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

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Abstract: The current research examines some of the basic morphological hematological indicators (erythrocyte count, leukocyte count, hemoglobin, differential blood formula) in adult, fertile species within a population of the marsh frog *Pelophylax ridibundus*, inhabiting two heavily polluted rivers in Bulgaria. One of the two rivers is Sazliika River, which is polluted with nitrates, phosphates, biological oxygen demand in five days and insoluble substances, and the other one is Topolnica River, polluted with heavy metals. The paper explores the use of these indicators as biological markers for the quality of the environment in the two rivers. Statistically significant differences were found between the examined parameters in the two rivers and the control group. The living in toxic conditions resulted in a single type change in the blood toxicology (increased erythrocyte count and amount of hemoglobin). The changes in the differential blood formula differ considerably – the samples from Sazliika exhibit adaptive characteristics (neutrophilia, monocytosis and lymphopenia in general leukocytosis), whereas the samples from Topolnica showed lymphopenia accompanied with neutropenia and monocytosisin general leukocytosis. This is an evidence of the degenerative effects of toxicity.

Key words: erythrocytes, leukocytes, anthropogenic pollution, bioindication, *Pelophylax ridibundus.*

Introduction

It has been shown that ecological factors including various forms of anthropogenic pollution affect the physiology of animals in a given habitat. Studying the changes in the parameters in blood, which dynamically react to external stressors, makes it possible to use the data in the system of biomonitoring (Cabagna *et al.* 2005, Card & Hippardy 2007, Lajmanovich *et al*. 2012, Zhelev 2012a). Haematological indicators are very specific and they fluctuate within narrow parameters (Cül *et al.* 2011, Machapatra *et al.* 2012), which allows their use as markers in different physiological and pathological processes taking place at the levels of the organism and the ecosystem (Peskova 2001, Zhelev *et al.* 2006, Lajmanovich *et al*. 2008). Amphibians, especially the members of the *Ranidae* (Rafinesque-Schmaltz, 1814) family have fully developed cardiovascular and immune systems (Manning & Horton 1982), they can withstand high levels of urbanization and pollution (Vershinin 2007) which makes them suitable bioindicators

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for an anthropogenically transformed environment (Venturino *et al*. 2003, Venturino & D'Angelo 2005, Zhelev *et al*. 2012). However, there has not been a lot of research investigating the changes in the morphological content of blood from amphibians in relation to a specific pollutant in the habitat and using the blood as a tool for biomonitoring.

The aim of the current research is to acquire data for some basic haematological morphological parameters (erythrocyte count – RBC, leukocyte count – WBC amount of haemoglobin – Hb, differential blood formula) in blood samples of adult, fertile species of marsh frogs *Pelophylax ridibundus* (Pallas, 1771) (taxonomy according to Speybroeck *et al*. 2010), inhabiting two heavily polluted (with different pollutants) rivers in Bulgaria (Sazliyka and Topolnitsa) and using the samples as bioindicators for the quality of life in the environment.

Materials and Methods

The collecting of the required materials took place in the spring of 2011 in biotopes along the Sazliyka River (up the river stream at Rakitnitsa village – 7 April 2011, in the middle of the city of Radnevo after flowing into the Blatnitsa River – 9 April 2011). The rest of the materials were collected from Topolnitsa River at Poibrene village – 12 April 2011 (where Medetska River flows into the Topolnitsa Reservoir) (Fig. 1).

The rivers Sazliyka (flowing out of the city of Stara Zagora) and Topolnitsa are two of the most heavily polluted rivers in Bulgaria. The Sazliyka River is mainly polluted by sewage waters and industrial wastewater from the cities of Stara Zagora and Nova Zagora coming from the rivers Bedechka and Blatnitsa. The pollution in the Topolnitsa River comes from the Copper Production Plant "Aurubis", Assarel-Medet JSC Mining and Processing Complex and "Chelopech Mining".

Equal number of adult (L > 60.0 mm), fertile specimens of *P. ridibundus* were taken from each of the rivers – 30 from each population. The animals were captured in the night with an electrical torch in the water and along a 1 km long and 4m wide transect of the river bank after each populated station (according to Sutherland 2000). In the literature there is data suggesting a correlation between the age, size and gender of amphibians with the haematological parameters (Grenat *et al*. 2009, Peskova & Zhelev 2009, Cül *et al*. 2011, Machapatra *et al*. 2012, Das & Mahapatra 2012), but the evidence regarding the *Ranidae* family is inconclusive. Due to gender differences in the morphological haematologycal indicators in tailless amphibians from both *Ranidae* family and *P. ridibundus* that inhabit anthropologically polluted habitats containing specimens with morfa *striata,* the gender and phonetic characteristics of the animals were not indicated when being examined. The various levels of pollution of the biotope as well as the

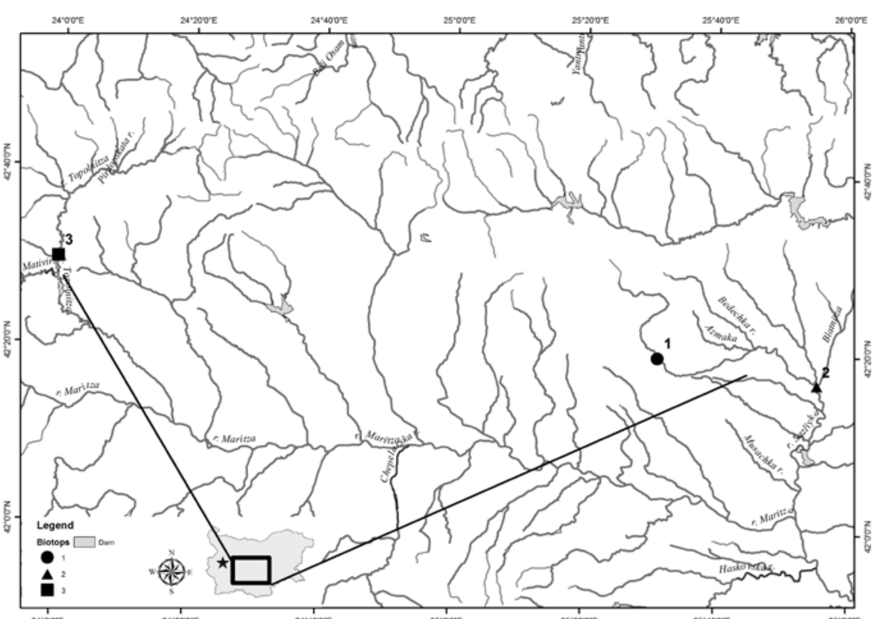

Fig. 1. Geographical location of the studied water ecosystems.

nature of the pollutants of the rivers were the factors initially considered. There was no evidence of anthropogenic pollution in the biotope in the upper part of the Sazliyka River (marked as № 1). The other two biotopes: Sazliyka River downstream of the Radnevo city - № 2 and Topolnitsa River at the Topolnitsa dam - № 3, were polluted with an increase of the concentration of pollutants above the accepted norms in the country for Category 1 (clean) and the projected for the two rivers waters of Category 2 (slightly polluted) and 3 (with average level of pollution). In biotope № 2, the main pollutants were: nitrite nitrogen, phosphates, biological oxygen demand in five days - BOD_5 and insoluble substances, while in biotope № 3, the main pollutants were heavy metals (copper, iron, manganese, lead, arsenic). The level of pollution in the water basins of the studied biotopes and the nature of the pollutants is based on data from the Annual report for the condition of the environment (environmental waters) in the Republic of Bulgaria for the 2001-2011 period of the EEA (http://eea.goverment.bg), and data from the physicochemical analysis of water in the rivers Sazliyka and Topolnitsa for the same period from the reports of the East Aegean Sea River Basin Directorate – Plovdiv (http://www.bg-ibr.org), Ministry of Environment and Water of Bulgaria (Table 1).

The haematological indicators of the peripheral blood (0.20 ml) were analyzed in laboratory conditions immediately after the animals had been captured; the blood was taken through cardiac punctures and after the manipulation, the animals were released back in the wild. Erythrocyte and leukocyte count was determined using the Wierord method, in a Burker chamber. The amount of haemoglobin was determined by the cyan-haemoglobin method at λ= 540 nm wavelength (Pavlov *et al*. 1980). The differential blood formula was determined on the basis of 100 leukocytes from microscope slide (3000 cells in a population), using the microscopic method of Shiling (Ivanova 1982, Ibrishimov & Lalov 1984).

The comparisons of the haematological indicators for the samples from the biotopes with varying degree and nature of pollution were performed by standard statistical methodology using the Statistical 7.0. Software (Statistica 2004). The preliminary analysis (one-way ANOVA), showed high statistically reliable difference between the analyzed features in the populations. The grouping was performed on the basis of the level of pollution of the biotopes and the results were confirmed with a post-hoc LSD - test ($p < 0.05$). The normality in the distribution of the studied parameters was checked with Shapiro-Wilk - test (Shapiro *et al*. 1968), which indicated normal distribution: $p > 0.05$. Results with $p < 0.05$ [$\alpha = 5\%$] were considered significant.

Results and Discussion

In the literature, it is reported that even a short stay of *P. ridibundus* in polluted industrial wastewater leads to the decrease of the amount of haemoglobin and erythrocytes (Vafis & Peskova 2009). In the case of permanent stay of the species in the conditions of anthropogenic pollution, long-term adaptations in the opposite direction are observed: an increase of the amount of haemoglobin and erythrocytes (Peskova & Zhukova 2005, Sedalishtev 2005, Cabagna *et al*. 2005, Romanova & Egorikhina 2006), changes in the shape of the erythrocyte cells (Zhelev *et al.* 2006), as well as an increase in the frequency of the occurrence of erythrocyte pathologies (Mineeva & Mineev 2010, Zhelev 2012b).

In our research, the data indicates a statistically reliable increase in the number of erythrocytes, leukocytes and amount of haemoglobin for the two samples from the anthropogenically polluted biotopes (№ 2 and № 3), when compared with the control group - № 1 (1.4 - 1.8 times) and at the same time reliably highest values of the three indicators for the samples from the population inhabiting Topolnitsa River (Tables 2 and 3). The most common reason for the erythrocytosis caused by a long-term stay in the conditions of anthropogenic pollution is the hypoxia – the increase of the number of erythrocytes, shown by the increase of the oxygen capacity of the blood. It is a compensatory reaction of the lasting effect of the toxicants. Usually the erythrocytosis of the *P. ridibundus* is accompanied by a parallel increase of the amount of haemoglobin as well, and this is found in the case of inhabiting areas with metallurgical, chemical (Tarasenko & Tarasenko 1988) and copper-processing (Toktamysova 2005) companies. Rarely, the erythrocytosis in polluted conditions is not linked to changes in the amount of haemoglobin (Zhelev *et al.* 2005), and most frequently it is accompanied by hypochromy because of the effect of high doses of pesticides (Peskova 2001). In the literature, there is significant evidence that the representatives of the *P. ridibundus* residing in the conditions of an-

Table 1. Current data for the condition of the biotopes in the period of research (Physicochemical analysis – samples of surface water, Sazliyka River, Topolnitsa River).

Parameters	Units SI	Order №7/8.8.1986 categories			№ 1			№ 2			№ 3		
		I	III	II.	2009	2010	2011	2009	2010	2011	2009	2010	2011
pH	pH units	6.5-8.5	6.0-9.0	6.0-8.5	8.23	8.34	8.06	7.81	7.8	7.7	8.29	7.82	8.03
Temperature	°C	to 3° middle of the season			13.7	13.9	15.1	13.63	14.1	14.9	13.2	12.5	10.9
Insoluble substances	mg/dm^3	30	100	50	5.0	5.8	6	38.33	31.8	41.8	6.0	1.51	6.75
Electroconductivity	µS/cm	700	1600	1300	859	751.5	891	1140	643	596	639	468	611
Dissolved oxygen	mgO_2/dm^3	6	2	4	8.7	8.1	7.88	7.77	5.2	5.5	5.74	5.92	5.77
Oxygenation	%	75	20	40	83.3	81.5	82.2	53.3	52.3	56.5	63	63	52
Biological oxygen demand. five days – BOD_5	mgO_2/dm^3	5	25	15	3.0	3.2	1.8	6.7	18.8*	9.3	–	–	–
Chemical oxygen demand – COD	mgO_2/dm^3	25	100	70	4.4	4.9	6.7	19.73	66.2	42.2		–	–
Nitrate ammonium $N – NH_4$	mg/dm^3	0.1	5	2	0.03	0.07	0.079	0.326	2.2*	1.3	–	–	–
Nitrate nitrogen $N – NO_3$	mg/dm^3	5	20	10	2.4	2.1	1.2	4.28	2.2	1.2	–	–	–
Nitrite nitrogen $N – NO_2$	mg/dm^3	0.002	0.06	0.04	0.011	0.016	0.012	0.136**	0.2**	0.149**	–	0.005	0.032
Orthophosphates P	mg/dm^3	0.2	2	1	0.023	0.045	0.316	0.429	0.46	0.443	0.012	0.004	0.004
Total nitrogen	mg/dm^3	1	5	10	1.5	2.4	1.8	5.9*	5.3*	5.2*	–	–	–
Total phosphorus - as P	mg/dm^3	0.4	3	2	0.02	0.07	0.303	0.406	0.734	0.43	–	–	–
Sulphates (SO_4^{2-})	mg/dm^3	200	400	300	–	–	–	306.7	55.7	57.8	151.2	111	218
Iron – total (Fe)	mg/dm^3	SKOS – 0.1									0.22″	0.21″	0.26″
Manganese (Mn)	mg/dm^3	SKOS – 0.05									0.46″	0.22″	0.18″
Copper (Cu)	mg/dm^3	SKOS – 0.022									0.140	0.06″	0.053″
Arsenic (As)	mg/dm^3	SKOS – 0.25			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.03	0.003	0.006
Lead and its compounds (Pb)	mg/dm^3	SKOS – 0.0072									–	0.005	0.00701″
Nickel and its compounds (Ni)	mg/dm^3	SKOS – 0.02									0.02	0.05″	0.007

Note * – above TLV for category II; ** – above TLV for category III; ″ – above SKOS: very poor condition; – not studied.
Sample taking: – № 1 – Sazliyka River (Syutliyka) close to Rakitnitsa village; № 2 Sazliyka River close to the town of Radnevo (after the Blatnitsa River flows into it); № 3 – Topolnitsa River close to the village of Poibrene

thropogenic pollution receive an increase both in the absolute as well as in the relative size of their bodies. At the same time, in the conditions of toxicosis, in some tailed amphibians, where the main haemopoietic organ is the spleen and to a smaller degree the bone marrow and the liver (Carmena 1973), it is proven that there is a significant increase of the erythrocyte count and at the same time a reliable decrease in the spleen mass (Frangioni & Borgioli 1991). In tailless amphibians, it is found that the main haemopoietic organ is the liver and to a smaller degree, the spleen and the bone marrow (Carmena 1971). In the conditions of urbanization, with the growth of the anthropogenic pressure, a reliable decrease is observed in the erythroid predecessors in the young frogs who have just completed their metamorphosis and erythropeniain the peripheral blood of adult *P. ridibundus* (Sils 2008). The reason for that is explained by the author with pathological processes occurring in the liver. In some tailless amphibians *Bufo arenarum* (Hensel, 1867), a suppression of the erythropoiesis was found as a result of the accumulation of heavy metals in the liver (Chiesa *et al.* 2006). The results of our research on the population from the Topolnitsa River, where the pollutants are predominantly heavy metals, indicate an opposite direction of changes - erythrocytosis accompanied with hyperchromia, a reaction with mostly adaptive rather than pathological nature. In a previous research (Peskova & Zhelev 2009) we found that there is a reliable positive correlation not only between the two haematological indicators (number of erythrocytes and amount of haemoglobin) in *P. ridibundus* with the linear size and bodily proportions, but also with the absolute and relative size of the organs. Of particular importance is the positive correlation between the index of the liver with the haemoglobin level, as seen from the context of the statements (Moiseenko 2000), that for fish living in polluted conditions where the correlation is negative, the animals with low haemoglobin concentration retain more successfully their viability. The results of this research and the data from the literature visibly indicate that the adaptation to an increased level of toxicants in the environment of fish and reptiles occurs in different ways.

Since the determining of the reference range of the haematological indicators in the poikilotermic animals is a difficult task, the comparison with the data from the literature is the only way for evaluating the fluctuations in their values and their dependence on the environmental factors and the anthropogenic pollutants. For example, our data for the erythrocyte count in population № 1 living in the conditions of a stable environment without anthropogenic pollution is close to that reported for *P. ridibundus* from relatively clean water basins in Bulgaria situated in the city of Plovdiv region – 380.000 x 10^6/μl ± 11.24 (Tatchev *et al.* 1975) and the town of Harmanli – 297.400 x 10^6/μl ± 21.22 (Zhelev *et al.* 2005). On the other hand, the data for that same indicator (Table 3) in both populations living in conditions of anthropogenic pollution are comparable (№ 2) or slightly higher (№ 3) than those that were found for species living in analogical conditions in the environment of the "Rozov Kladenets" Reservoir from the region of "Maritsa-Iztok" – 1 Thermal Power Plant (the town of Galabovo) – 437.100 x 10^6/μl ± 29.27 and Maritsa River in the region of the "Neohim" chemical plant (the town of Dimitrovgrad) – 426.700 x 10^6/μl ± 24.70 (Zhelev *et al.* 2005). Based on the comparisons that were made, we find that the erythrocyte count is a sufficiently indicative feature that indicates anthropogenic transformation of the biotope and is visibly affected by the nature of the toxicants. So, in the present research, we found a clearly increasing trend in the populations from the polluted biotopes, in connection with the type of pollution (in population № 3 the values are the highest) based on the values achieved earlier for the indicator for the species in Bulgaria both when living in relatively clean (2.01 – 9.95 g/dl), as well as in polluted (2.30 – 3.87 g/dl) biotopes (Tatchev *et al.* 1975, Zhelev *et al.* 2005). However, our results on the amount of haemoglobin and the literature data discussed above, do not allow us to firmly declare that the amount of haemoglobin when observed outside of the context of the other morphophysiological indicators is a sufficiently reliable bioindicative marker for the anthropogenic pollution.

The analysis of the differential blood formula in the excerpts of the studied populations of *P. ridibundus* indicates that regardless of the environmental parameters in the three biotopes, the blood of all animals has lymphoid nature, but the amount of the biggest cell group is statistically reliable in the relatively clean biotope and it is the lowest in population № 2 (Tables 2 and 3). The established reliable lymphopenia in the populations from the anthropogenically polluted biotopes occurs in the conditions of statistically reliable changes in the numeric ratio of all other mature white blood cells in the animal

Table. 2. Results from the comparison of the haematological parameters in the groups of *Pelophilax ridibundus* from the three biotopes in Southern Bulgaria, examined using one-way ANOVA; post-hoc LSD - test.

Parameters	SS Effect	Df Effect	MS Effec	SS Error	Df Erro	MS Error	F	P
RBC	2818378	2	1409189	225270.3	87	2589.314	544.2326	0.00~1
WBC	342	2	197	263.3	87	3.119	54.2542	0.00~1
Hb	11341	2	5670	6146.7	87	70.652	80.2592	0.00~1
St	93	2	46	185.0	87	2.126	21.8297	0.00~1
Sg	874	2	437	273.0	87	3.138	139.3055	0.00~1
Ba	372	2	186	284.0	87	3.265	57.0437	0.00~1
Eo	291	2	146	271.4	87	3.120	46.7105	0.00~1
Mo	1197	2	599	649.7	87	7.467	80.1808	0.00~1
Ly	3345	2	1673	1960.5	87	22.534	74.2317	0.00~1

Table 3. Descriptive statistics: morphological haematological parameters (RBC: erythrocyte count; WBC: leukocyte count; Hb: hemoglobin; St: Stab neutrophils; Sg: Segmented nuclei neutrophils Ba: Basophils; Eo: Eosinophils; Mo: Monocytes; Ly: Lymphocytes) in the populations of *Pelophylax ridibundus* from the investigated biotopes (Means ± standard errors of means; Range).

<table>
<tr><th rowspan="2">Parameters</th><th colspan="3">Biotopes</th><th rowspan="2">one-way ANOVA; LSD - test</th></tr>
<tr><th>River Sazliyka near the village Rakitnitsa. Population 1 (n=30)</th><th>River Sazliyka near the town Radnevo. Population 2 (n=30)</th><th>River Topolnitsa near the village Poibrene. Population 3 (n=30)</th></tr>
<tr><td>RBC
(x 10^6/µl)</td><td>366.670±10.40
(270.000–490.000)</td><td>482.670±12.12
(380.000–590.000)</td><td>629.670±26.84
(400.000–920.000)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td>WBC
(x 10^6/µl)</td><td>2.396±0.08
(1.500–3.400)</td><td>3.400±0.09
(2.600–4.400)</td><td>4.123±0.09
(3.100–4.850)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td>Hb
(g/dl)</td><td>5.53±1.38
(4.57–7.10)</td><td>6.88±2.04
(4.87–8.64)</td><td>8.28±1.02
(7.18–9.25)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td>St</td><td>2.40±0.23
(1–6)</td><td>3.03±0.30
(1–8)</td><td>4.80±0.26
(3–8)</td><td>1/2 - ns; 1/3***; 2/3***</td></tr>
<tr><td>Sg</td><td>7.20±0.29
(4–10)</td><td>11.10±0.38
(5–15)</td><td>3.46±0.29
(1–7)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td>Ba</td><td>2.13±0.18
(1–4)</td><td>5.60±0.52
(2–10)</td><td>0.77±0.08
(0–3)</td><td>1/2***; 1/3**; 2/3***</td></tr>
<tr><td>Eo</td><td>1.83±0.14
(1–3)</td><td>4.70±0.53
(1–11)</td><td>0.37±0.10
(0–2)</td><td>1/2***; 1/3**; 2/3***</td></tr>
<tr><td>Mo</td><td>3.40±0.35
(1–9)</td><td>7.17±0.49
(3–13)</td><td>12.30±0.62
(6–19)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td>Ly</td><td>83.04±0.80
(72–90)</td><td>68.40±1.03
(57–78)</td><td>78.30±0.74
(72–87)</td><td>1/2***; 1/3***; 2/3***</td></tr>
<tr><td colspan="5">Note: * - p < 0.05 (significant); ** - p < 0.01 (more significant); *** - p < 0.001 (most significant); ns - p > 0.05 (not significant)</td></tr>
</table>

peripheral blood (the only exception is that for the band cells between populations № 1 and № 2; p > 0.05), which is an illustration for a total mobilization of the defense mechanisms of the organism as a result of the development of infectious processes.

Thus, under the circumstances of neutrophilia in population № 2 and neutropenia in population № 3 when compared with the control group, many significant differences in the changes among the neutrophil granulocytes in the populations from the two polluted biotopes can be found. At an unchanged ratio of the band cells (when compared with the control group), the amount of segmented cells in the population inhabiting Sazliyka River in the vicinity of the town of Radnevo increases 1.5-times. In the population in Topolnitsa River, the number of band cells is increased reversely statistically reliably when compared with the control group and the population № 2 (in approximately close ratios), while the number of segmented cells simultaneously reliably decreased, the reduction compared with the control group is 2-times, and compared with population № 2 is over 3-times.

The main role of the neutrophils is the participation in the nonspecific immune response and the defense of the organism against infections and toxicosis. As a rule, these cells, being active ferment makers, participate in the phagocytosis of bacteria and products from the tissue dissolution, a function they start to perform from the stage of a band cell. It is proven that *P. ridibundus* when compared with other representatives of the genus possesses high exit level of neutrophil granulocytes (Sils 2008), which is one of the prerequisites for their high biological tolerance when living in urban and anthropogenically polluted environment. A general stimulation of the neutrophil granulocytosis with an increase of the relative part of the segmented cellsis was found when *P. ridibundus* had a short-term stay in high concentrations of solutions of waste water coming from sugar companies. In the case of a longer stay in the conditions of toxicosis, the part of the band cells increases and young undifferentiated cells appear (Vafis & Peskova 2009). Neutrophilia, combined with left-hand movement of the granulocytosis was found when the species lived in the conditions of urbanization and anthropogenic pollution (Sils 2008). It is considered that the stimulation of the general neutrophil granulocitopoiesis in the amphibians can be viewed as an adaptive mechanism which increases the defense function of the blood when living in a polluted environment (Fournier *et al*. 2005). Continuous living in the conditions of high anthropopression leads to neutropenia, resulting in total weakening of the defense mechanisms of the organism (Chernishova & Starostin 1994, Cabagna *et al*. 2005). It is indicated that the contact with high doses of the toxicant initially leads to a sharp increase of the band neutrophils, which is accompanied by a decrease of the segmented neutrophils (Vafis & Peskova 2009) and is followed by the disappearance of the young cells in the blood and a sharp decrease of the band neutrophils (Peskova & Vafis 2007).

In the context of the reviewed literature data, the situation observed for population № 2 (neutrophilia, mostly at the expense of the segmented cells) can be considered as an adaptive reaction to the toxicants present in the water. At the same time, the population living in the Topolnitsa River (a relative increase in the number of band cells in the conditions of general neutropenia) is an indication for deep degenerative malfunctioning in the amphibian bodies under the effect of the toxicants.

The presence of basophil granulocytes in the peripheral blood of the individuals from population № 2 is with the highest statistical reliability both compared with the control group, and with population № 3. On the other hand, when compared with the other two groups, the population living in the Topolnitsa River has statistically reliable basopenia.

The functional role of the basophil cells and their cytochemical functions in the amphibian organism is not very clear. It is considered that they, being cells that emit biologically active substances, participate in the desintoxicating processes (Chernysheva & Starostin 1994). In Bulgaria, unlike in other parts of the area, it was found that there is relatively high basophilia in the blood of the *P. ridibundus*, even when they reside in relatively clean water basins (Nikolov & Darakchiev 1988, Zhelev 2007). In the literature, it is reported that when *P. ridibundus* lives in an environment with a low level of toxicants, there is basophilia in their blood (Romanova & Romanova 2003). The same was found in relatively high levels of urbanization (Sils 2008). In experimental conditions, under the influence of medium to high doses of some toxicants such as petrol (Peskova & Vafis 2009), in some cases basopenia was found, and in others (petroleum) there was total lack of basophils in the peripheral blood of the *P. ridibundus* (Peskova & Sharpan 2007).

Regarding the eosinophil granulocytes in the peripheral blood of the samples studied, the highest value is in the population № 2, and the increase is reliable when compared with the control group and population № 3. At the same time, the population from the Topolnitsa River has statistically reliable eosinopea when compared with the other two.

It is considered that the increase of the eosinophil cells in the amphibian blood most often is caused by parasite invasions (Elkan 1976) and it is usually observed in natural populations, rather than in those living in urbanized environment (Kiesecker 2002, Sils 2008, Davis *et al*. 2010). On the other hand, there is the notion that the stimulation of the eosinophil granulocitopoiesis can be viewed as a type of a defense (antitoxic and antimicrobial) reaction of the organism (Chernysheva & Starostin 1994, Rohr *et al*. 2008, Shutler & Marcogliese 2010), a situation like the one we have in our research in population № 2.

In the conditions of the previously discussed lymphopenia in the populations living in the conditions of anthropogenic pollution, when compared with the control group, we find in the peripheral blood of *P. ridibundus* for the other type of mature granulocytes statistically reliable monocytosis, and the increase of the cell macrophages is the biggest in population № 3 (Tables 2 and 3).

The monocytes, being the basic cells of the mononuclear phagocyte system, actively participate in the phagocytosis of foreign agents and products of the tissue dissolution. Statistically reliable monocytosis was found in the peripheral blood of *P. ridibundus* inhabiting waste waters from chemical companies (Isaeva & Vyazov 1997), as well as the areas of companies from the mining industry. There, in the conditions of general lympho- and monocytosis when compared with the control group, seasonal stimulation of the myeloid branch of the haemopoiesis and a reliable increase of the monocyte count were found (Zhelev 2007).

Peskova (2001), upon using the analysis of her data and literature data, offers the distinguishing of two types of changes in the differential blood formula in the tailless amphibians living in the conditions of anthropogenic pollution. The first type of changes in the conditions of leukocytosis is the reliable increase of either the total neutrophil count or just the segment of the band neutrophils while there is a simultaneous decrease of the number of segmented neutrophils. Thus, the neutrophilia is not always required but it is always linked with "left-shifting" of the formula. Besides, it is almost always accompanied with monocytosis. In this general type of reaction, we can observe various changes in the lymphocyte, eosinophil and basophil count: lymphocytosis, basophilia and eosinophilia (Romanova & Romanova 2003, Romanova & Egorikhina 2006, Zhelev 2007, Shutler & Marcogliese 2011); lymphocytosis with basopenia and eosinopenia (Peskova & Sharpan 2007); lymphopenia, myelocytopenia and basopenia (Peskova & Vafis 2007). The changes in the white blood cell count of the first type, in the conditions of anthropogenic pollution, strengthen the defense of the organism against deteriorated living conditions and are considered to be adaptive.

In our research, we find this type of change of the differential blood formula (neutrophilia and monocytosis, combined with basophilia and eosinophilia in the conditions of general lymphopenia when compared with the control group) in the case of general leukocytosis in *P. ridibundus* from the population, inhabiting Sazliyka River in the region of the town of Radnevo.

In the second type of change of the differential blood formula in the tailless amphibians, again in the case of general leukocytosis, it is observed clear neutropenia and monocytosis, accompanied most often with eosinophilia or eosinopenia. It is considered that such type of change in the white blood cell count is an indication of serious disorder in the animal organisms and a total decrease of their viability. This type of reaction is found most frequently in a living environment with a high concentration of pesticides, heavy metals or ionizing radiation (Isaeva & Vyazov 1997, Zhykova & Peskova 1999, Peskova 2001, Cabagna *et al.* 2005, Davis *et al*. 2008, Shutler & Marcogliese 2011, Lajmanovich *et al.* 2004, 2012).

In our research we find changes in the differential blood formula close to the second type of reaction (neutropenia and monocytosis combined with basopenia, eosinopenia and lymphopenia compared with the control group) in the case of general leukocytosis in the population of *P. ridibundus* from Topolnitsa River in the region of the village of Poibrene.

Conclusions

Based on the data obtained in this study, we can make the following conclusions:

1. The variations of basic hematological parameters in the populations of *P. ridibundus*, inhabiting anthropogenically polluted ponds, reflect objectively the physiological reactions of the body towards the effects of the toxicants present in the environment, and allow the finding of changes of adaptive and pathological nature, which show the stability of population development.

2. The changes in blood morphological composition of the populations of *P. ridibundus* in anthropogenically polluted environment depend on both the degree of water contamination and the nature of the existing toxicants.

3. The morphological contents of blood in the

populations of *P. ridibundus* is a highly informative indicator reflecting the long-term effect of the environment to the biota components and it can be used as a bio-indicative marker for evaluating the conditions in water basins with various degrees and types of anthropogenic pollution, and it also can be used as an addition to the data from the physicochemical analysis or as an individual primary evaluation of the conditions in the ecosystems.

Achnowledgements: The marsh frog *Pelophylax ridibundus* is listed in Encl. No. 4 of the Biological Diversity Act (Prom. SG. 77, August 9th 2002) in Bulgaria. According to Articles 41, Article 42, and Appendix 2 of the same law, capture permits for the marsh frog *Pelophylax ridibundus* are not required when the specimens are used for scientific research purposes.

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