of green frogs under conditions of anthropogenetic load. *Journal of Evolutionary, Biochemistry and Physiology, 39* (4), 480-484. doi: 10.1023/B:JOEY.0000010246.27310.85.

Romanova, E.B., & Egorikhina, M.N. (2006). Changes in hematological parameters of *Rana* frogs in a transformed urban environment. *Russian Journal of Ecology, 37*(3), 188-192. doi: 10.1134/S10674136030078.

Romanova, E.B. (2005). Hematological aspects of adaptation mechanisms of natural populations of green frogs under anthropogenic environmental stress. In Bakiev, A.G. (Ed.). *Actual problems of Herpetology and toxicology.* (8th Edition, 169-176) Togliatti, Russian Federation: IEVB Publishing House. (In Russian).

Romero, L.M. & Romero, R.C. (2002). Corticosterone responses in wild birds: the importance of rapid initial sampling. *Condor, 104*(1), 129-135. doi: 10.1650/0010-5422(2002)104[0129:CRIWBT]2.0.CO;2.

Romero, L.M. & Reed, J.M. (2005). Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 140*(1), 73-79. doi: 10.1016/j.cbpb.2004.11.004.

Ruiz, G.,. Rosenmann, M., & Veloso, A. (1989). Altitudinal distribution and blood values in the toad, *Bufo spinulosus* Wiegmann. *Comparative Biochemistry and Physiology Part A, 94*(4), 643-646. doi: 10.1016/0300-9629(89)90609-9.

Salinas, Z.A., Baraquet, M., Grenat, P.R., Martino, A.L., & Salas, N.E. (2017). Morphology and size of blood cells of *Rhinella arenarum* (Hensel, 1867) as environmental health assessment in disturbed aquatic ecosystem from central Argentina. *Environmental Science and Pollution Research, 24*(32), 24907-24915. doi: 10.1007/s11356-017-0107-y.

Sedalishchev, V.T. (2005). Siberian frog (*Rana amurensis*) as an indicator of anthropogenic impacts. In Ruchin, A.B. (Ed.): *Current problems of environmental physiology, biochemistry and genetics of animals* (5th Edition, 209-211). Saransk, Russian Federation: Publishing House of the Mordovian State University. (In Russian).

Seiverd, C.E. (1972). *Hematology for medical technologists. 4th edition.* Philadelphia, USA: Lea and Febiger.

Selye, H. (1956). *The Stress of Life.* New York USA: McGraw-Hill.

Shutler, D., & Marcogliese, D.J. (2011). Leukocyte profiles of Northern Leopard

Frogs, *Lithobates pipiens*, exposed to pesticides and hematozoa in agricultural wetlands. *Copeia, 2,* 301-307.

Sils, E.A. (2006). Features leukogram peripheral blood lake frog Rana ridibunda in the territory of a large industrial city. In Bolshakov, V.N., & Ivanter, E. (Eds.). *Population ecology of animals: Proceedings of the International Conference "Problems of population ecology of animals", dedicated to the memory of Academician Shilov, I.A.,* (1st Edition, 349-351). Tomsk, Russian Federation: Publishing House of the Tomsk State University. (In Russian).

Sils, E.A. (2008). Specific of amphibian (genus *Rana*) peripheral blood leucogram under condition of anthropogenic load. In Ananjeva, N.B. (Ed.). *The Problems of Herpetology.* (1st Edition, 369-374) Saint-Petersburg, Russian Fedaration: Russian Collection publishing. (In Russian).

Sils, E.A., & Vershinin, V.L. (2005). Population ecology of amphibians inhabiting the city based on hematological parameters. In Bolshakov, V.N. (Ed.). *Fundamental and applied Ecology: Proceedings of the International scientific and practical conference* (2nd Edition, 310-311). Yekaterinburg, Russian Federation: Publishing House of the Ural State University. (In Russian).

Silva, M.B., Fraga, R.E., Nishiyama, P.B., Silva, I.S.S., Costa, N.L.B., Oliveira, L.A.A., Rocha, M.A., & Juncá, F.A. (2020). Leykocyte profiles in *Odontophrynus carvalhoi* (Amphibia: Odontopharynidae) tadpoles exposed to organophosphate chlorpyrifos pesticides. *Water, Air & Soil Pollution, 231,* 372. doi: 10.1007/s11270-020-04726-4.

Şişman, T., Keskin, M.Ç., Dane, H., Adil, Ş., Geyikoğlu, F., Çolak, S., & Canpolat, E. (2021). Marsh Frog (*Pelophylax ridibundus*) as a Bioindicator to Assess Pollution in an Agricultural Area. *Pakistan Journal of Zoology, 53*(1), 337-349. doi: 10.17582/journal.pjz/20190103130130.

Spirina, E.V. (2009). Morphophysiological adaptations in *Rana ridibunda* Pall. under the influence of pollution. *Bulletin of the Altai State Agrarian University, 12*(62), 64-68. (In Russian).

StatSoft Inc. (2004). *STATISTICA (Data analysis software system),* Version 7. Retrieved from statsoft.com.

Steven, J., Jacobson, E.R., Lillywhite, H.B. & Zamundo, K. (2004). *Guidelines for use of Amphibians and Reptiles in field and laboratory research. Second Edition, Revised by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists*. Retrieved from asih.org.

Stetter, M.D. (2001). Fish and amphibian anesthesia. *Veterinary Clinics of North America: Exotixc Animal Practice, 4*(1), 69-82. doi: 10.1016/s1094-9194(17)30052-x.

Stojanov, A.J., Tzankov, N., & Naumov, B. (2011). *Die Amphibien und Reptilien Bulgariens*. Frankfurt am Main, Deutschland: Chimaira.

Sures, B., Nachev, M., Selbach, C., & Marcogliese, D.J. (2017). Parasite responses to pollution: what we know and where we go in Environmental Parasitology. *Parasites & Vectors, 10*(1), 65. doi: 10.1186/s13071-017-2001-3.

Sutherland, W.J. (2000). *The Conservation Handbook: Research, Management and Policy*. Oxford, England: Blackwell.

Tarasenko, S.N. (1981). Hematological aspects of adaptation marsh frog to the extreme conditions of industrial pollution. In Darevskii, I.S. (Ed.). *Questions of Herpetology* (4th Edition, 129-130). Saint Petersburg, Russian Federation: Science. (In Russian).

Tarasenko, S.N., & Tarasenko, S.V. (1988). Comparative characteristics of blood parameters in anurans of different degree of familiarity of human ecosystems. In Volskis, R.S. (Ed.). *Kind and productivity in the habitat* (1st

Edition, 137-138). Vilnius, Lithuania: Biosphere. (In Russian).

Thrall, M.A., Baker, D.C., & Lassen, E.D. (2004). *Veterinary Hematology and Clinical Chemistry.* Iowa, USA: Iowa State University Press.

Thrall, M.A., Baker, D.C., Lassen, E.D., Campbell, T.W., de Nicola, D., Rebar, A., Fettman, M.J., & Weiser, G. (2006). *Veterinary Hematology and Clinical Chemistry.* Ames, Iowa, USA: Blackwell Publishing.

Toktamysova, Z.S. (2005). Morphological indicators of marsh frog populations exposed to anthropogenic impact. In Pakhomov, A.E. (Ed.). *Biodiversity and role of zoocenoses in natural and anthropogenic ecosystems* (2nd Edition, 380-381). Dnepropetrovsk Ukraine: DNU Publishing House. (In Russian).

Tyler, R.D., & Cowell, R.L. (1996). Classification and diagnosis of anaemia. *Comparative Haematology International, 6,* 1-16. doi: 10.1007/BF00368096.

Vafis, A.A., & Peskova, T.Yu. (2009). The reaction of the blood of marsh frog *Rana ridibunda* Pal. The effect of waste water of sugar factories. *Problems of Contemporary Science and Practice, 2,* 8-18. (In Russian).

Valenzuela, A.E., Silva, V.M., & Klempau, A.E. (2006). Qualitative and quantitative effects of constant light photoperiod on rainbow trout (*Oncorhynchus mykiss*) peripheral blood erythrocytes. *Aquaculture, 251*(2-4), 596-602. doi: 10.1016/j.aquaculture.2005.06.012.

Vallejo, C.A., Hernándes-Gallegos, O., Von Herbing, J.H., López-Moreno, A.E., Ruiz-Gómez, M.de L., Grandos-Gonzalez, G., Garundño-Paz, M.V., Méndez-Sánchez, J.F., Banda-Leal, J., & Davis, A.D. (2015). Assessing population health of the Toluca axolotl *Ambystoma rivulare* (Taylor, 1940) from Mexico, using leukocyte profiles. *Herpetological Conservation and Biology, 10*(2), 592-601.

Venturino, A., Rosenbaum, E., Caballero de Castro, A., Anguiano, O.L., Gauna, L., Fonovich de Schroeder, T., & Pechen de D'Angelo, A.M. (2004). Biomarkers of effect in toads and frogs. *Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals, 8*(3-4), 167-186. doi: 10.1080/1354700031000120116.

Vinogradov, A.E., & Anatskaya, O.V. (2006). Genome size and metabolic intensity in tetrapods: A tale of two lines. *Proceedings of the Royal Society B: Biological Sciences, 273*(1582), 27-32. doi: 10.1098/rspb.2005.3266.

Webster, M., Witkin, K.L., & Cohen-Fix, O. (2009). Sizing up the nucleus: Nuclear shape, size and nuclear-envelope assembly. *Journal of Cell Science, 122*(Pt 10), 1477-1486. doi: 10.1242/jcs.037333.

Wei, J., Li1, Y.Y., Wei, L., Ding, G.H., Fan, X.L., & Lin, Z.H. (2015). Evolution of erythrocyte morphology in amphibians (Amphibia: Anura). *Zoologia, 32*(5), 360-370. doi: 10.1590/S1984-46702015000500005.

Wells, R.M.G., & Baldwin, J. (1990). Oxygen transport potential in tropical reef fish with special reference to blood viscosity and haematocrit. *Journal of Experimental and Marine Biology and Ecology, 141*(2-3), 131-142. doi: 10.1016/0022-0981(90)90219-3.

Wintrobe, M. (1933). Variation s in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. *Folia Haematologyca, 51*(1), 32-49.

Wojtaszek, J., & Adamowicz, A. (2003). Haematology of the fire-bellied toad, *Bombina bombina* L. *Comparative Clinical Pathology, 12*(3), 129-134. doi: 10.1007/s00580-003-0482-2.

Wright, K. (2001). Amphibian hematology. In Wright, K., & Whitaker, B. (Eds). *Amphibian medicine and captive husbandry* (2nd Edition, 129-146). Malabar, Florida, USA: Krieger Publishing.

Xiong, J., Zhang, Y., Chen, W., Min, Y., & Gou, J. (2018). Some haematological parameters of wild caught warty toothed toad *Oreolalax rugosus* (Liu, 1943) (Anura: Megophryidae) *Acta zoologica bulgarica, 70*(1), 69-74.

Zamaletdinov, R.I., Nazarov, N.G., Svinin, A.O., Drobot, G.P., & Sal'nikova, E.Yu. (2019). Biochemical peculiarities of the peripheral blood of the *Pelophylax lessonae* pond frog (Camarano, 1882) of the Kazan city populations. *University Proceedings of the Volga region, 25*(1), 41-49. doi: 10.21685/2307-9150-2019-1-5.

Zhelev, Zh., Petkov, M., & Adzalijski, Z. (2005). Blood composition in *Rana ridibunda* (*Anura, Amphibia*) from an area of highly developed chemical industry. *Acta zoologica bulgarica, 57*(2), 229-236.

Zhelev, Zh.M., Angelov, M.V., & Mollov, I.A. (2006). A study of some metric parameters of the erythrocytes in *Rana ridibunda* (Amphibia: Anura) derived from an area of highly developed chemical industry. *Acta zoologica bulgarica, 58*(2), 235-244.

Zhelev, Zh. (2007). Investigation on the blood differential formula in *Rana ridibunda* (*Amphibia: Anura*) from the area of the Maritsa-Istok-1 Steam Power Plant. *Acta zoologica bulgarica, 59*(2), 181-190.

Zhelev, Zh.M., Popgeorgiev, G.S., & Angelov, M.V. (2013). Investigating the changes in the morphological content of the blood of *Pelophylax ridibundus* (Amphibia: Ranidae) as a result of anthropogenic pollution and its use as an environmental bioindicator. *Acta zoologica bulgarica, 65*(2), 187-196.

Zhelev, Zh.M., Popgeorgiev, G.S., Mehterov, N.H. (2015). Haematological Parameters of *Pelophylax ridibundus* (Amphibia: Ranidae) from the Region of the Lead and Zinc Plant "Kardzhali" (South Bulgaria) and their use in the Environmental Quality Assessment. *Acta zoologica bulgarica, 67*(2), 271-282.

Zhelev, Zh.M. (2016a). *Biomonitoring on polluted water ecosystems in Bulgaria. Fluctuating asymmetry and hematological indices in Pelophylax ridibundus (Amphibia: Anura: Ranidae).* Saarbrucken: Deutschland: LAP LAMBERT Academic publishing.

Zhelev, Zh.M., Mehterov, N.H., & Popgeorgiev, G.S. (2016b). Seasonal changes of basic erythrocyte-metric parameters in *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria and their role as bioindicators. *Ecotoxicology and Environmental Safety, 124*, 406-417. doi: 10.1016/j.ecoenv.2015.11.011.

Zhelev, Zh.M., Georgieva, K.N., Todorov, O.B., & Peeva, K.G. (2017a). Haematological parameters of *Bufotes viridis* (Laurenti, 1768) (Anura: Bufonidae) from southern Bulgaria. *Acta zoologica bulgarica, 69*(3), 335-343.

Zhelev, Zh., Popgeorgiev, G., Ivanov, I., & Boyadzhiev, P. (2017b). Changes of erythrocyte-metric parameters in *Pelophylax ridibundus* (Amphibia: Anura: Ranidae) inhabiting water bodies with different types of anthropogenic pollution in Southern Bulgaria. *Environmental Science and Pollution Research, 24*(21), 17920-17934. doi: 10.1007/s11356-017-9364-z.

Zhelev, Z., Tsonev, C., Georgieva, K., & Arnaudova, D. (2018). Health status of *Pelophylax ridibundus* (Amphibia: Ranidae) in a rice paddy ecosystem in Southern Bulgaria and its importance in assessing environmental state: Haematological parameters. *Environmental Science and Pollution Research, 25*(8), 7884-7895. doi: 10.1007/s11356-017-1109-5.

Zhelev, Zh.M., Arnaudova, D.N., Popgeorgiev, G.S., & Tsonev, S.V. (2020). *In situ* assessment of health status and heavy metal bioaccumulation of adult *Pelophylax ridibundus* (Anura: Ranidae) individuals inhabiting polluted area in southern Bulgaria. *Ecological Indicators, 115*, 106413. doi: 10.1016/j.ecolind.2020.106413.

Zhelev, Z.M., Arnaudova, D.N., Popgeorgiev, G.S., & Tsonev, S.V. (2021). Determinations of erythrocyte sizes in adult *Pelophylax ridibundus* (Amphibia: Anura: Ranidae) inhabiting industrial area in southern Bulgaria. *Water, Air, & Soil Pollution, 232*(4), 125. doi: 10.1007/s11270-021-05072-9.

Zhukova, T.I., & Peskova, T.Yu. (1999). The reaction of the blood of anurans in pesticide contamination. *Ecology, 4*, 288-292.

Received: 08.10.2021
Accepted: 20.12.2021