Open Journal of Animal Sciences, 2014, 4, 206-216
Published Online July 2014 in SciRes. http://www.scirp.org/journal/ojas
http://dx.doi.org/10.4236/ojas.2014.44026

Haematology of *Pelophylax ridibundus* (Amphibia: *Ranidae*) of *Striata* and *Maculata* Morphs in Populations Living in Conditions of Anthropogenic Pollution in Southern Bulgaria

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Received 23 May 2014; revised 8 July 2014; accepted 24 July 2014

Abstract

Basic haematological parameters were studied in adult, sexually mature individuals of colour morphs (*striata* and *maculata*) in the populations of *Pelophylax ridibundus* living in biotopes with various degrees of anthropogenic pollution (control, domestic sewage pollution and heavy metal pollution) in Southern Bulgaria. We found that in the polluted biotopes in individuals of both morphs of *P. ridibundus* the blood parameters: erythrocyte (RBC) and leukocyte (WBS) count, haemoglobin concentration (Hb), haematocrit (PCV) were statistically reliably higher, the number of lymphocytes (Ly) decreased, and the parameters: mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), mean cell volume (MCV) and differential blood formula changed considerably in comparison with the control group. In terms of anthropogenic pollution, the average values of RBC and Hb were higher in *P. ridibundus* of *striata* morph.

Keywords

***Striata* and *Maculata* Morphs, *Pelophylax ridibundus*, Anthropogenic Pollution, Bio-Indication,**

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How to cite this paper: Zhelev, Z.M., Arif, M., Popgeorgiev, G.S., Rauf, M. and Mehterov, N.H. (2014) Haematology of *Pelophylax ridibundus* (Amphibia: *Ranidae*) of *Striata* and *Maculata* Morphs in Populations Living in Conditions of Anthropogenic Pollution in Southern Bulgaria. *Open Journal of Animal Sciences*, **4**, 206-216.
http://dx.doi.org/10.4236/ojas.2014.44026

1. Introduction

The growing negative human impact on the environment calls for continuous monitoring of its condition, for the assessment of which more bio-indication research is increasingly being used. At the end of the 20th and in the beginning of the 21st century, a number of researchers, such as Cabagna *et al*. [1], Davis *et al.* [2], Korzh *et al*. [3], Lajmanovich *et al*. [4], Zhelev [5], have substantiated the successful application of haematological parameters of tailless amphibians (Order Anura) in biomonitoring. They are one of the first life-forms to react to changes caused by negative environmental factors, including anthropogenic ones.

Nowadays, despite the controversial attitude towards using them as good biomarkers, the changes in the values of haematological parameters in the marsh frog *Pelophylax ridibundus* Pallas, 1771 are used successfully; one of the three species of *Pelophylax* kl. *esculentus* Linnaeus, 1758. They are very attached to a specific breeding basin (They rarely leave it and spend almost their entire lives in it), they reproduce quickly in large quantities and have an extensive range. This makes it possible to conduct research on large areas using the same signs, obtaining comparable results [6]-[9]. From *Pelophylax* kl. *esculentus* the marsh frog *P. ridibundus* is a widely spread species in Bulgaria being found throughout the country, *P. esculentus* Linnaeus, 1758 can be found in the north-along the Danube, while *P. lessonae* Camerano, 1882, despite its presumed presence, has not been reliably documented [10].

The presence of alternative morphs of *P. ridibundus* by the presence or absence of a central light stripe on the back has been used since fairly recently as a bio-indicative marker for evaluation of the condition of the outer environment. Analysing the genetic nature of this morph in Ranidae Rafinesque-Schmaltz, 1814 family showed that the *striata* morph is a monogenic mutant. The dominant allele of the diallelic autosomal *striata* gene determines the presence of a stripe (complete dominance). Such mode of inheritance has been found for *P. ridibundus* [11] and *Rana arvalis* Nilsson, 1842 [12]. For the various species from the Ranidae family it has been found that in the individuals with *striata* morph the total level of the reduction-oxidation processes and the amount of haemoglobin are higher, and the sodium penetrability of the skin is twice lower than that of the *maculata* morph [13]. We found that in juvenile [14] and adult [5] [15] individuals of *P. ridibundus* of *striata* morph the hepatic-somatic index (HSI) was higher than that of *maculate* morph. Studies on the bioaccumulation of heavy metals in *R. arvalis* found that in animals of *striata* morph strontium values (Sr-90) are five times lower than those in animals of *maculata* morph [16]. According to these authors' opinion, the relatively weak ability to bioaccumulate is associated with a higher frequency of occurrence in populations of *R. arvalis*, living in areas with natural and artificial geochemical anomalies in individuals of *striata* morph. According to the views of Sils and Vershinin [17], the lower skin permeability in individuals of *striata* morph leads to an increasing role of pulmonary breathing, which, in turn, is linked with hemopoiesis intensification (the number of erythrocytes and the amount of haemoglobin get higher).

A new large-scale study was conducted in 2012 in the same biotopes 3 along the rivers Sazliyka and Topolnitsa in populations of *P. ridibundus*, in view of analysing a wider range of haematological parameters in individuals of both morphs *striata* and *maculate*.

We set the following objectives:

• Obtaining data on the main haematological parameters (including any seasonal variations) in adult, sexually mature individuals of both colour morphs (*striata* and *maculata*) in the populations of *P. ridibundus*, inhabiting biotopes with different levels of anthropogenic pollution in Southern Bulgaria.

• Assessment of the most informative haematological parameters that objectively reflect the changes in the functional status of animals in habitats with anthropogenic stress.

• Output of possible differences in the values of the parameters in individuals of both morphs in *P. ridibundus* in the water basins polluted with different types of toxins and assessment of their role in the survival and environmental adaptation of the morph to the peculiarities of the environment.

• Analysis of the possibilities of using these haematological parameters in individuals of both morphs of *P. ridibundus* as markers for assessing the ecological status of the environment in the system of biomonitoring.

A schematic overview of the work-flow showing the design of the study is present in **Figure 1**.

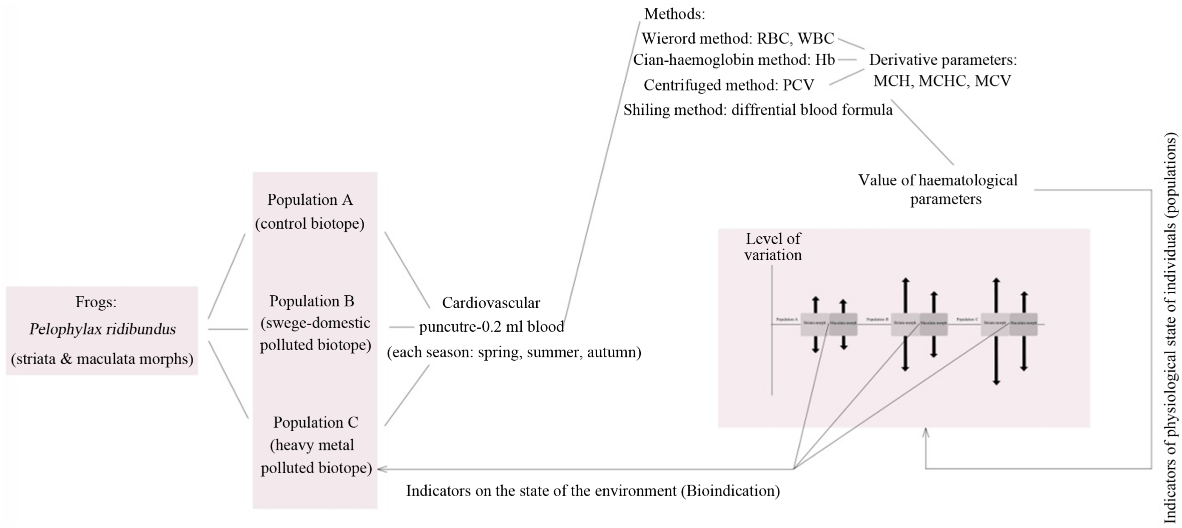

Figure 1. Schematic overview of the work-flow showing the design of the study.

2. Materials and Methods

2.1. The Area of Investigation

The samples were gathered during three seasons (spring, summer and autumn) of 2012 in three biotopes (for convenience labeled as A, B and C) located along the courses of two rivers in Southern Bulgaria: the river Sazliyka in its upper reaches near the village of Rakitnitsa (N 42˚19'58.8", E 25˚31'1.2", 200 m above sea level—biotope A), in the middle part in the region of the town of Radnevo, after it flows into the river Blatnitsa (N 42˚18'0", E 25˚55'58.8", 113 m above sea level-biotope B) and the river Topolnitsa near the village of Poibrene, below where the river Medetska enters the Topolnitsa water reserve (N 42˚30'0", E 24˚0'0", 511 m above sea level-biotope C). Physiographic map of the investigated biotopes are presented in Zhelev *et al.* [18].

2.2. Data from Physicochemical Analysis of the Water Ecosystems Studied

The average annual data relating to the water basins studied for 2012 are extracted from the reports of the Basin Directorate for water management—East Aegean Region—Plovdiv (http://www.bg-ibr.org) and the Ministry of the environment and water resources (data not shown). Physico-chemical monitoring is based on 21 indicators. Details of the physicochemical analysis for the period 2009-2011 in each of the biotopes are presented in Zhelev *et al*. [18]. For Biotope A there is no data about anthropogenic pollution (all 21 indicators are standard for Category I (clean) and Category II (slightly polluted) water basins, under Order No. 7/8.8.1986 (State Gazette, No. 96.12.12.1986). In our study it is viewed as control. The remaining two biotopes B and C are polluted. In the biotope B the main pollution is of sewage-domestic type (higher Biologic Consumption of Oxygen (BOD: 17.2 mg O_2/dm^3, MAC (maximum admissible concentrations) for Category I, II and III (normally contaminated) respectively 5.0, 15.0 and 25.0 mg O_2/dm^3),), Nitrite-Nitrogen (N-NO_2: 0.2 mg/dm^3, MAC for Category I, II and III respectively 0.002, 0.04 and 0.06 mg/dm^3) and Total Nitrogen (N 5.3 mg/dm^3, MAC for Category I, II and III respectively 1.0, 5.0 and 10.0 mg/dm^3)), while the biotope C is polluted with heavy metals (Iron-total (Fe): 0.20 mg/dm^3, above 0.1 mg/dm^3—very poor condition, Manganese (Mn): 0.33 mg/dm^3, above 0.05 mg/dm^3—very poor condition, Copper (Cu): 0.031 mg/dm^3, above 0.022 mg/dm^3—very poor condition, Lead (Pb): 0.015 mg/dm^3, above 0.0072 mg/dm^3—very poor condition, Nickel (Ni): 0.09 mg/dm^3, above 0.02 mg/dm^3—very poor.

2.3. Subject of Study and Methods of Analysis

The subject of our study is the marsh frog *P. ridibundus*. To achieve maximum objectivity in the research and to avoid selectivity in terms of the number of individuals of the two morphs in the test group (30 animals per season), the catches were performed in the following way: once in the spring, summer and autumn of 2012 (the dates are listed below), in the evening after sunset, we spotlighted each of the habitats using 2 Petromax lanterns placed on the shore at a few square metres. Then in the spotlighted area we caught adult individuals randomly in the water (using fishing net) and on land-along the bank. (Snout-Vent Length > 60.0 mm) [19], there was no selectivity with respect to the morph. We caught altogether 270 individuals from the three biotopes and the animals were divided as follows: Biotope A [spring (24 April 2011)-*striata* = 14, *maculata* = 16; summer (17 July 2011)-s = 12, m = 18; autumn (4 October 2011)-s = 11, m = 19]; Biotope B [spring (28 April 2011)-s = 23, m = 7; summer (22 July 2011)-s = 21, m = 9; autumn (9 October 2011)-s = 22, m = 8]; Biotope C [spring (4 May 2011)-m = 5, s = 25; summer (28 July 2011)-m = 7, s = 23; autumn (17 October 2011)-s-24, m-6]. To prevent eventual recapture in spring and summer the animals were marked through taking individual photographs [20].

The frogs were transported from the place of capture to the laboratory. The live frogs were anaesthetised with ether and blood (0.20 ml) was drawn by means of cardiac ventricular puncture using small heparinized needles (20 mm length) via heparinized haematocrit capillaries [1]. The erythrocyte (RBC) and leukocyte (WBC) count was determined according method of Wierord using a Burker chamber [21]. For dilution standardised Hayem solution was used for erythrocytes via Thoma pipettes, while for the leucocytes we used Turck's solution. Dilution was carried out 200 times for the erythrocyte count and for 20 times for the leukocyte count. The haemoglobin concentration (Hb) was determined with the cyan-haemoglobin method-reading 540 nm [21]. The haematocrit value (PCV) was determined with heparinized haematocrit capillaries. Blood was centrifuged for 5 min at 3000 rpm constant-rotation, in thin-walled capillary tubes and the value obtained was read from the scale and recorded in L/l [22]. The derivative haematological parameters (MCH-mean cell haemoglobin, MCHC-mean cell haemoglobin concentration, and MCV-mean cell volume) were calculated mathematically from the results above, according to Wintrobe's formula [23]. MCV was calculated by dividing haematocrit per litre of blood by total RBC count. 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, using Shiling's microscopic method [20]. After the analysis, the animals were released back to the same locations in the biotopes.

2.4. Statistical Procedures

The mathematical data processing was performed using standard statistical procedures with the help of the software package STATISTIKA 7.0 [24]. The normality in the distribution of the examined haematological parameters was checked using a Shapiro-Wilk test that indicated normal distribution: $p > 0.05$. The haematological parameters have been analysed (in total) in the individuals of both morphs of *P. ridibundus* using principal component analysis-PCA and standard discriminant analysis—DA. The statistical reliability of the differences in the values of the haematological parameters received for the individuals from both morphs in the compared biotopes was proven with a one-way ANOVA. As a post-hoc test was used an LSD-test. Results with $p < 0.05$ [α = 5%] were considered significant.

3. Results and Discussion

In preliminary testing of our data (individuals of the homonymous morphs-♂_s/♀_s и ♂_m/♀_m) with one-way ANOVA, we found only two statistically significant differences during spring in the control group: RBC in ♂_s was higher than ♀_s, and the number of Ba cells in ♀_m was higher than in ♂_m. There were no gender differences in the haematological parameters in the populations of the two anthropologically polluted biotopes ($p > 0.05$). For this reason, comparing the biotopes with various levels and types of pollution, the sample of the relevant population was analysed as a whole (♂ + ♀) for the individuals of the respective morph.

3.1. Multi-Variational Statistics—PCA

The sum of the first three variables includes 75.46% of the variation in the individuals from the two morphs of *P. ridibundus*: eigenvalue was fixed ≥ 1. The parameters: RBC (−0.941), Hb (−0.859), PCV (−0.891), WBC (−0.805) and Mo (−0.883) show a high degree of correlation in reference to the first axis. The parameters Sg (−0.875), Eo (−0.826), Ba (−0.671), Ly (0.796) have a high correlation in reference to the second axis. The positive dependence on F3 is indicated only by MCV (0.796). The grouping of parameters in reference to the first two main axes is shown in **Figure 2**.

3.2. Multi-Variational Statistics—DA

DA-discriminant analysis defines the difference between the comparable groups of individuals from the two

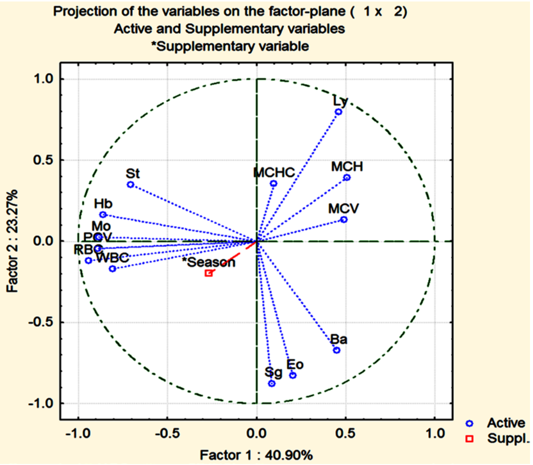

Figure 2. Graph of the correlations of the 12 haematological parameters (factor weights) in the individuals from the two morphs of *Pelophylax ridibundus* and the first two main axes of the biotopes with various degrees of anthropogenic pollution in Southern Bulgaria.

morphs in the biotopes with various degrees of anthropogenic pollution in Southern Bulgaria as statistically reliable, using the parameters: RBC (Wilks' Lambda = 0.012; F = 3.681; p = 0.003), Hb (Wilks' Lambda = 0.013; F =9.621; p = 0.000), PCV (Wilks' Lambda = 0.014; F = 16.916; p = 0.000), Sg (Wilks' Lambda = 0.015; F = 18.908; p = 0.000), Ba (Wilks' Lambda = 0.012; F = 3.678; p = 0.003), Eo (Wilks' Lambda = 0.012; F =5.230; p = 0.000) and Mo (Wilks' Lambda = 0.011; F = 3.628; p = 0.003). The derived Mahalanobis distances are shown in **Table 1**. The graph of the factor weights of the individuals of both morphs in the six comparable groups, from the biotopes with various degree of anthropogenic pollution, depicts three differentiated clouds (**Figure 3**). The animals of both morphs from the biotope polluted with heavy metals were clearly distinguished from those in the other two biotopes, while for the animals that inhabit the relatively clean biotope and that of the domestic sewage pollution type, there was an overlapping zone, where most of the individuals are with the *striata* morph; in our opinion, it is an expression of its higher flexibility in the conditions of specific type of toxins.

3.3. Descriptive Statistics and ANOVA

Due to the insignificant influence of the season factor on the change of the values of the haematological parameters (**Figure 2**), the data for each of the biotopes was combined (year-round). The ratio between the individuals of the *maculata* and *striata* morphs is: Biotope A: 1/0.7; Biotope B: 1/2.8; Biotope C: 1/4.0.

Our findings (descriptive statistics) for the haematological parameters in the groups of *P. ridibundus* from the three biotopes in Southern Bulgaria, with one-way ANOVA, are shown in **Tables 2-4**. The differences with a high degree of statistical reliability were found for all examined haematological parameters of the *P. ridibundus* individuals and from both morphs (*striata* and *maculata*) in the groups inhabiting the biotopes with different degree and nature of anthropogenic pollution (**Table 5** and **Table 6**). Statistically significant differences in comparisons of the individuals from the two morphs (*striata* and *maculata*):

a) In each type of biotope

In the relatively clean biotope (biotope A), the three parameters RBC, St and Sg of the thirteen we studied, have higher values in the individuals of the *striata* morph, and the parameter Eo—in those of the *maculata* morph. In the biotope with domestic sewage pollution (biotope B), the parameters RBC, Hb and Ly have higher values in the individuals of the *striata* morph and it is the same for MCHC, Ba and Eo in those of *maculata* morph. In the biotope polluted with heavy metals (biotope C), the values of RBC, Hb and PCV are higher in the individuals of the *striata* morph, while MCH and MCV are higher in those of the *maculata* morph.

b) In the polluted biotopes (domestic sewage pollution biotope-B and heavy metal pollution biotope-C) compared with the control sample (biotope A)

There was an increase of RBC, Hb and PCV in the individuals of both morphs in the two polluted biotopes. MCH value was lower in both morphs in the biotope B, while in the individuals of the *striata* morph only it was

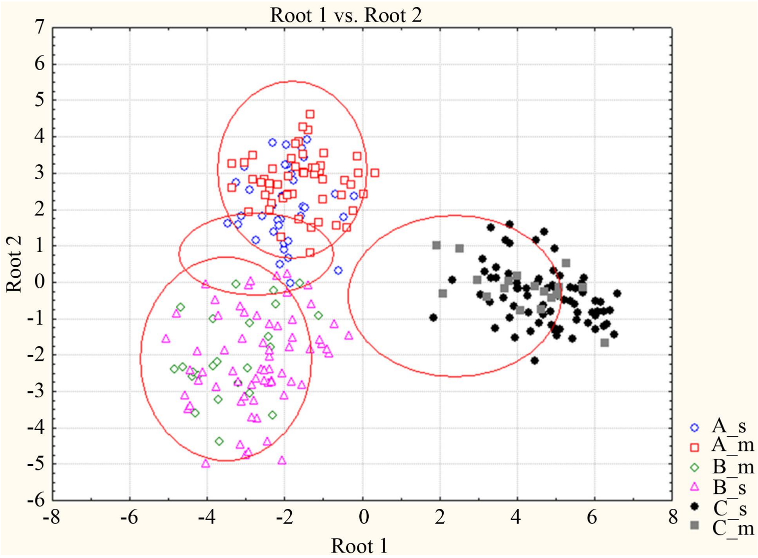

Figure 3. Discriminant coordinates for the ten haematological parameters for the individuals of both morphs of *Pelophylax ridibundus* from the six comparable groups inhabiting biotopes in Southern Bulgaria with various degree of anthropogenic pollution. [Biotopes: A—the river Sazliyka below the village of Rakitnitsa, B—the river Sazliyka below the town of Radnevo; C—the river Topolnitsa below the village of Poibrene and morphs s-*striata*, m—*maculata* of individuals].

Table 1. The Mahalanobis distance between the six groups of individuals of both morphs (*striata* and *maculata*) of *Pelophylax ridibundus* from the biotopes in Southern Bulgaria with various degree of anthropogenic pollution using 10 haematological parameters and the corresponding statistically reliable differences between them.

	1					
1	A_s	A_m	B_m	B_s	C_s	C_m
A_s	0.000	1.795*	21.784*	19.076*	52.218*	45.631*
A_m	1.795*	0.000	27.423*	25.741*	50.358*	43.015*
B_m	21.784*	27.423*	0.000	4.194*	69.894*	61.247*
B_s	19.076*	25.741*	4.194*	0.000	58.608*	53.365*
C_s	52.218*	50.358*	69.894*	58.608*	0.000	3.238*
C_m	45.631*	43.015*	61.247*	53.365*	3.238*	0.000

A_s, A_m (Population A—*striata* and *maculata* morphs of individuals), B_s, B_m (Population B—*striata* and *maculata* morphs of individuals); C_s, C_m (Population C—*striata* and *maculata* morphs of individuals). $^{*}p < 0.00\sim1$.

lower than those of the control sample biotope with the maculate morph in the biotope C. MCHC decreases in both morphs in the biotope with domestic sewage pollution. MCV value is lower in the individuals of the *maculata* morph in the biotope B and in these of the *striata* morph in the biotope C, when compared with the control ones of the maculate morph. In the polluted biotopes there was an increase of WBC in the individuals of both morphs, compared with the control group, regardless of the nature of toxins, however, there were various changes in the character of the differential blood formula: St neutrophils increase proportionally in both morphs; in Biotope B that increase refers only to the individuals of the *maculata* morph in Biotope A, while in Biotope C to the individuals of both morphs of the control group; the number of Sg neutrophils in the biotope C is greatly reduced in the individuals of both morphs, whereas their number increases in both morphs in the biotope B; the number of Ba cells in biotope B is higher in the individuals of both morphs, but those of the *maculata* morph are more than these of both morphs in the control group, and these of the *striata* morph are more only than those of the *maculata* morph in the control group. In the biotope C, the number of Ba cells decreases in the individuals of

Table 2. Results from the comparison of the haematological parameters in the groups of *Pelophylax ridibundus* from the three biotopes in Southern Bulgaria, with one-way ANOVA.

Variable	SS Effect	DF Effect	MS Effect	SS Error	DF Error	MS Error	F Error
RBC	3,923,678	5	784735.7	2,472,492	264	9365.50	83.790*
Hb	35,930	5	7186.1	15,521	264	58.79	122.230*
PCV	1	5	0.2	0	264	0.00	159.815*
MCH	24,237	5	4847.3	151,143	264	572.51	8.466*
MCHC	25,137	5	5027.4	148,858	264	563.86	8.916*
MCV	213,689	5	42737.8	2,692,438	264	10198.63	4.190*
WBC	175	5	35.0	214	264	0.81	43.096*
St	616	5	123.1	525	264	1.99	61.968*
Sg	4632	5	926.4	896	264	3.39	272.982*
Ba	774	5	154.8	750	264	2.84	54.501*
Eo	1088	5	217.6	816	264	3.09	70.444*
Mo	5083	5	1016.6	2080	264	7.88	129.029*
Ly	14,339	5	2867.8	6894	264	26.11	109.813*

Regression SS = Total SS-Residual SS; Regression DF = regression degrees of freedom = number of independent variables (factors); Regression MS = Regression SS/Regression D; Regression F = Regression MS/Residual MS, Standard Error = (Residual MS) $^{0.5}$ *p < 0.00~1.

Table 3. Descriptive statistics of haematologic parameters of circulating blood in the populations of *Pelophylax ridibundus* from the researched biotopes [RBC: erythrocyte count (N/μl); Hb: haemoglobin concentration (g/dl); PCV: haematocrit value (L/l); MCH: mean cell haemoglobin (pg); MCHC: mean cell haemoglobin concentration (g/l); MCV: mean cell volume (fl)].

Parameters	The river Sazliyka below the village of Rakitnitsa A (control) Number of individuals: n = 70 Morphs: *striata*-37; *maculata*-53				The river Sazliyka below the town of Radnevo B (domestic sewage pollution) Number of individuals: n = 90 Morphs: *striata*-66; *maculata*-24				The river Topolnitsa below the village of Poibrene C (heavy metal pollution) Number of individuals: n = 90 Morphs: *striata*-72; *maculata*-18			
	Morph	Mean	SD	Range	Morph	Mean	SD	Range	Morph	Mean	SD	Range
RBC	S	382,703	664,851	260,000 - 500,000	s	582,576	985,637	390,000 - 730,000	S	642,639	1,278,312	380,000 - 920,000
	M	331,321	556,444	250,000 - 460,000	m	533,333	865,607	380,000 - 660,000	M	532,778	1,052,619	410,000 - 740,000
Hb	S	5.59	7.19	4.49 - 7.17	s	7.47	9.76	5.21 - 8.87	S	8.32	5.32	6.73 - 9.25
	M	5.43	7.39	4.57 - 7.19	m	6.36	8.91	4.88 - 7.64	M	7.60	6.86	6.41 - 8.68
PCV	S	0.25	0.04	0.20 - 0.34	s	0.35	0.03	0.29 - 0.43	S	0.38	0.03	0.30 - 0.43
	M	0.24	0.03	0.18 - 0.32	m	0.34	0.03	0.30 - 0.38	M	0.34	0.03	0.31 - 0.41
MCH	S	152.43	28.85	109.59 - 220.17	s	141.93	20.95	103.94 - 186.25	S	143.57	25.75	98.55 - 224.43
	M	162.19	23.11	105.17 - 232.87	m	132.92	18.63	105.38 - 179.53	M	162.08	24.02	122.46 - 191.05
MCHC	S	224.09	31.82	149.77 - 298.45	s	215.88	23.61	157.97 - 264.36	S	220.30	18.06	141.72 - 284.10
	M	227.96	27.95	169.32 - 297.48	m	190.78	20.64	152.41 - 224.65	M	221.16	12.32	205.88 - 246.52
MCV	S	678.25	105.93	517.44 - 983.33	s	662.84	97.21	476.14 - 856.00	S	646.44	105.20	430.71 - 912.50
	M	712.63	92.29	521.74 - 950.00	m	704.55	103.21	529.17 - 931.25	M	728.76	108.62	547.57 - 887.50

Table 4. Descriptive statistics of haematologic parameters of circulating blood in the populations of *Pelophylax ridibundus* from the researched biotopes [WBC: white blood cell count (N/μl); blood differential formula (N/100 WBC). St: stab neutrophils; Sg: Segmented nuclei neutrophils; Ba: Basophils; Eo: Eosinophils; Mo: Monocytes; Ly: Lymphocytes].

Parameters	The river Sazliyka below the village of Rakitnitsa A (control) Number of individuals: n = 70 Morphs: *striata*-37; *maculata*-53				The river Sazliyka below the town of Radnevo B (domestic sewage pollution) Number of individuals: n = 90 Morphs: *striata*-66; *maculata*-24				The river Topolnitsa below the village of Poibrene C (heavy metal pollution) Number of individuals: n = 90 Morphs: *striata*-72; maculate-18			
	Morph	Mean	SD	Range	Morph	Mean	SD	Range	Morph	Mean	SD	Range
WBC	S	2692	0.56	1600 - 3800	s	4118	1.29	2600 - 6400	S	4396	0.69	3300 - 5700
	M	2526	0.64	1600 - 3700	m	4200	1.18	2800 - 6200	M	4517	0.64	3600 - 5500
St	S	2.86	1.25	1.00 - 6.00	s	3.39	1.28	1.00 - 8.00	S	5.93	1.86	3.00 - 11.00
	M	2.15	0.86	1.00 - 4.00	m	3.50	1.47	1.00 - 6.00	M	6.22	1.26	3.00 - 8.00
Sg	S	7.97	1.24	5.00 - 10.00	s	13.06	2.64	6.00 - 18.00	S	2.72	1.29	1.00 - 7.00
	M	6.30	1.35	4.00 - 9.00	m	12.33	2.39	8.00 - 16.00	M	3.39	1.58	1.00 - 7.00
Ba	S	3.73	1.79	1.00 - 7.00	s	4.20	2.21	2.00 - 10.00	S	0.68	0.78	0.00 - 3.00
	M	3.55	1.69	1.00 - 7.00	m	5.33	2.26	2.00 - 10.00	M	0.33	0.59	0.00 - 2.00
Eo	S	2.51	1.17	1.00 - 5.00	s	4.68	2.33	1.00 - 11.00	S	0.28	0.51	0.00 - 2.00
	M	3.40	1.69	1.00 - 7.00	m	6.46	3.16	1.00 - 11.00	M	1.06	0.94	0.00 - 3.00
Mo	S	3.27	1.98	1.00 - 9.00	s	9.97	3.35	3.00 - 16.00	S	13.43	2.91	6.00 - 19.00
	M	3.23	1.23	1.00 - 7.00	m	10.42	3.89	3.00 - 16.00	M	14.67	3.25	8.00 - 19.00
Ly	S	79.68	4.63	72.00 - 89.00	s	64.36	6.67	40.00 - 78.00	S	76.96	3.89	66.00 - 87.00
	M	81.45	3.65	74.00 - 90.00	m	61.96	7.49	51.00 - 76.00	M	74.33	3.16	70.00 - 82.00

Table 5. Summary presentation of statistically reliable differences in the comparison of the haematological parameters in the groups of *Pelophylax ridibundus* from the three biotopes in Southern Bulgaria with one-way ANOVA and LSD-test (the signs > and < compare mean values of haematological parameters in **Table 3**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Parameters	In the separate biotopes	Between the biotopes	
		Polluted/control sample	Domestic sewage pollution/heavy metal pollution
RBC	$A\_s > A\_m^{**}$; $B\_s > B\_m^{**}$; $C\_s > C\_m^{***}$	$B\_s > A\_s^{***}$; $B\_m > A\_m^{***}$; $B\_s > A\_m^{***}$; $B\_m > A\_s^{***}$; $C\_s > A\_s^{***}$; $C\_s > A\_m^{***}$; $C\_m > A\_m^{***}$; $C\_m > A\_s^{***}$	$C\_s > B\_s^{***}$, $C\_s > B\_m^{***}$
Hb	$B\_s > B\_m^{***}$; $C\_s > C\_m^{***}$	$B\_s > A\_s^{***}$; $B\_s > A\_m^{***}$; $B\_m > A\_m^{***}$; $B\_m > A\_s^{***}$; $C\_s > A\_s^{***}$; $C\_s > A\_m^{***}$; $C\_m > A\_m^{***}$; $C\_m > A\_s^{***}$	$C\_s > B\_s^{***}$, $C\_s > B\_m^{***}$; $C\_m>B\_m^{***}$
PCV	$C\_s > C\_m^{***}$	$B\_s > A\_s^{***}$; $B\_s > A\_m^{***}$; $B\_m > A\_m^{***}$; $B\_m > A\_s^{***}$; $C\_s > A\_s^{***}$; $C\_s > A\_m^{***}$; $C\_m >A\_m^{***}$; $C\_m > A\_s^{***}$	$C\_s > B\_s^{***}$; $C\_s > B\_m^{***}$
MCH	$C\_m > C\_s^{***}$	$B\_s < A\_s^{***}$; $B\_s < A\_m^{***}$; $B\_m < A\_m^{***}$; $B\_m < A\_s^{**}$; $C\_s <A\_m^{***}$	$C\_m > B\_s^{***}$; $C\_m > B\_m^{***}$
MCHC	$B\_s < B\_m^{***}$	$B\_m < A\_m^{***}$; $B\_s < A\_m^{***}$; $B\_m < A\_m^{***}$	$B\_s < C\_m^{***}$; $B\_s < C\_s^{***}$
MCV	$C\_s < C\_m^{***}$	$B\text{-}m < A\text{-}m^{***}$; $C\_s < A\_m^{***}$	$B\_s > C\_s^{***}$; $B\_m > C\_s^{***}$

both morphs; in the biotope B, the number of Eo cells increases in the individuals of both morphs, but their number sharply decreases in the individuals of both morphs in the biotope C; the number of Mo cells increases proportionally in the individuals of both morphs in the biotopes B and C; in the polluted biotopes, the number of Ly decreases in the individuals of both morphs.

C) In the two polluted biotopes—B and C

Table 6. Summary presentation of statistically reliable differences in the comparison of the haematological parameters in the groups of *Pelophylax ridibundus* from the three biotopes in Southern Bulgaria with one-way ANOVA and LSD-test (the signs > and < compare mean values of haematological parameters in **Table 4**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *ns* − $p > 0.05$.

Parameters	In the separate biotopes	Between the biotopes	
		Polluted/control sample	Domestic sewage pollution/heavy metal pollution
WBC	Ns	$B_s > A_s^{***}$; $B_m > A_m^{***}$; $B_s > A_m^{***}$; $B_m > A_s^{***}$; $C_s > A_s^{***}$; $C_m > A_m^{***}$; $C_s > A_m^{***}$; $C_m > A_s^{***}$	Ns
St	$A_s > A_m^{**}$	$B_m > A_m^{***}$; $B_s > A_m^{***}$; $C_s > A_s^{***}$; $C_m > A_m^{***}$; $C_s > A_m^{***}$; $C_m > A_s^{***}$	$B_s < C_s^{***}$; $B_m < C_m^{***}$; $B_s < C_m^{***}$; $B_m < C_s^{***}$
Sg	$A_s > A_m^{***}$	$B_s > A_s^{***}$; $B_m > A_m^{***}$; $B_s > A_m^{***}$; $B_m > A_s^{***}$; $C_s < A_s^{***}$; $C_m < A_m^{***}$; $C_s < A_m^{***}$; $C_m < A_s^{***}$	$B_s > C_s^{***}$; $B_m > C_m^{***}$; $B_s > C_m^{***}$; $B_m > C_s^{***}$
Ba	$B_s < B_m^{***}$	$B_m > A_m^{*}$; $B_s > A_m^{***}$; $B_s > A_s^{***}$; $C_s < A_s^{***}$; $C_m < A_m^{***}$; $C_s < A_m^{***}$; $C_m < A_s^{***}$	$B_s > C_s^{***}$; $B_m > C_m^{***}$; $B_s > C_m^{***}$; $B_m > C_s^{***}$
Eo	$A_s < A_m^{**}$; $B_s < B_m^{***}$	$B_s > A_s^{***}$; $B_m > A_m^{***}$; $B_s > A_m^{***}$; $B_m > A_s^{***}$; $C_s < A_s^{***}$; $C_m < A_m^{***}$; $C_s < A_m^{***}$; $C_m < A_s^{***}$	$B_s > C_s^{***}$; $B_m > C_m^{***}$; $B_s > C_m^{***}$; $B_m > C_s^{***}$
Mo	Ns	$B_s > A_s^{***}$; $B_m > A_m^{***}$; $B_s > A_m^{***}$; $B_m > A_s^{***}$; $C_s > A_s^{***}$; $C_m > A_m^{***}$; $C_s > A_m^{***}$; $C_m > A_s^{***}$	$B_s < C_s^{***}$; $B_m < C_m^{***}$; $B_s < C_m^{***}$; $B_m < C_s^{***}$
Ly	Ns	$B_s < A_s^{***}$; $B_m < A_m^{***}$; $B_s < A_m^{***}$; $B_m < A_s^{***}$; $C_s < A_s^{***}$; $C_m < A_m^{***}$; $C_m < A_s^{***}$; $C_s < A_m^{***}$	$B_s < C_s^{***}$; $B_m < C_m^{***}$; $B_s < C_m^{***}$; $B_m < C_s^{***}$

RBC, Hb and PCV values are higher in the individuals of the *striata* morphs in the biotope C. MCH value in the individuals of *maculata* morph in the biotope C is higher than this in the individuals of both morphs in the biotope B. MCHC value is higher in the individuals of both morphs in the biotope C, compared with B. MCV value is higher in the individuals of both morphs in the biotope B than that of the *striata* morph in the biotope C. The number of St neutrophils is lower in the individuals of both morphs in the biotope B. The number of Sg neutrophils is lower in the individuals of both morphs in the biotope C. The number of Ba and Eo cells is lower in the individuals of both morphs in the biotope C. The number of Mo and Ly cells is higher in the individuals of both morphs in the biotope C.

The results of this research support the view from our previous work that the increasing values of RBC, Hb and WBC in populations of *P. ridibundus* (for individuals of both morphs) are sufficiently reliable markers of anthropogenic pollution. The same is true for the changes in PCV values, and partially in MCV. Also the changes in the differential blood formula are sufficiently informative, but the enhanced specificity of their alterations in individuals of the two different morphs to a specific type of toxin must be taken into account.

4. Conclusions

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

• In populations of *P. ridibundus* (for individuals of both morphs *striata* and *maculata*), living in conditions of long-term anthropogenic pollution, there is an increase in the values of the haematological parameters: RBC, Hb, PCV, WBC, St and Mo, as well as reducing Ly and diverse changes in individuals of both morphs in the values of MCH, MCHC, MCV, Sg, Ba and Eo. In the anthropogenically polluted biotopes, they do not undergo seasonal changes and they have constant high values compared to the control group.

• The haematological parameters: RBC, Hb, PCV, Sg, Ba, Mo, to a lesser degree MCV, WBC, Eo, and Ly are most informative, regarding the establishment of changes (of adaptive nature or damages as a result of toxicosis) in the individuals of both morphs (*striata* and *maculata*) in *P. ridibundus* living in conditions of anthropogenic pollution.

• The higher values of RBC (at a high exit level of the parameter in the control group), as well as of the other two basic haematological parameters (Hb and PCV) in individuals of *striata* morph, in comparison with those of *maculata* morph, in the two polluted biotopes, are probably one of the reasons for the increased resistance and better survival of the individuals of this morph in anthropogenically polluted biotopes. The adaptive changes in the basic haematological parameters in individuals of *striata* morph are more distinctive in conditions of domestic sewage pollution.

• The haematological parameters RBC, Hb, PCV, WBC and differential blood formulae in adult *P. ridibundus*, of both sex and both morphs (*striata* and *maculata*), are suitable as markers for assessing water basins' conditions with a different level and nature of anthropogenic pollution; they can also complement the data from physicochemical analysis and be applied for performing an independent initial general assessment of ecosystems.

Acknowledgements

The *P. ridibundus* is listed in Appendix 4 to the Bulgarian Biodiversity Act (Prom. SG. 77, August 9th 2002). According to Article 42, Article 41 and Appendix 2 for Article 41 of the same law, capture permits for *P. ridibundus* are not issued if in use for scientific research.

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