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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

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Abstract: Basic haematological parameters such as erythrocyte count, leukocyte count, haemoglobin concentration, haematocrit value, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume, and differential blood formula of adults, of *Pelophylax ridibundus* from the Studen Kladenets Reservoir near the Lead and Zink Plant "Kardzhali" were studied. The data were compared with those of populations from biotopes with various levels of anthropogenic pollution in Southern Bulgaria. Based on the results, an evaluation of the ecological status of the Studen Kladenets Reservoir was made. The information value of the haematological parameters as bioindicators was confirmed by a general assessment of changes occurring in *P. ridibundus* populations from polluted biotopes, which showed specificity depending on the type and concentration of toxic agents.

Keywords: Haematological parameters, erythrocytes, leukocytes, anthropogenic pollution, bioindication, *Pelophylax ridibundus*

Introduction

Currently, bioindicational research is getting increasingly useful for evaluating the negative human impact on the environment (Ashikhmina 2005). Over the last decade, the great interest in the haematological research on amphibians of the order Anura has grown (Davis *et al*. 2008, Shutler, Marcogliese 2011, Baraquet *et al.* 2013, Arikan, Çiçek 2014, Zenklusen *et al*. 2014). Amphibians have fully developed circulatory and immune systems (Manning, Horton 1982). Changes in their parameters, especially under the influence of anthropogenic toxicants, give valuable information on the physiological status of animals and the developmental stability of their populations. Those parameters are successfully used for monitoring assessments of the environment (Cabagna *et al*. 2005, Venturino, Pechen de D'Angelo 2005, Sils 2008, Lajmanovich *et al*. 2008, 2012, Mineeva, Mineev 2010, 2011, Zhelev 2012a). Representatives of the family Ranidae, especially the European green frog complex, have turned into very successful test subjects. These frogs are strongly attached to the water basin (rarely move away and usually spend their lives not far from the breeding place), reproduce quickly and can sustain high levels of urbanisation (Vershinin 2007) and anthropogenic pollution (Misyura, Marchenkovskaya

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2001, Romanova, Romanova 2003, Romanova, Egorikhina 2006, Zhelev *et al*. 2005, 2006, Zhelev 2007, 2012b, Peskova, Zhelev 2009, Korzh *et al*. 2012).

Among the species of the *Pelophylax esculentus* complex, the marsh frog *P. ridibundus* (Pallas, 1771) is widespread in Bulgaria; *P. esculentus* (L., 1758) occurs only along the Danube River while *P. lessonae* (Camerano, 1882), despite its presumable presence, has not been reliably documented in this country (Biserkov *et al*. 2007, Stojanov *et al*. 2011).

There are data suggesting a correlation between the age, size and sex in some species of amphibians and their haematological parameters (Grenat *et al*. 2009, Cül *et al*. 2011, Machapatra *et al*. 2012) but the evidence for this in regard to the family Ranidae is inconclusive (Pesskova 2001, Peskova, Zhelev 2009). The changes of haematological parameters in *P. ridibundus* under conditions of anthropogenic pollution have similar effect on both sexes, with non-significant differences between them (Zhelev 2012a). The most sensitive parameters influenced by the anthropogenic pollutions are the total number of erythrocyte (RBC) and leukocyte (WBC) counts, haemoglobin (Hb) concentration and differential blood formula (Peskova 2001, Cabagna *et al*. 2005, Romanova, Egorikhina 2006, Davis *et al*. 2008, Sils 2008, Shutler, Marcogliese 2011, Zhelev *et al*. 2005, 2006, Zhelev 2007, 2012a, Zenklusen *et al*. 2014).

In our previous study (Zhelev *et al*. 2013), some statistically reliable differences in the values of main haematological parameters were found (RBC and WBC counts, Hb concentration, differential blood formula) in *P. ridibundus* populations that inhabit three biotopes with different types of anthropogenic pollution in South Bulgaria (reference conditions, domestic sewage pollution and heavy metal pollution). In parallel, there were possibilities for an objective assessment of the ecological status of each biotope on the grounds of the changes in the values of the haematological parameters. Our study continued in the populations of *P. ridibundus* from the region of the Lead and Zinc Plant “Kardzhali”. The haematological investigations in that region were part of our extensive study, which also included an indicator for developmental stability such as the fluctuating asymmetry in *P. ridibundus* populations (see Zhelev *et al*. 2014).

This article aims to report basic haematologic parameters in *P. ridibundus* from the region of Lead and Zinc Plant “Kardzhali” (South Bulgaria). We compare these results with those from anthropogenically polluted biotopes, which have been acquired in a previous study (Zhelev *et al*. 2013). On the basis of these comparative analyses, we assess the ecological status of the Studen Kladenets Reservoir, parallel and independently from the physicochemical analyses. We also evaluate the information value of the observed haematological parameters in reflecting changes in the physiological status of *P. ridibundus* under conditions of anthropogenic pollution. In this way, we test two working hypotheses: (1) there are alterations in haematological parameters of *P. ridibundus* populations in the Studen Kladenets Reservoir compared to those in reference water basin and these variations are results of the permanent pollution of the reservoir with heavy metals, and (2) under the conditions of anthropogenic pollution, the nature and concentration of toxic agents determine the specificity of changes in haematological parameters of *P. ridibundus* populations.

Material and Methods

The area of investigation

Samples were collected on 26 April 2011 from a biotope located at the tail of the Studen Kladenets Reservoir (29 km^2, 580 m altitude) in the vicinity of Lead and Zinc Plant (LZP) “Kardzhali” (Fig. 1).

Data from physical and chemical analysis of the Studen Kladenets Reservoir

Data on the water physical and chemical parameters for the period 2009-2011 were obtained from the newsletters of the Basin Directorate for Water Management in the Eastern Aegean region (http://www.bg-ibr.org) and the National Reports on the State of Environment in Bulgaria issued by the Executive Environment Agency of Bulgaria (http://eea.goverment.bg). The data showed that heavy metals in the reservoir exceeded the permissible standards in Bulgaria, Order No 7/08.08.1986 (State Gazette, No 96. 12.12.1986) (see Table 1).

Subject of study and methods for analyses

Marsh frogs *P. ridibundus* were identified based on morphological characteristics (Biserkov *et al*. 2007). A total of 30 adult individuals (Snout-Vent Length > 60.0 mm, see Bannikov *et al*. 1977) of both sexes were captured. The animals were caught during their time of reproduction in shallow coastal areas of the reservoir, at night, with the help of an electric torch (see Sutherland 2000).

The marsh frog *Pelophylax ridibundus* is listed in Annex 4 of the Bulgarian Biodiversity Act (Promulgated, State Gazette No 77/9.08.2002) al-

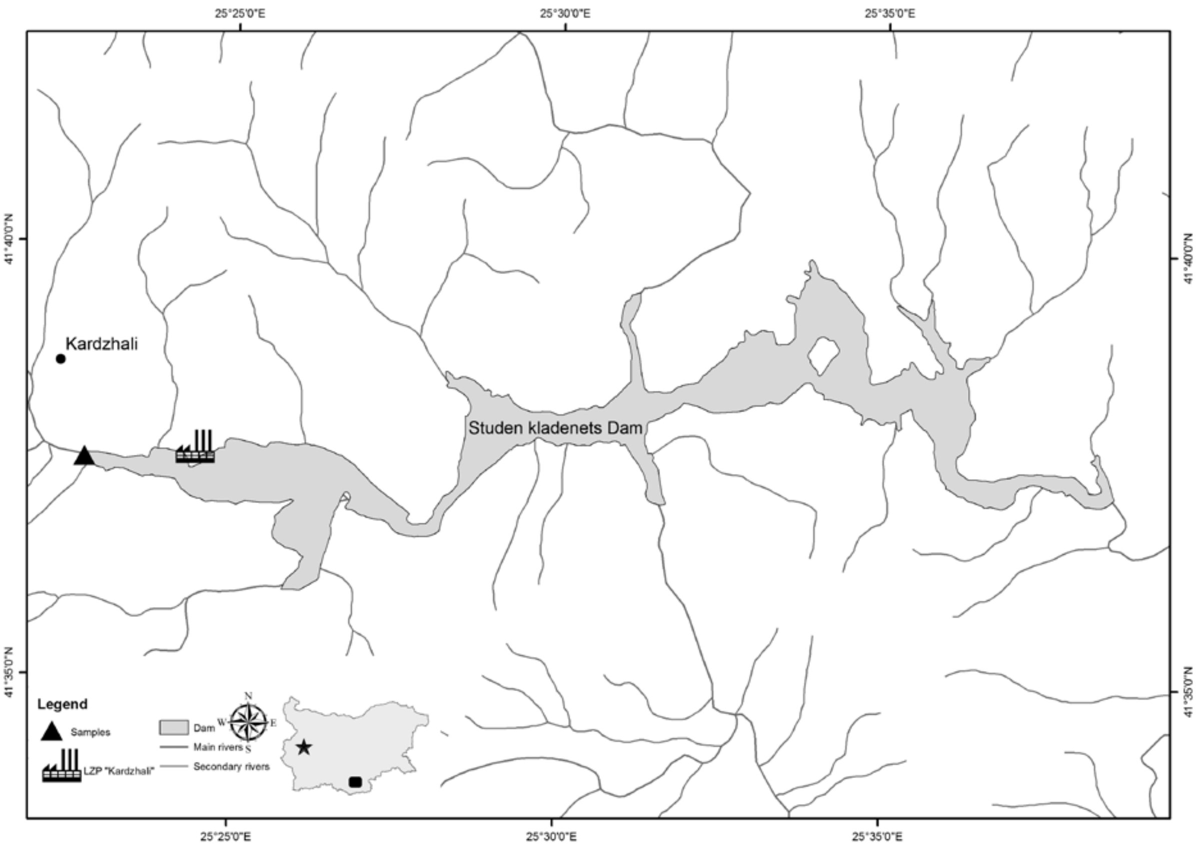

Fig. 1. Map of the studied biotope

Table 1. Data on the status of the Studen Kladenets Reservoir in the study period (physical and chemical analysis for surface water sample). * – above SKOS (assessment index): very poor condition

Parameters	**Units SI**	**Order No 7/8.8.1986 categories**	**Average annual**		
		I	2009	2010	2011
pH	pH units	6.5-8.5	8.28	7.98	7.79
Temperature	°C	to 3° middle of the season	17.03	16.9	16.5
Insoluble substances	mg/dm^3	30.0	19.78	33.33	6.74
Electroconductivity	µS/cm	700.0	250.0	245.0	313.0
Dissolved oxygen	mgO_2/dm^3	6.0	10.30	9.77	9.64
Oxygenation	%	75.0	109.44	102.4	101.98
BOD_5	mgO_2/dm^3	5.0	2.71	1.4	2.68
COD	mgO_2/dm^3	25.0	18.25	11.62	18.59
Nitrate ammonium, $N – NH_4$	mg/dm^3	0.1	0.12	0.07	0.08
Nitrate nitrogen, $N – NO_3$	mg/dm^3	5.0	0.41	0.43	0.33
Nitrite nitrogen, $N – NO_2$	mg/dm^3	0.002	0.001	0.002	0.03
Orthophosphates	mg/dm^3	0.2	0.15	0.05	0.02
Total nitrogen	mg/dm^3	1.0	0.43	1.73	0.93
Total phosphorus - as P	mg/dm^3	0.4	0.03	0.12	0.10
Sulphates (SO_4^{2-})	mg/dm^3	200.0	33.59	50.08	68.64
Cadmium (Cd)	mg/dm^3	SKOS – 0.005	0.012*	0.015*	0.014*
Copper (Cu)	mg/dm^3	SKOS – 0.05	0.011	0.007	0.015
Zinc (Zn)	mg/dm^3	SKOS – 1.0	0.619	3.366*	4.905*
Lead (Pb)	mg/dm^3	SKOS – 0.02	0.0225*	0.034*	0.027*
Nickel (Ni)	mg/dm^3	SKOS – 0.02	0.006	0.001	0.002
Chromium (Cr)	mg/dm^3	SKOS – 0.02	0.003	< 0.0005	< 0.0005

lowing (Articles 41-42 and Annex 2) capturing *P. ridibundus* for scientific purposes without issuing special permits. The Ethics Board for Experimental Animals, Faculty of Biology at Plovdiv University, approved the animal handing and laboratory methodology. All manipulations were conducted in accordance with national and international guidelines of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/EU). The manipulations with the animals, one day after their capturing, were conducted under laboratory conditions, in accordance with the ethical standards for research work with live animals (Fellers *et al*. 1994, Steven *et al*. 2004). To reduce the stress, animals were transported to the laboratory as soon as possible in buckets full of water. The frogs were anaesthetised with ether (see Stetter 2001). Blood (0.20 ml) was taken through cardiac ventricular punctures using small heparinised needles 20 mm long (Wright 2001; Cabagna *et al*. 2005). Due to the sex differences in haematological indicators of species of the family Ranidae, particularly in *P. ridibundus* in polluted habitats containing specimens of the *striata* morph*,* the sex of the animals was not indicated. Data were analysed as a whole, not separating males and females or morphs *striata* and *maculata*.

The erythrocyte (RBC) and leukocyte (WBC) counts were determined according to the method of Wierord, using Burker chamber (Pavlov *et al*. 1980, Ibrishimov, Lalov 1984). The standard Hayem's solution was used as a diluting solution for erythrocytes, while the Turck's solution was used for leucocytes. Dilution of 200 times was carried out in the erythrocyte count and of 20 times in the leukocyte count. The haemoglobin concentration (Hb) was determined by means of cyan-haemoglobin method reading 540 nm (Pavlov *et al*. 1980). The differential blood formula (St - Stab neutrophils; Sg - Segmented nuclei neutrophils; Ba - Basophils, Eo - Eosinophils; Mo - Monocytes; Ly - Lymphocytes) was determined on the basis of 100 leukocytes per slide (Ivanova 1982) using the microscopic method of Shiling (Pavlov *et al*. 1980, Ibrishimov, Lalov 1984).

The data on haematological parameters obtained for *P. ridibundus* from the region of the Studen Kladenets Reservoir were compared with the data for the species populations that inhabit biotopes in Southern Bulgaria with different levels and types of anthropogenic pollution (Sazliyka River near the village of Rakitnitsa – reference site; Sazliyka River near the town of Radnevo – domestic sewage pollution; Topolnitsa River near the village of Poibrene – heavy metal pollution) acquired in a previous study (Zhelev *et al*. 2013).

Along with RBC, WBC, Hb and the differential blood formula that were used in the analyses in Zhelev *et al.* (2013), the haematocrit values (PCV) as well as three derivative parameters, i.e. mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV), were studied. The PCV was determined by using heparinised haematocrit capillaries. The blood was centrifuged for 5 min at a 3000 rpm constant-rotation centrifuge, in thin-walled capillary tubes and the value obtained was read from the scale and recorded in L/l (Blaxhall, Daisley 1973, Pavlov *et al.* 1980). The three derivative haematological parameters (MCH, MCHC, MCV) were calculated from the above results. MCH and MCHC were calculated according to Wintrobe (1933) while MCV by dividing the haematocrit per litre of blood by the total RBC count (Pavlov *et al.* 1980, Perry *et al*. 1998).

Statistical procedures

We applied standard statistical methodology using the Statistical 7.0. Software (Statistica 2004). The normality in distribution of the parameters was tested with Shapiro-Wilk-test (Shapiro *et al*. 1968), which indicated normal distribution: $p > 0.05$. The preliminary analysis (one-way ANOVA) showed a high statistically reliable difference between the features analysed. The grouping was performed on the basis of the level of pollution in the biotopes, and the results were confirmed with a post-hoc LSD-test. Results with $p < 0.05$ [$\alpha = 5\%$] were considered significant. The degree of informativeness and the participation of each of the haematological parameters in differentiating the individuals from the populations in the four compared biotopes were assessed by a Principal Component Analysis - PCA). Using standard discriminant analysis (DA), the relation between the parameters of the environment and the complex of haematological parameters in the *P. ridibundus* populations from the region of the LZP "Kardzhali" as well as of those in the three compared populations was evaluated. Discrimination between the populations was performed on the basis of the extent and nature of the anthropogenic pollution in the biotopes, as follows: Population 1 (P-1) – relatively unpolluted – the Sazliyka River downstream of the village of Rakitnitsa; Population 2 (P-2) – with domestic sewage pollution – the Sazliyka River downstream of the town of Radnevo; Population 3 (P-3) – the Topolnitsa River downstream of the village of Poibrene; and Population 4 (P-4) – with heavy metal pollution - the Studen Kladenets Reservoir.

Results

Descriptive statistics and ANOVA

The results obtained (descriptive statistics) for the haematological parameters in the *P. ridibundus* population from the Studen Kladenets Reservoir, as well as their comparisons with the data for the other three populations in Southern Bulgaria, by using one-way ANOVA, are presented in Tables 2, 3 and 4.

RBC - Red blood cell counts. The value of this parameter in the population from the Studen Kladenets Reservoir (P-4) was higher than that of the control group (P-1) and of the population living in the water with domestic sewage pollution (P-2); however, this value was lower than that of the population inhabiting the basin contaminated with heavy metal pollution (P-3). All differences were significant.

Hb - haemoglobin concentration. The value of this parameter in P-4 was higher than that in the reference group and lower than that of P-3. There was no difference with P-2.

PCV - haematocrit value. The value of this parameter was significantly the lowest in P-1 and the highest in P-3. There was no difference when comparing P-2 with P-4.

MCH - mean corpuscular haemoglobin. The lowest value of this parameter was found in P-4; its differences with P-1 and P-2 were significant, and there were no difference with P-3. The highest value of this parameter was found in P-2; its differences with P-3 and P-4 were significant (no differences when comparing with P-1). There was no significant difference of this parameter between P-1 and P-3.

MCHC - mean corpuscular haemoglobin concentration. The value of the parameter was the lowest in P-2. Statistically significant differences were recorded with P-1 and P-3. There was no significant difference with P-4. The highest value of this parameter was found in P-1; only the difference with P-3 was not statistically significant. There was no significant difference between P-3 and P-4.

MCV - mean corpuscular volume. The value of the parameter was the statistically significantly highest in P-2. In P-4 the value was the lowest, but there were no significant differences in the comparisons with P-1 and P-3. The difference between P-1 and P-3 was not statistically significant, either.

WBC - leukocyte count. The value of the parameter in P-4 was higher than that in P-1 and lower than that in P-3 (the differences were statistically significant). There was no difference with P-2.

St - Stab neutrophils. The value of the parameter in P-4 was higher than that in P-1 and lower than that in P-3 (the differences were statistically significant). There was no difference with P-2.

Sg - Segmented nuclei neutrophils. In P-4 the parameter had the statistically significantly highest value.

Ba – Basophils. The value of the parameter in P-4 was lower than that in P-2 and higher than that in P-3 (the differences are statistically significant). No significant difference with P-1.

Table. 2. Comparison of the haematological parameters (RBC: erythrocyte count, WBC: leukocyte count, Hb: haemoglobin concentration, PCV: haematocrit value, MCH: mean corpuscular haemeglobin, MCHC: mean corpuscular haemeglobin concentration, MCV: mean corpuscular volume, St: Stab neutrophils, Sg: Segmented nuclei neutrophils, Ba: Basophils, Eo: Eosinophils, Mo: Monocytes, and Ly: Lymphocytes) in the groups of *Pelophilax ridibundus* from the four biotopes in Southern Bulgaria, using one-way ANOVA. * (p<0.00~1)

Parameters	SS Effect	Df Effect	MS Effect	SS Error	Df Error	MS Error	F
RBC	1181462	3	393820.7	980271.0	116	8450.612	46.6026*
WBC	11498	3	3832.8	9073.8	116	78.223	48.9989*
Hb	93	3	30.9	222.3	116	1.917	16.1449*
PCV	1742	3	580.7	360.4	116	3.107	186.8957*
MCH	374	3	124.7	309.4	116	2.667	46.7483*
MCHC	338	3	112.6	292.9	116	2.525	44.5798*
MCV	1292	3	430.5	820.3	116	7.072	60.8768*
St	4319	3	1439.7	2276.5	116	19.625	73.3599*
Sg	0	3	0.1	0.1	116	0.001	92.3850*
Ba	10856	3	3618.8	58587.7	116	505.066	7.1650*
Eo	8129	3	2709.5	67131.1	116	578.717	4.6820*
Mo	302881	3	100960.4	875474.3	116	7547.192	13.3772*
Ly	48	3	15.9	23.6	116	0.203	78.2500*

Table 3. Descriptive statistics: morphological haematological parameters (RBC: erythrocyte count, Hb: haemoglobin, PCV: haematocrit value, MCH: mean corpuscular haemeglobin, MCHC: mean corpuscular haemeglobin concentration, and MCV: mean corpuscular volume) in the *Pelophylax ridibundus* populations (P) from the investigated biotopes (Means ± standard errors of means, Range)
* (p<0.05), ** (p<0.01), *** (p<0.001), ns (p>0.05)
The data from the comparisons of the mean values for the parameters RBC and Hb in populations 1, 2 and 3 are presented in Zhelev *et al*. (2013)

Parameters	**Biotopes**				One-way ANOVA, post-hoc LSD-test
	The Sazliyka River near the village of Rakitnitsa. P-1 (n=30)	The Sazliyka River near the town of Radnevo. P-2 (n=30)	The Topolnitsa River near the village of Poibrene. P-3 (n=30)	The Studen Kladenets Reservoir near the LZP "Kardzhali" P-4 (n=30)	
RBC (x 10^6/µl)	366.670±10.40 (270.000–490.000)	482.670±12.12 (380.000–590.000	629.670±26.84 (400.000–920.000)	571.633±10.72 (480.000–690.000)	1/4***, 2/4***, 3/4*
Hb (g/dl)	5.53±1.38 (4.57–7.10)	6.88±2.04 (4.87–8.64)	8.28±1.02 (7.18–9.25)	7.16±1.83 (5.21–8.97)	1/4***, 2/4ns, 3/4***
PCV (L/l)	0.25±0.005 (0.21–0.31)	0.34±0.005 (0.30–0.40)	0.37±0.006 (0.31–0.43)	0.34±0.005 (0.30–0.41)	1/2***, 1/3***, 2/3***, 1/4***, 2/4ns, 3/4***
MCH (pg)	153.32±5.02 (105.17–220.17)	160.30±2.77 (129.02–186.25)	146.08±5.09 (98.55–191.05)	134.59±2.92 (110.04–172.85)	1/2ns, 1/3ns, 2/3*, 1/4***, 2/4***, 3/4ns
MCHC (g/l)	225.05±5.28 (149.77–298.45)	203.98±4.27 (152.41–245.12)	221.18±3.74 (141.72–265.19)	211.65±4.13 (157.97–254.53)	1/2***, 1/3ns, 2/3***, 1/4*, 2/4ns, 3/4ns
MCV (fl)	682.57±17.82 (517.44–937.50)	774.44±9.83 (670.00–931.25)	650.91±20.82 (452.78–912.50)	652.82±12.59 (540.50–806.25)	1/2***, 1/3ns, 2/3***, 1/4ns, 2/4***, 3/4ns

Table 4. Descriptive statistics: morphological haematological parameters (WBC: leukocyte count, St: Stab neutrophils, Sg: Segmented nuclei neutrophils, Ba: Basophils, Eo: Eosinophils, Mo: Monocytes, and Ly: Lymphocytes) in the populations (P) of *Pelophylax ridibundus* from the investigated biotopes (Means ± standard errors of means, Range)
* (p<0.05), ** (p<0.01), *** (p<0.001), ns (p>0.05)
The data from the comparisons of the mean values for the parameters WBC and differential blood formula in populations 1, 2 and 3 are presented in Zhelev *et al*. (2013)

Parameters	Biotopes				One-way ANOVA, post-hoc LSD-test
	The Sazliyka River near the village of Rakitnitsa. P-1 (n=30)	The Sazliyka River near the town of Radnevo. P-2 (n=30)	The Topolnitsa River near the village of Poibrene. P-3 (n=30)	The Studen Kladenets Reservoir near the LZP "Kardzhali" P-4 (n=30)	
WBC (x 10^6/µl)	2.396±0.08 (1.500–3.400)	3.400±0.09 (2.600–4.400)	4.123±0.09 (3.100–4.850)	3.647±0.06 (3.200–4.300)	1/4***, 2/4ns, 3/4***
St	2.40±0.23 (1–6)	3.03±0.30 (1–8)	4.80±0.26 (3–8)	3.43±0.21 (2–6)	1/4**, 2/4ns, 3/4***
Sg	7.20±0.29 (4–10)	11.10±0.38 (5–15)	3.46±0.29 (1–7)	13.47±0.32 (10–17)	1/4***, 2/4***, 3/4***
Ba	2.13±0.18 (1–4)	5.60±0.52 (2–10)	0.77±0.08 (0–3)	2.57±0.17 (2–6)	1/4ns, 2/4***, 3/4***
Eo	1.83±0.14 (1–3)	4.70±0.53 (1–11)	0.37±0.10 (0–2)	0.87±0.16 (0–3)	1/4*, 2/4***, 3/4ns
Mo	3.40±0.35 (1–9)	7.17±0.49 (3–13)	12.30±0.62 (6–19)	9.67±0.44 (5–16)	1/4***, 2/4***, 3/4***
Ly	83.04±0.80 (72–90)	68.40±1.03 (57–78)	78.30±0.74 (72–87)	70.00±0.60 (65–75)	1/4***, 2/4ns, 3/4***

Eo – Eosinophils. The value of the parameter in P-4 was lower than those in P-1 and P-2 (the differences were statistically significant). No difference with P-3.

Mo – Monocytes. The value of the parameter in P-4 was higher than those in P-1 and P-2, and less than that in P-3 (statistically significant differences).

Ly – Lymphocytes. The value of the parameter in P-4 was statistically significantly lower than in P-1 and P-3. No difference with P-2.

Multi-variational statistics - PCA

The sum of the first four variables includes 79.41% of the variables in individuals of both sexes in the *P. ridibundus* populations from the four biotopes studied: eigenvalue was fixed ≥ 1 (Fig. 2).

The parameters RBC (0.928), Hb (0.833), PCV (0.855), Mo (0.827) and WBC (0.752) showed high degree of correlation in reference to the first axis (Factor 1). The parameters Ly (-0.872), Ba (-0.796), Eo (-0.664), and Sg (-0.634), had a high correlation in reference to the second axis (Factor 2). The negative dependence on Factor 3 was indicated only by MCH (-0.848). The positive dependence on Factor 4 was indicated only by MCHC (0.708). The grouping of parameters in reference to the first two main axes is shown in Fig. 3.

Standard discriminant analysis - DA

The discriminant analysis (on the basis of the parameters RBC, Hb, PCV, Mo, WBC, Ly, Ba, Eo and Sg) defined as statistically significant the difference between the compared groups of individuals from the four populations in the biotopes with various degrees of anthropogenic pollution in Southern Bulgaria. The parameters used were as follows: RBC (Wilks' Lambda = 0.009; F= 2.942; p = 0.003), PCV (Wilks' Lambda = 0.011; F = 13.783; p = 0.000), WBC (Wilks' Lambda = 0.011; F = 13.296; p = 0.000), Sg (Wilks' Lambda = 0.012; F = 18.451; p = 0.000), Ba (Wilks' Lambda = 0.009; F = 5.599; p = 0.001) and Eo (Wilks' Lambda = 0.010; F =10.339; p = 0.000). The Mahalanobis distances received are presented in Table 5.

The spatial distribution of factor weights separates the individuals of *P. ridibundus* from the reference group (P-1) and those from the Topolnitsa River (P-3) into two clearly differentiated groups. The individuals from P-2 and P-4 jointly outlined a third group (Figure 4).

The results of the discriminative analysis distinguished most clearly the individuals of *P. ridibundus* from P-3. According to the rate of increase in the Mahalanobis distances, it is differentiated from the others as follows: 3/4 (40.465), 3/2 (50.942) and 3/1 (52.942). For the individuals from the population

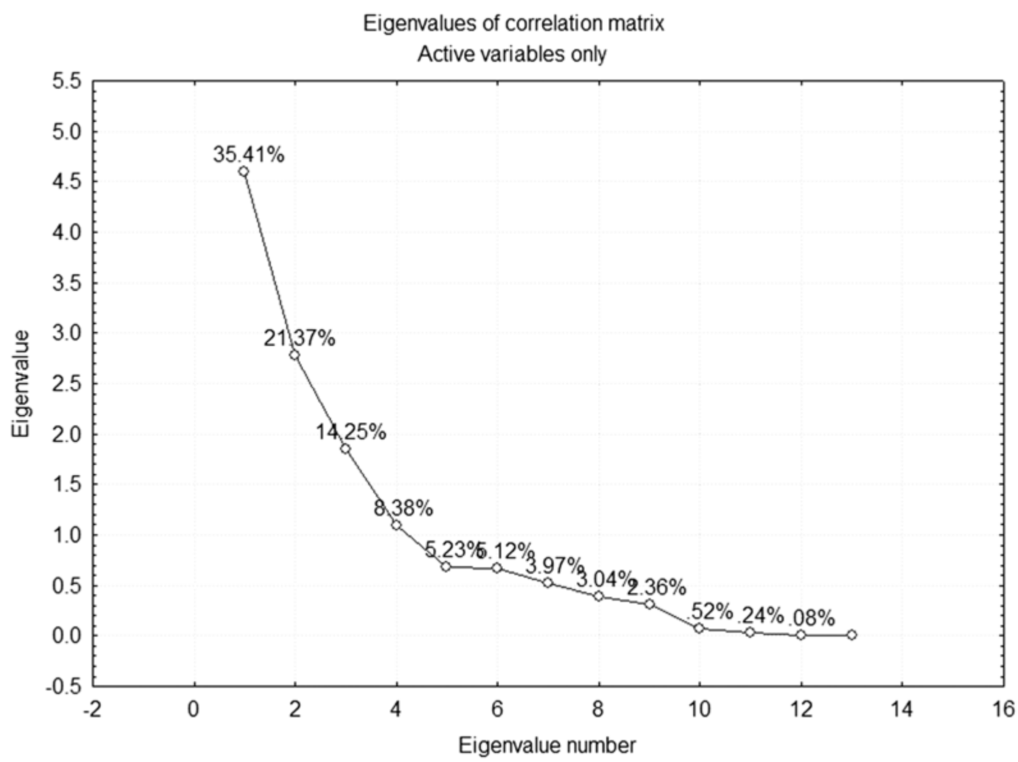

Fig. 2. Graphic of the variation for 13 haematological parameters in the *Pelophylax ridibundus* populations from the biotopes with different levels of anthropogenic pollution in Southern Bulgaria

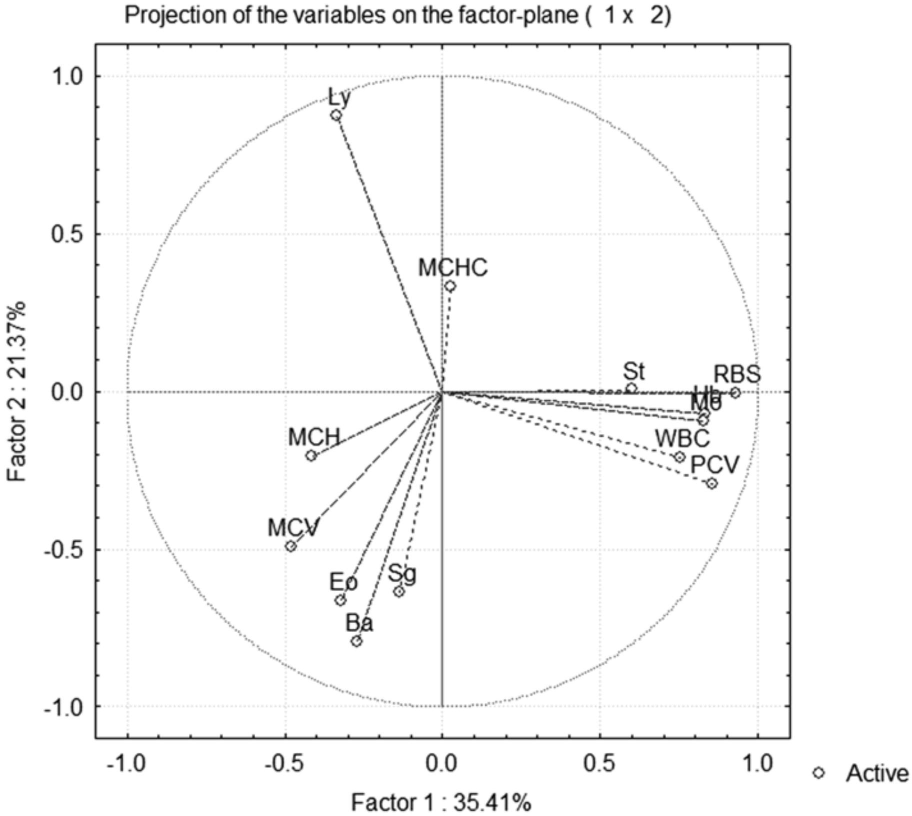

Fig. 3. Graphic of the correlations of 13 haematological parameters (factor weights) in the *Pelophylax ridibundus* individuals from the biotopes with various degrees of anthropogenic pollution in Southern Bulgaria, to the first two main axes

Table 5. Mahalanobis distances on six haematological parameters: RBS, PCV, WBC, Sg, Ba, and Eo, between the individuals from the four compared populations (P) of *Pelophylax ridibundus* from the biotopes with different levels of anthropogenic pollution in Southern Bulgaria

Type	P-1	P-2	P-3	P-4
P-1	0.000	27.164	52.942	32.722
P-2	27.164	0.000	50.254	12.869
P-3	52.942	50.254	0.000	40.465
P-4	32.722	12.869	40.465	0.000
F-values; df = 9.108; p-levels < 0.00~1				
P-1	0.000	42.151	82.151	50.776
P-2	42.151	0.000	77.980	19.969
P-3	82.151	77.980	0.000	62.791
P-4	50.776	19.969	62.791	0.000

under the conditions of heavy metal pollution (P-4), the Mahalanobis distance is the least with the individuals from P-2, followed by that of the individuals from the reference group P-1 (Table 5). The individuals that live in the conditions of domestic sewage pollution (P-2), in accordance with the Mahalanobis distances for the six comparable haematological parameters, showed the biggest closeness to those from P-4. There was a greater Mahalanobis distance to those from P-1, and the most significant differences were those to the individuals from P-3 (Table 5).

Discussion

This study found the following changes in the *P. ridibundus* population from the Studen Kladenets Reservoir when comparing with the reference group: erythrocytosis, hyperchromia, leukocytosis, neutrophilia (increasing St and Sg cells), monocytosis, eozinopeniya and lymphopenia.

On analysing PCV and the three derivative parameters, i.e. MCH, MCHC, and MCV, we found: an increase in the PCV values in each population that

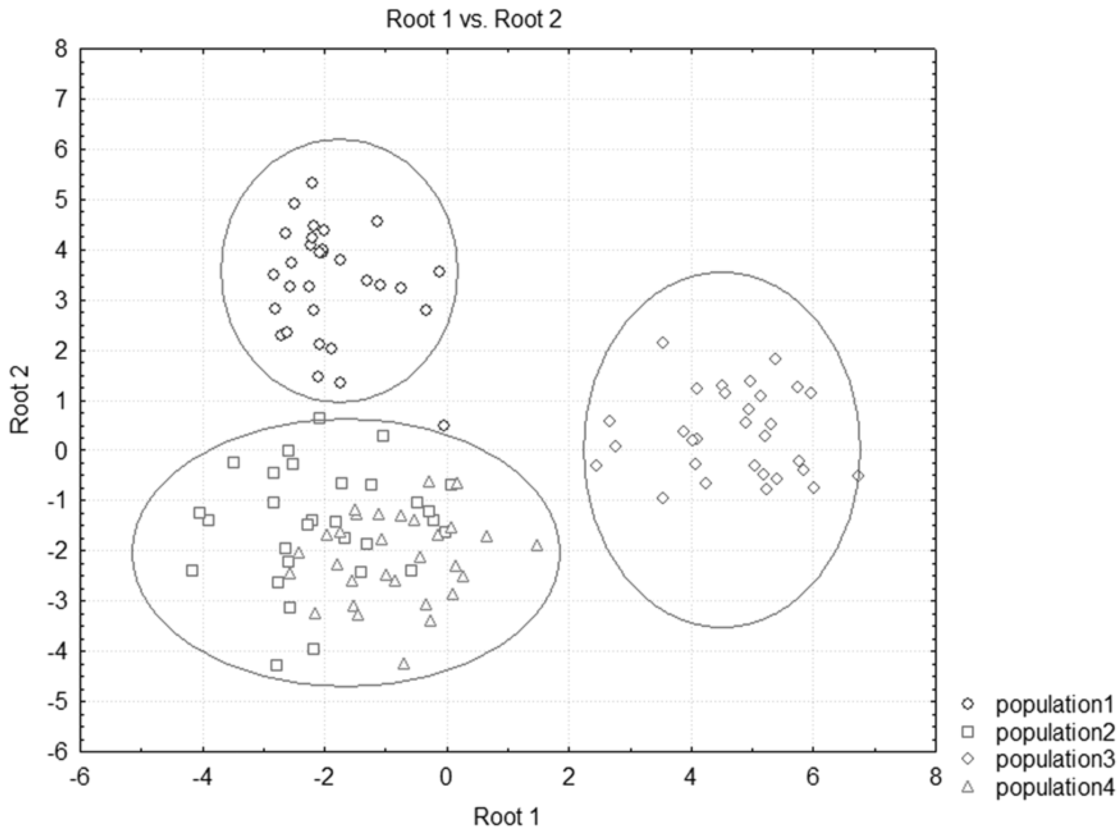

Fig. 4. Discriminatory coordinates by six haematological parameters (RBC, PCV, WBC, Sg, Ba, and Eo) of individuals from the four compared *Pelophylax ridibundus* populations from the biotopes with different levels of anthropogenic pollution in Southern Bulgaria

lives in conditions of anthropogenic pollution, a decrease in the MCH values (in P-4) and MCHC (in P-2 and P-4), and an increase in the MCV in P-2. In the literature, there are scarce data on the indicators PCV, MCH, MCHC and MCV in Anura; most studies are focused on the identification of reference values in different species (Arserim, Mermer 2008, Gül *et al*. 2011, Mahapatra *et al*. 2012). There are few data on changes of PCV and the three derivative indicators in populations of amphibians, which live in conditions of anthropogenic pollution. In a previous study (Zhelev *et al*. 2005), we found a statistically significant increase in PCV in the *P. ridibundus* populations living in a region of high-developed energy industry – Maritsa-East 1 TPP, near the town of Galabovo, and there were no differences found in the values of indicators MCH, MCHC and MCV as compared with the reference group from the region of the town of Harmanli (a relatively pure biotope). At the same time, in the *P. ridibundus* populations that inhabit another anthropogenically polluted region (town of Dimitrovgrad, in the area of the Neochim Chemical Plant), there was a statistically significant decrease in the values of all four indicators (PCV, MCH, MCHC and MCV) compared to the reference group.

Basing on above results, we consider that the changes in the haematological parameters in P-4 could be referred rather to the so-called first type of reaction, according to the classification of Peskova's (2001). The results of discrimination (Fig. 4) put P-2 and P-4 in the group of a common multiple, while separate them distinctly not only from P-1, but also from P-3. This is certainly an interesting fact in terms of the nature of toxicants (heavy metals) in the Studen Kladenets Reservoir. The same type of pollutants was presented in the biotope inhabited by P-3 (the Topolnitsa River downstream of the village of Poibrene). Therefore, the following conclusion can be made: anomalies in the physiological condition of the animals from the respective *P. ridibundus* populations are different in the presence of the same toxicants: those anomalies are the most serious in the Topolnitsa River. This opinion is also supported by the data obtained on the values of an integral indicator for developmental stability (the fluctuating asymmetry), which was studied in parallel, in *P. ridibundus* populations in both biotopes. In the population living in the Studen Kladenets Reservoir, the grade rate for developmental stability in the period 2009-2011 ranged between 3 (average degree of anomalies and animals in a satisfactory condition) and 4 (high degree of anomalies and an unfavourable body condition) (Zhelev *et al*. 2014). At the same time, the value of the integral indicator for developmental stability in the population, inhabiting the Topolnitsa River near Poibrene in 2010, was 5 (very serious anomalies and a critical body condition) (Zhelev *et al.* 2012). Another important fact that supports the presence of serious functional disorders in the body of amphibians from the population that inhabit the Topolnitsa River was the presence of high percentage of erythrocyte pathologies (a total of 11 different types of erythrocyte abnormalities were recorded) in the blood of animals in this group (Zhelev 2012b). The reason for the differences reported on the condition of the *P. ridibundus* populations can be found in the type of water body and the concentration of toxicants. In a basin of a closed type (such as Studen Kladenets Reservoir), the absence of currents, as well as the large size of the water body, create conditions for the accumulation of the heavy metal in the bottom sediments, while in more shallow flowing rivers this is not always the case. It is possible that the blood changes in the *P. ridibundus* populations that inhabit the Topolnitsa River (P-3) were preceded by some changes similar to those found in the populations from the anthropogenically transformed biotopes of the Sazliyka River (P-2) and the Studen Kladenets Reservoir (P-4). In this context, it is important to establish the thin borderline between the changes that characterise the peculiar "fighting phase" in the blood of amphibians, expressed in the mobilisation of body defences, and the threshold of toxicants that lead to predominance of degenerative changes of pathological nature. Naturally, this task becomes more complicated in regard to the natural water basins of different types, where the effect from a large number of combined environmental factors must be considered along with that of the anthropogenic factor alone. Such direction outlines perspectives for further research not only of fundamental, but also of strictly applied character, for the needs of bioindication and biomonitoring.

Conclusions

Basing on the changes found in the values of the haematological parameters studied in the *P. ridibundus* populations from the Studen Kladenets Reservoir, near the LZP "Kardzhali", the following conclusions can be made:

• The conducted bioindicational analyses confirm that the ecological conditions in the Studen Kladenets Reservoir are disturbed as a result of its permanent pollution with heavy metals. The living conditions in the reservoir are deteriorated, though better than those in another river ecosystem polluted with heavy metals - the Topolnitsa River. The changes that occur in the blood of *P. ridibundus* increase body defences. They help the population to better adapt to the specific living conditions in an environment with a higher content of toxicants.

• The present research confirms the opinions stated in our previous studies (Zhelev *et al*. 2005, 2006, 2013, Zhelev 2007, 2012a) about the high informativeness of the haematological parameters RBC, Hb, WBC, and the differential blood formula of *P. ridibundus* in determining the changes that occur in the body of amphibians as a result of living in conditions of anthropogenic pollution. The mentioned parameters are good bioindication markers. These results give us grounds to relate the PCV indicator to the same category. MCH and MCHC have a lower degree of informativeness.

• The parameters RBS, PCV and WBC as well as the changes in the differential blood formula (Sg, Ba and Eo) are good diagnostic markers for the blood of *P. ridibundus* showing the alterations related to the type of toxicants and their concentration (which depends on the type of water basin as well).

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