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Leukogram of Adult *Pelophylax ridibundus* (Pallas, 1771) (Anura: Ranidae) in the Sedimentation Lake of the Brikel Thermal Power Station in Southern Bulgaria: Evaluation of Biomarkers for Ecological Stress Assessment

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Abstract: Evaluation of changes in the leukogram of adult marsh frogs *Pelophylax ridibundus* inhabiting the polluted sedimentation lake of the Brikel Thermal Power Station (TPS), southern Bulgaria, as biomarkers for ecological stress is presented. For a comparison, data for frogs inhabiting a less disrupted habitat (reference site, RS) are presented. Leukopenia in the blood of individuals of *P. ridibundus* inhabiting the sedimentation lake of the Brikel TPS was detected but not in frogs from the reference site. Furthermore, the differential leukocyte count of the affected frogs was characterised by neutrophilia, eosinophilia, monocytosis, basopenia and lymphopenia. The values of the neutrophil-lymphocyte (N/L) ratio of the frogs from the lake of the Brikel TPS were higher than that of the ones inhabiting the RS. These changes in the leukogram are indicators of the exhaustion of the immune system and of activation of the organism's defences in response to stress. The changes in the leukogram are interpreted as caused by the deteriorated water quality in the sedimentation lake as it contained toxicants (reactive water-soluble forms of nitrogen and phosphates) and industrial ash. The results from this study confirm the role of parameter changes in the leukogram of *P. ridibundus* as diagnostic biomarkers for ecological stress.

Key words: *Pelophylax ridibundus*, environmental pollution, white blood cells, differential leukocyte count, ecotoxicology

Introduction

Anuran amphibian populations have been declining globally over the last decade (Ficetola 2015). The strong anthropogenic pressure in combination with global climate changes and habitat loss is named as among the main reasons for the worldwide decline in anuran populations (Whittaker 2013). Many anuran amphibians are unable to survive in an environment with increased xenobiotic content and, therefore, die (Stuart et al. 2004). However, there are anuran species that adapt surprisingly well and survive in conditions of anthropogenic stress (Narayan et al. 2013, Nazarov et al. 2022). In Eurasia, such an anuran amphibian species is the marsh frog *Pelophylax ridibundus* (Pallas, 1771). The numerous field studies conducted with *P. ridibundus* show that it is a suitable biomonitoring test subject

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because the physiological responses of its organism make it possible to draw a connection with the toxicants present in the environment (Snegin & Barkhatov 2019, Zamaletdinov et al. 2019, Mani et al. 2021, Şişman et al 2021, Zhelev et al. 2021, 2022a). The marsh frog has a relatively large area of distribution in Eurasia and a population density sufficient for field tests and it is not under a strict protection regime in many countries (Kuzmin et al. 1996). In this context, the determination of different morphological, physiological, biochemical and molecular biomarkers for this anuran species is a priority in modern ecotoxicology because it can help assess the health status of the populations of the species inhabiting conditions of pollution, acting as sublethal endpoints of intoxication (Falfushinska et al. 2008, Prokić et al. 2017, Zhelev et al. 2020, 2022a). Furthermore, biomarkers, with their integrated assessment of the effect of pollutants on the organism's health, provide an addition and an improvement to the reliability of the data from chemical monitoring of the environment (Venturino et al. 2004, Şişman et al. 2021, Zhelev et al. 2022b).

Amphibians (including tailless amphibians) have a fully developed and differentiated circulatory system with blood components similar to the ones of other vertebrates (Glomski et al. 1997). A leukogram is a test determining the total number of leukocytes (WBC); with differential count, it determines the number of different leukocyte types and their percentages. WBC count can be used for assessment of the hematopoietic productivity and the level of activation of the immune system (Davis et al. 2008, Franco-Belussi et al. 2022). In recent years, this type of analysis has found broader application in different *in situ* studies, in "wild" anuran populations inhabiting conditions of anthropogenic stress (Davis & Durso 2009, Shutler & Marcogliese 2011, Peterson et al. 2013, Das & Mahapatra 2014, Zhelev & Popgeorgiev 2021). Usually, lymphocytes and neutrophils constitute a large part of leukocytes and the ratio of these cells is frequently used as a reliable indicator of the state of the immune system (Davis et al. 2008, Silva et al. 2020).

This study is focused on the assessment of the changes in the leukogram of adult specimens of the marsh frog *P. ridibundus*. The individuals inhabiting the sedimentation lake of the Brikel Thermal Power Station (TPS) near the town of Galabovo, southern Bulgaria, are considered potential bioindicators of ecological stress (the parameters of their leukogram, as biomarkers, respectively). Our working hypothesis is based on the assumption that changes in the leukogram are expected in frogs inhabiting conditions of chronic pollution with industrial wastewater in comparison with ones from a reference site.

Materials and Methods

Sampling area and data collection

This study was conducted in April 2019 during the breeding season of *P. ridibundus* at two locations in southern Bulgaria: Site 1 (42.1610° N, 25.8812° E, 90 m a.s.l.), the sedimentation lake of the Brikel TPS, near the town of Galabovo; Site 2 (42.0343° N, 24.4704° E, 190 m a. s. l.), Vacha River, near the town of Krichim (Fig. 1). In our study, site 1 was treated as a polluted area (PS – polluted site) because it is located near the Brikel TPS. It receives water with a high ash concentration due to burning of lignite in the process of electricity production. The water used in the technological process is inflated with pumps from the Rozov Kladenets Reservoir, which is filled from the Sazliyka and Sokolitsa Rivers. The sedimentation lake covers an area of over 1000 Decares and is located near the town of Galabovo. Due to the specific parameters of the technological process that includes sedimentation of industrial ash in different areas of the sedimentation lake, it never runs out of water. This ensures the life conditions necessary for the marsh frogs, for the population of which we assume to live in isolation because of the high embankment surrounding the sedimentation lake and the industrial terrain around it. Site 2 is located south of the town of Krichim in a protected area, far from industrial or agricultural anthropogenic activities. In our study, site 2 is regarded as a less disrupted habitat: RS – reference site. This study uses data from the physicochemical monitoring of the water in the Rozov Kladenetc Reservoir and the Vacha River ordered by the Basin Directorate for Water Management– East-Aegean Sea, Region Plovdiv, Ministry of the Environment and Waters (https://earbd.bg) at the moment of capturing the frogs at site 1 and at site 2. These data are presented in Table 1.

According to the dispositions of Chapter 3, Section 3, Articles 41 and 42 and Appendix 4 to the Article 41 of the Bulgarian Law on Biological Diversity, capture permits for *P. ridibundus* are not required for the aims of scientific research (BDA 2002). Sixty (30 ♂ and 30 ♀) adult and sexually mature individuals of *P. ridibundus* (snout-vent length, SVL > 60 mm) were caught from each site: on 17 April 2019 for site 1 and on 26 April 2019 for site 2. The frogs were captured in the water using a flashlight at night-time. The

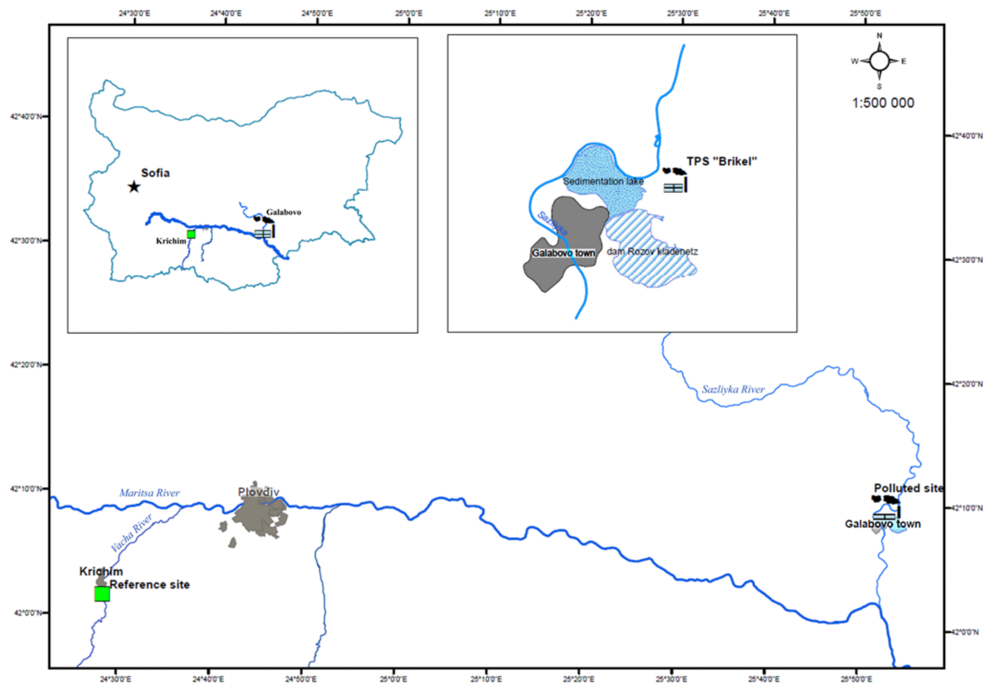

Fig. 1. An indicative map of the sites in southern Bulgaria where individuals of *Pelophylax ridibundus* were captured in 2019.

Table 1. Physicochemical priority substances determining the ecological status on the Rozov Kladenets Reservoir, near the town of Galabovo and the Vacha River, near the town of Krichim, according to the data of the newsletters of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, Ministry of the Environment and Waters (http://www.bg-ibr.org).

Parameters and standards for high water quality* April 2019		Rozov Kladenets Reservoir (Polluted site) Moderate surface water status**		Vacha River (Reference site) Good surface water status**	
		Average annual for 2019	April 2019	Average annual for 2019	April 2019
Temp °C	–	19.2	–	12.8	–
pH units	–	8.1	8.3	8.0	8.1
EC µS/cm	700.0	1088.0	994.0	201.0	192.6
DO mgO$_2$/dm^3	9.0–7.0	7.9	7.8	10.4	10.1
BOD$_5$ mgO$_2$/dm^3	< 2.0	2.4	1.9	1.0	0.8
COD mgO$_2$/dm^3	25.0	23.0	–	9.0	–
Ox %	100–105	88.0	–	117.0	–
NH$^+_4$-N mg/dm^3	< 0.10	0.35	0.53	0.04	0.08
NO$^-_2$-N mg/l	< 0.03	0.08	0.14	0.01	0.01
NO$^-_3$-N mg/l	< 0.7	1.14	1.12	0.57	0.55
TN mg/l	< 0.7	2.5	2.4	< 1.0	< 1.0
PO$^{3-}_4$ mg/l	< 0.07	0.25	0.34	0.04	0.06
TP mg/l	< 0.15	0.27	0.45	0.01	0.02

Legend: Parameters, abbreviations: Temperature (Temp), electrical-conductivity (EC), dissolved oxygen (DO), oxygenation (Ox), biological oxygen demand five days (BOD$_5$), chemical oxygen demand (COD), ammonium nitrogen (NH$^+_4$-N), nitrite nitrogen (NO$^-_2$-N), nitrate nitrogen (NO$^-_3$-N), total nitrogen (TN), orthophosphates (PO$^{3-}_4$), total phosphorus, as P (TP). (*) Standards for high water quality of inland surface water: flat-type rivers and reservoirs, (**) surface water status (according to Annex V of EU water framework directive 2000/60/EC (EC, 2000) and Ordinance № H-4 of 14.09.2012 (State Gazette, № 22. 5.03.2013) on the characterisation of surface waters in Bulgaria.

frogs were captured by hand. The grouping by sex was done based on secondary sexual characteristics: resonator bubbles in the corners of the mouth and "marital corns" on the first finger in male individuals.

Data analysis

The full analysis was performed in field conditions. According to the method used by Stetter (2001), the live frogs were anaesthetised with ether. We measured the SVL, to the nearest 0.1 mm with a Digi-Max™ slide calliper, (Z503576-1EA, Germany). Blood was taken directly from the heart with small heparinised needles (20 mm long). Total WBC counts were manually quantified using Turck's solution and a Burker haemocytometer counting chamber. Differential leukocyte counts (DLC) were determined on two blood smears per each individual, with Giemsa–Romanowsky staining (Pavlov et al. 1980). After the analyses, the frogs were brought back to their natural habitat.

Statistical processing of the data was performed, using the STATISTICA 7.0 Software (Stat Soft 2004). A Shapiro-Wilk test was used to evaluate the normal distribution of data, which proved to be statistically significant. Female individuals of *P. ridibundus* are bigger than males. For this reason, the SVL was included in the statistical analyses. A statistical significance among the differences in the values of all studied parameters between male and female frogs from RS and PS was verified using a parametric one-way analysis of variance (ANOVA) with a post-hoc Fisher's least significant difference (LSD) test. Results with $p < 0.05$ [$\alpha = 5\%$] were considered significant. Data were given as mean ± standard deviation and minimum–maximum values. Multivariate statistical analyses (principal component analysis PCA and standard discriminant analysis DA) were applied as an integrated assessment of the immune status of *P. ridibundus* from the two sites in southern Bulgaria.

Results

The descriptive statistics and results from the comparisons through one-way ANOVA analysis of the studied leukogram parameters in individuals of *P. ridibundus* from the two sites are presented in Table 2.

Table 2. Mean ± standard deviation and minimum–maximum values for the studied parameters in **individuals** of *Pelophylax ridibundus* from two sites in southern Bulgaria and results from their comparisons (one-way ANOVA). The signs > and <are used to compare the mean values of the parameters.

Parameters	**Polluted site: Sedimentation lake of the Brikel TPS**		**Reference site: Vacha River near the town of Krichim**		***F* and *LSD* (post-hoc) tests**
	Female (1) ***n* = 30**	**Male (2)** ***n* = 30**	**Female (3)** ***n* = 30**	**Male (4)** ***n* = 30**	
SVL (cm)	8.31±0.82 7.20–10.20	6.71±0.35 6.10–7.20	7.78±0.64 7.10–9.60	6.61±0.38 6.10–7.20	$F_{3, 116} = 60.68$ $1>2^{***}$, $1>3^{***}$, $1>4^{***}$, $2<3^{***}$, $2/4_{ns}$, $3>4^{***}$
WBC (x 10⁶/µl)	4.266±5.39 3.200–5.300	4.063±4.65 3.200–4.900	2.280±3.89 1.600–3.400	2.226±4.71 1.600–3.300	$F_{3, 116} = 166.83$ $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$
Ne (%)	10.00±1.76 8.00–15.00	9.93±1.26 8.00–15.00	18.96±1.90 15.00–22.00	20.17±1.66 18.00–23.00	$F_{3, 116} = 335.65$ $1>2^{*}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3/4_{ns}$
Ba (%)	4.10±1.92 1.00–7.00	4.30±1.18 2.00–6.00	0.86±0.77 0.00–2.00	0.90±0.80 0.00–3.00	$F_{3, 116} = 69.79$ $1/2_{ns}$, $1>3^{***}$,$1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$
Eo (%)	1.23±1.04 0.00–3.00	1.03±0.81 0.00–3.00	4.57±1.13 1.00–6.00	5.23±1.14 1.00–7.00	$F_{3, 116} = 133.84$ $1>2^{*}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3/4_{ns}$
Mo (%)	3.73±1.55 1.00–9.00	4.57±0.81 3.00–6.00	13.17±1.72 9.00–17.00	11.73±1.70 9.00–15.00	$F_{3, 116} = 314.37$ $1<2^{**}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3>4^{*}$
Ly (%)	80.93±3.44 71.00–88.00	80.17±2.10 76.00–84.00	62.43±2.21 60.00–66.00	61.96±2.27 60.00–69.00	$F_{3, 116} = 512.96$ $1/2_{ns}$, $1>3^{***}$, $1>4^{***}$, $2>3^{***}$, $2>4^{***}$, $3/4_{ns}$
N/L	0.12±0.02 0.10–0.20	0.12±0.01 0.10–0.17	0.30±0.04 0.24–0.37	0.32±0.34 0.26–0.38	$F_{3, 116} = 404.79$ $1/2_{ns}$, $1<3^{***}$, $1<4^{***}$, $2<3^{***}$, $2<4^{***}$, $3/4_{ns}$

Legend: SVL: snout-vent length, Ne: neutrophils, Ba: basophils, Eo: eosinophils, Mo: monocytes, Ly: lymphocytes, N/L: neutrophil-lymphocyte ratio. Significance codes: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns $p > 0.05$.

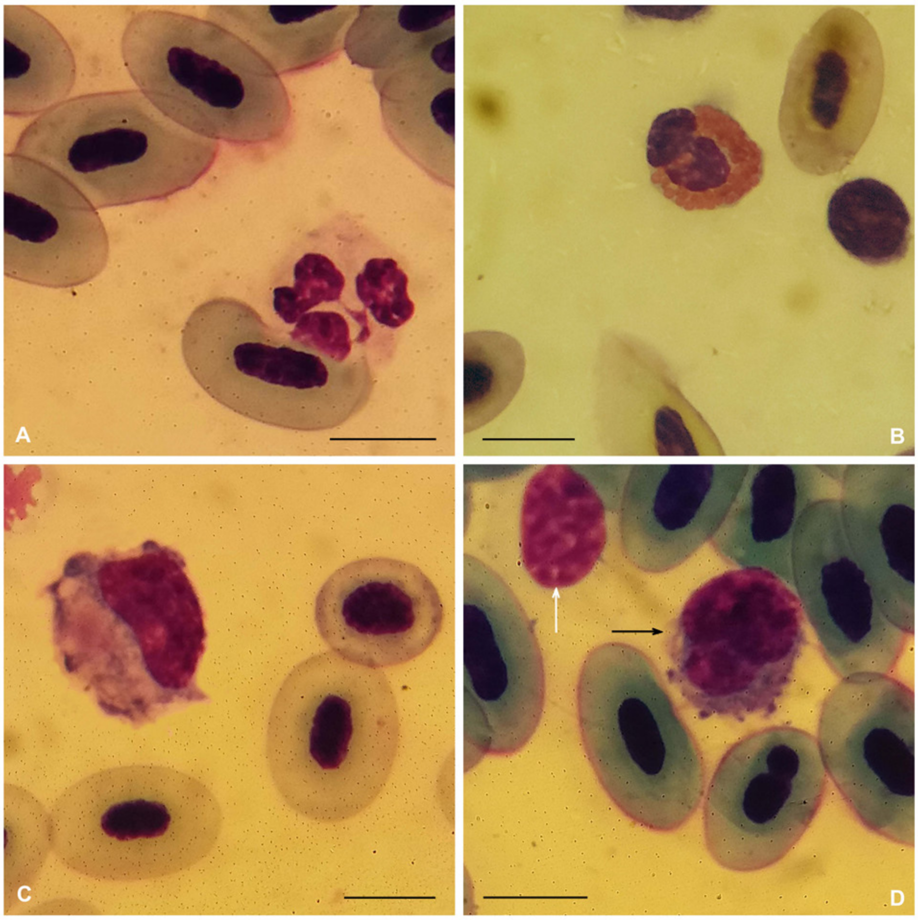

Fig. 2. Leukocytes encountered in blood samples from individuals of *Pelophilax ridibundus*: (A) neutrophil, (B) eosinophil, (C) basophil, (D) lymphocyte (white arrow) and monocyte (black arrow). Scale bar = 10 µm; Giemsa–Romanowsky stain.

The values of the SVL parameter for female frogs inhabiting RS and PS were significantly higher than those of males ($p < 0.001$). This is normal because female marsh frogs are larger than males.

The WBC count of frogs of both sexes inhabiting the RS was significantly higher when compared to the respective values of the frogs from the PS ($p < 0.001$). At the same time, in female or male individuals inhabiting the same site, there were no significant differences in WBCs ($p > 0.05$). In DLC, statistically significant differences were found between male and female individuals inhabiting the RS (Fig. 2). The number of neutrophils (Ne) and eosinophils (Eo) was significantly higher in females ($p < 0.05$), while the number of monocytes (Mo) was higher in males ($p < 0.01$). The comparison among the individuals of the two sexes inhabiting the PS found only one statistically significant difference, namely the number of Mo was higher in females ($p < 0.05$). The numbers of Ne, Eo, Mo and the values of the neutrophil-lymphocyte (N/L) ratio of frogs of both sexes inhabiting the PS were significantly higher than these in female and male individuals of *P. ridibundus* inhabiting RS ($p < 0.001$). Conversely, the number of lymphocytes (Ly) and basophils (Ba) was significantly higher in the blood of frogs of both sexes inhabiting the reference site ($p < 0.001$).

The results of the PCA analysis showed that the first two principal components explained 88.56% of the data variance (Fig. 3). The first component (Factor 1) explained 76.36% of the variation and was composed of all leukogram parameters (eigenvectors = 6.107). Four parameters correlated strongly positively: Ne (0.971), Eo (0.905), Mo (0.928) and N/L ratio (0.978), while the three parameters correlated strongly negatively with this axis: WBC (- 0.915), Ba (- 0.826) and Ly (- 0.977). The second component (Factor 2) explained 12.20% of the data variation. The strongest positive correlation with the second axis indicatedthe parameter SVL (0.970).

The results of discriminant analysis found that the first canonical ($\chi^2 = 26.444$, $df = 21$, $p = 0.001$) and the second canonical function ($\chi^2 = 1.571$, $df = 12$, $p = 0.001$) were significant. Four parameters significantly contributed to the differentiation between the groups of individuals of *P. ridibundus* from the two sites (RS and PS) subjected to comparison: SVL, WBC, Eo and Mo (Table 3).

On the basis of the values of Mahalanobis distances (D^2), the following data were recorded: differences among the individuals of *P. ridibundus* of both sexes from the two sites: RS_female / RS_male—7.736 ($F_{7, 110}$ = 15.721, p = 0.001); PS_male / PS_female—6.510 ($F_{7, 110}$ = 13.229, p = 0.000); PS_male / RS_male—98.306 ($F_{7, 110}$ = 199.761, p = 0.000); PS_male / RS_female—113.670 ($F_{7, 110}$ = 230.979, p = 0.000); PS_female / RS_male—102.554 ($F_{7, 110}$ = 209.003, p = 0.000); PS_female / RS_female—107.893 ($F_{7, 110}$ = 219.242, p = 0.000). A scatter plot of canonical functions separated two clearly differentiated 'clouds': the individuals of *P. ridibundus* from RS as well as those from RF (Fig. 4).

Discussion

Changes in leukogram parameters are used separately (Davis et al. 2008, Silva et al. 2020) or in combination with other haematological parameters

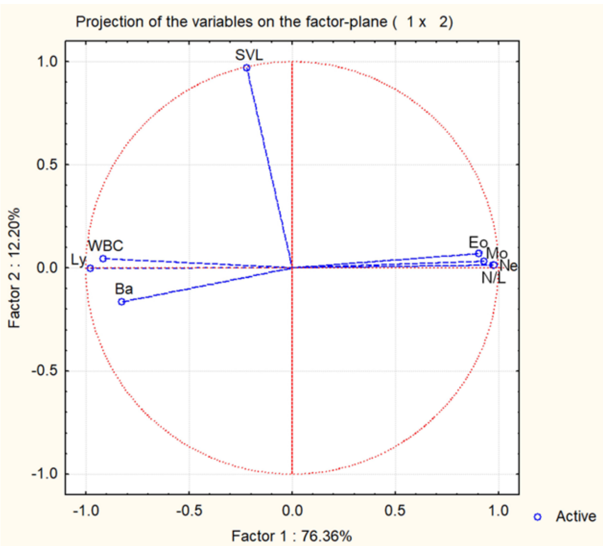

Fig. 3. Ordination on the two canonical variables (Factor 1) and (Factor 2) for tested biological parameters in individuals of *Pelophylax ridibundus*. The distinguishing force of the parameter is indicated by the arrow length; a large importance is shown by the long arrow and it is strongly correlated with the ordination axes.

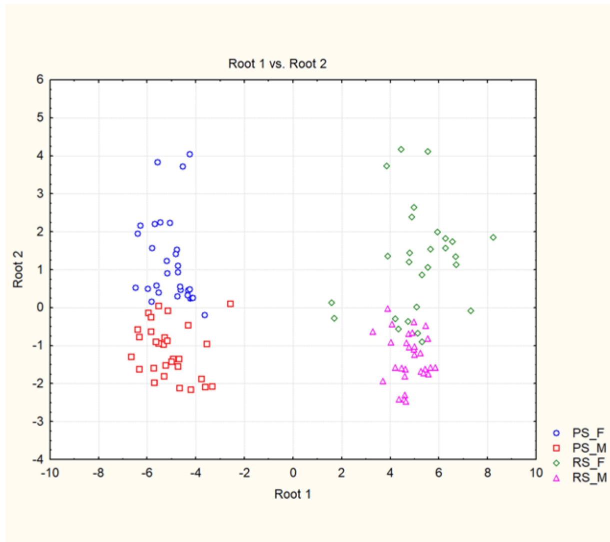

Fig. 4. Canonical discriminant functions scatter plot of tested biological parameters of individuals of *Pelophylax ridibundus* from the studied sites. Legend: Polluted site (PS): the sedimentation lake of the Brikel TPS, Reference site (RS): the Vacha River, F: females, M: males

Table 3. Discriminant functions statistics, correlations between original variables (seven biological parameters of specimens of *Pelophylax ridibundus* from the studied sites) and summary of discriminant functions.

Parameters	**Statistics**				**Discriminant functions and discriminant function coefficients (row / standardised)**	
	Wilks' Lambda	**Partial Lambda**	**$F_{(3, 110)}$**	**p**	**Canonical 1**	**Canonical 2**
SVL*	0.028	0.403	54.200	0.001	0.064 / 0.037	1.715 / 1.000
WBC*	0.013	0.848	6.561	0.001	0.001 / 0.397	-0.001 / -0.053
Ne	0.011	0.967	1.236	0.300	-0.427 / -0.711	-0.121 / -0.200
Eo*	0.014	0.777	10.513	0.001	-0.528 / -0.548	-0.102 / -0.105
Mo*	0.018	0.632	21.305	0.001	-0.634 / -0.948	0.161 / 0.240
Ly	0.012	0.955	1.704	0.170	-0.185 / -1.475	0.030 / 0.045
N/L	0.011	0.994	0.220	0.881	- 2.656 / - 0.081	6.006 / 0.182
Eigenvalue	–				26.444	1.571
Cumulative	–				0.936	0.991

Legend: * The marked parameters showed large absolute correlations with any discriminant function.

(Salinas et al. 2017, Zhelev & Popgeorgiev 2021) in field analyses to evaluate the immune status or general health status of anurans inhabiting environments in conditions of anthropogenic stress.

The results of this study established well-expressed leukopenia in the population of *P. ridibundus* from the sedimentation lake of the Brikel TPS. The changes in DLC of these frogs were characterised by neutrophilia, eosinophilia, monocytosis, basopenia and lymphopenia. Furthermore, the values of the N/L ratio of frogs at that site were significantly higher compared to those from the RS. These changes in the leukogram are indicators of the exhaustion of the immune system and of activation of the organism's protective forces in response to stress (Priyadarshani et al. 2015, Salinas et al. 2017, Silva et al. 2020). We believe that the reason for this stress is of anthropogenic origin and is the result of the industrial wastewater entering the sedimentation lake from the Brikel TPS. The water in the Rozov Kladenets Reservoir is polluted from the Sazliyka and Sokolitsa Rivers (see Table 1). The two rivers are permanently polluted with high doses of reactive water-soluble forms of nitrogen (ammonium nitrogen NH^{+}_{4}-N; nitrate nitrogen NO^{-}_{3}-N; nitrite nitrogen NO^{-}_{2}-N; and total nitrogen TN), phosphates (orthophosphates PO^{3-}_{4} and total phosphorus, as P) and sewage domestic pollutants (Zhelev et al. 2013a, 2019). According to Rouse et al. (1999), Schuytema & Nebeker (1999) and Earl & Whiteman (2009), all water-soluble reactive forms of nitrogen are toxic to aquatic organisms (including water frogs). The comparison with the data from our previous study of the populations of *P. ridibundus* inhabiting these rivers (Zhelev et al. 2013b) shows a severely deteriorated immune status of the frogs from the sedimentation lake. In our opinion, this is the result of the additional pollution of this water with industrial ash from the Brikel TPS. The changes in the leukogram of the marsh frogs from the sedimentation lake of the Brikel TPS were very similar to the ones we found in the populations of the species inhabiting the Chaya River near the Plovdiv non-ferrous metals plant, where pollution with heavy metals had been observed (Zhelev et al. 2020). Such changes in the leukogram of anurans indicating a highly deteriorated immune status of their organism have been reported by authors working in different parts of the world (Carvalho et al. 2016, Pollo et al. 2017, De Assis et al. 2018, Gonçalves et al. 2019). The results of this study confirm that it is possible to collect significant information from the changes in the parameters in the leukogram of *P. ridibundus* as biomarkers for ecological stress. They support the results reported in the works of Davis et al. (2008), Shutler & Marcogliese (2011) and Priyadarshani et al. (2015) and outline perspectives for the direct application of leukogram parameters as a tool for biodiagnostics in anuran populations. On the other hand, this type of analysis can be used as an instrument for the assessment of the ecological quality of habitats. The state agency (Basin Directorate for Water Management – East-Aegean Sea, Region Plovdiv) does not perform physicochemical monitoring of the water of the sedimentation lake of the Brikel TPS. The changes in the leukogram of *P. ridibundus* indicated a highly deteriorated ecological quality of the water in their habitat. This confirms it is possible to apply the changes in the anurans leukogram as a tool in biomonitoring analyses. Of course, it could not completely replace routine physicochemical monitoring but it can be used as an instrument for the collection of additional information about environmental conditions. Chemical analysis is a "snapshot" of the condition of the waterbody at the moment of sampling, while bio tests and, in particular, the changes in the anurans leukogram show the long-term effects of exposure.

Conclusion

In this study, we confirmed leukopenia in the blood of individuals of *P. ridibundus* that inhabit the sedimentation lake of the Brikel TPS, comparing them with frogs from the reference site. Furthermore, the changes in differential leukocyte counts of these frogs are characterised by neutrophilia, eosinophilia, monocytosis, basopenia and lymphopenia. The values of the N/L ratio of frogs at this site were higher than the values of the ones inhabiting the RS. These changes in the leukogram could be indicators of the exhaustion of the immune system and of the activation of the organism's defences in response to stress. In our opinion, the above changes in the leukogram result from the deteriorated water quality of the sedimentation lake because it contains toxicants and industrial ash. The results confirm the role of the changes in the parameters in the leukogram of *P. ridibundus* as diagnostic biomarkers for ecological stress.

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