



Genotoxicity and Erythrocyte Nuclear Abnormalities in *Pelophylax ridibundus* (Pallas, 1771) (Anura: Ranidae) in an Industrial Area in Southern Bulgaria: Evaluation as Biomarkers for Ecological Stress Assessment

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Abstract: The genotoxicity and erythrocyte nuclear abnormalities in adult marsh frogs *Pelophylax ridibundus* inhabiting polluted industrial area at the city of Plovdiv, south Bulgaria, were evaluated as biomarkers for ecological stress. The present work is a part of a large-scale *in situ* study aimed at an assessment of the negative effects on *P. ridibundus* of the chronic impact of high doses of toxicants (mainly nitrates, nitrites, ammonium and heavy metals) entering its habitat with industrial waste waters. For a comparison, frogs from a less disrupted habitat (reference site) were used. Frequencies of micronuclei (MN), erythrocyte nuclear abnormalities (ENAs), mitosis and immature erythrocytes of frogs from the two sites were compared. Significantly higher MN and ENAs frequencies in frogs from the polluted site were registered. Industrial waste waters and the toxicants (reactive forms of nitrogen and heavy metals) in them are suggested as the reason for the genotoxic damages in erythrocytes of frogs from the polluted site.

Key words: *Pelophylax ridibundus*, environmental pollution, ecotoxicology, micronucleus, nuclear abnormalities.

Introduction

In the context of rising anthropisation, the continental freshwater ecosystems are particularly threatened by industrial activities (VUGTEVEEN et al. 2006). Aquatic frogs (Anura) are a key factor sustaining the “health” and biodiversity of freshwater wetlands (DUPLER et al. 2019). Polluting natural freshwater basins with wastewaters from industrial enterprises severely deteriorates the parameters of their habitat (SIEVERS et al. 2018). It is one of the reasons for the world-wide reduction in the populations of aquatic

anurans (WHITTAKER et al. 2013). Many frog species cannot survive under the ecological stress and perish but there are some that adapt and survive even in conditions of intense anthropogenic pollution (FRANCO-DE SÁ & VAL 2014, OTERO et al. 2018, HEGDE et al. 2019, Dönmez & ŞİŞMAN 2021). This allows the broader use of aquatic frogs for *in situ* bioindication analyses, thus providing information on the state of the habitat (SIMON et al. 2011, HEGDE & KRISHNAMURTHY 2014, POLLO et al. 2017). Physical and chemical analyses provide a “snapshot” of the state of the water body (they reflect the presence

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or absence of toxicants as of the moment of sample collecting), while the changes of biological parameters in aquatic frogs reflect the long-term effects of toxic agents (ZHELEV et al. 2020, 2021).

The micronucleus (MN) test and assessing other types of nuclear anomalies of erythrocytes (ENAs) are basic tools in contemporary ecotoxicology (ALNOAIMI et al. 2020). They are widely applied to evaluate the adverse effects of pollutants on animals living in stress conditions because of their simplicity, sensitivity and ability to detect alterations, which can lead to early prevention or remediation (BENVINDO-SOUZA et al. 2020).

This study was aimed at the evaluation of genotoxicity and erythrocyte nuclear abnormalities in adult marsh frog *Pelophylax ridibundus* (Pallas, 1771). Individuals inhabiting a polluted industrial area east of the city of Plovdiv, southern Bulgaria, were considered as potential biomarkers for ecological stress. Our working hypothesis is based on the assumption that larger genotoxic damages in the erythrocytes can be expected in frogs inhabiting conditions of chronic pollution with industrial wastewater in comparison to those from the reference site.

Materials and Methods

Study area, site selection criteria and data collection

This study was conducted in April 2018 (during the breeding season of the marsh frog) at two locations in southern Bulgaria: Site 1 (42.1561° N, 24.8973° E, 162 m a. s. l.), the Chaya River, near the confluence with the Maritsa River; Site 2 (42.0343° N, 24.4704° E, 200 m a. s. l.), the Vacha River (a map of the area can be seen in ZHELEV et al. 2020). In our study, Site 1 was treated as a polluted area (PS – polluted site) because it is located after the industrial zone of the city of Plovdiv, from where wastewaters flow into the Chaya River. 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 (for details on physical characteristics of the two sites see ZHELEV et al. 2020). The data on the ecological quality of the Chaya and Vacha Rivers (Table 1) were obtained from newsletters of the Basin Directorate for Water Management – East-Aegean Sea, Region Plovdiv, Ministry of the Environment and Waters (<https://earbd.bg>). Twenty (10 ♂ and 10 ♀) adult individuals of *P. ridibundus* (SVL > 60.0 mm) were caught from each site – on 12.04.2018 for site 1 and on 24.04.2018 for site 2.

Experimental analyses

All frogs were anaesthetised with ether in laboratory conditions one day after their capture. Blood for tests was taken with heparinised needles by a cardiac ventricular puncture and was used for analysing basic quantitative and qualitative blood parameters, presented in our previous publications (ZHELEV et al. 2020, 2021). For the purposes of this research, we prepared four blood smears for each sampled specimen on clean glass slides, fixed with methanol and then dyed with Giemsa–Romanowsky stain. One thousand erythrocytes per individual were examined at magnification $\times 1000$ using an Olympus stereo microscope (SZX16, resolve 900 line pair/mm, Germany). Only the cells clearly isolated from the surrounding cells were scored. The MN and ENAs were identified according to the criteria suggested by CARRASCO et al. (1990) and were consistent with methodologies described in other works performed with anurans (LAJMANOVICH et al. 2014, ŞIŞMAN et al. 2015, SAID et al. 2016, POLLO et al. 2017, RAGHUNATH et al. 2017, CORDUK et al. 2018). Moreover, the number of erythrocytes undergoing cell division / mitotic erythrocyte (ME) was estimated. Furthermore, the immature erythrocyte frequency (IE) was estimated in order to assess alterations on haematological dynamics according to POLLO et al. (2017).

All statistical procedures were performed using the statistical package R version 3.1.2. (R DEVELOPMENT CORE TEAM 2008). Data distributions for normality (Shapiro-Wilks test) and homogeneity of variances (Levene test) were assessed. According to the results of these tests, we compared the frequency of nuclear abnormalities in frogs from the two different sites using a non-parametric Kruskal-Wallis test. A Bonferroni post-hoc test was applied to establish the inter-sex differences in the frequencies of MN and ENAs in the frogs of both sites. A value of $p < 0.05$ was considered as significant. The associations (and their strength) between the frequencies of the manifestation of ENAs in the blood of frogs from both sites and the levels of bioaccumulation of heavy metals in frog tissues (muscle and liver), established in a previous study of ours (see ZHELEV et al. 2020) were tested with a Spearman rank correlation.

Results

The normal mature erythrocyte of *P. ridibundus* is ellipsoid with a centric nucleus (Fig. 1a). We observed nine types of nuclear abnormalities: lobed nucleus (LN – Fig. 1b), notched nucleus (NN – Fig. 1c), kidney-shaped nucleus (KSN – Fig. 1d), blebbed nucleus (BN – Fig. 1e), binucleate eryth-

Table 1. Physicochemical priority substances determining the ecological status on the studied sites of the Chaya and Vacha rivers, for the 2015-2017 period, 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>). Data are presented with average annual values and the lowest and highest measured values for the 2015-2017 periods (for April 2018: recent data at the time of the study).

Parameters and (standards) for high water quality	Reference site (Vacha River)		Polluted site (Chaya River)	
	2015–2017	April 2018	2015–2017	April 2018
Temp °C (–)	11.8 (5.0–17.0)	9.0	14.4 (5.0–22.0)	10.0
pH units (–)	7.9 (7.4–8.6)	7.9	7.7 (7.1–8.3)	8.0
EC µS/cm (700.0)	196.8 (169–220.0)	206.0	556.3 (250.0–1096.0)	390.0
DO mg/l (9.0–7.0)	10.3 (7.9–12.9)	9.8	9.4 (6.7–12.5)	12.2
BOD ₅ mg/l (<2.0)	1.2 (0.5–1.5)	0.8	1.7 (0.5–4.5)	2.0*
COD mg/l (25.0)	9.1 (6.0–14)	7.0	13.8 (6.0–41.0)	16.0
Ox % (100–105)	101.5 (89.0–124.0)	98.0	90.6 (68.0–114.0)	114.0
CCH mg CaCO ₃ /l (–)	94.6 (65.0–110.0)	94.0	197.9 (105.0–357.0)	143.0
NH ₄ ⁺ -N mg/l (<0.10)	0.16* (0.10–0.29)	0.15*	0.26* (0.17–0.56)	0.21*
NO ₂ ⁻ -N mg/l (<0.03)	0.02 (0.02–0.06)	0.01	0.04* (0.02–0.07)	0.03*
NO ₃ ⁻ -N mg/l (<0.7)	0.51 (0.26–0.72)	0.53	1.96* (0.49–4.1)	0.62
TN mg/l (<0.7)	0.09 (0.07–0.09)	0.23	2.62 (1.3–3.9)	1.3*
PO ₄ ³⁻ mg/l (<0.07)	0.02 (0.01–0.02)	0.02	0.05 (0.02–0.12)	0.03
TP mg/l (<0.15)	0.03 (0.02–0.07)	0.02	0.09 (0.05–0.21)	0.05
Pb µg/l [AAV (1.2); MPC (14.0)]	0.8 (0.3–1.0)	0.76	5.76' (1.12–55.0)	6.4'
Cu µg/l [AAV: 1.0 (CaCO ₃ 0-50 mg/l); 6.0 (CaCO ₃ 50-100 mg/l); 10.0 (CaCO ₃ 100-250 mg/l); 22 (CaCO ₃ > 250 mg/l); MPC: not applicable]	1.25 (0.3–1.8)	1.23	3.96 (1.81–12.1)	12.2'
Zn µg/l [AAV: 8.0 (CaCO ₃ 0-50 mg/l); 40.0 (CaCO ₃ 50-100 mg/l); 75.0 (CaCO ₃ 100-250 mg/l); 100.0 (CaCO ₃ > 250 mg/l); MPC: not applicable]	15.03 (1.0–26.0)	13.2	286.1'(52.0–1160.0)	57.0
Cd µg/l [AAV: ≤0.08 (class 1); 0.08 (class 2); 0.09 (class 3); 0.15 (class 4); 0.25 (class 5); MPC: ≤0.45 (class 1); 0.45 (class 2); 0.6 (class 3); 0.9 (class 4); 1.5 (class 5)]	0.05 (0.01–0.67)	0.02	4.21''(0.18–49.0)	1.11''
Ni µg/l [AAV: 4.0; MPC: 34.0]	0.19 (0.04–1.1)	0.22	0.93 (0.46–3.8)	0.67
As µg/l [AAV: 10.0; MPC: 25.0]	NM	0.02	4.23 (0.47–14.0)	0.46
Hg µg/l [AAV: not applicable; MPC: 0.07]	NM	0.01	0.01	0.02
Al µg/l [AAV: 15.0; MPC: 10 (pH<6.5); 25 (pH>6.5)]	NM	0.02	10.63 (4.5–39.0)	1.45

Legend: Parameters, abbreviations: Temperature (Temp), electrical-conductivity (EC), dissolved oxygen (DO), oxygenation (Ox), biological oxygen demand five days (BOD₅), chemical oxygen demand (COD), calcium carbonate hardness (CCH), ammonium nitrogen (NH₄⁺-N), nitrite nitrogen (NO₂⁻-N), nitrate nitrogen (NO₃⁻-N), total nitrogen (TN), orthophosphates (PO₄³⁻), total phosphorus, as P (TP), iron dissolved in water (Fe), lead (Pb), copper (Cu), zinc (Zn), cadmium (Cd), depending on the hardness classes of water, manganese (Mn), nickel (Ni), arsenic (As), mercury (Hg), aluminium (Al), not monitored (NM). Physicochemical data (water): * – The values deviating (<, >) Standards for high water quality according to Ordinance № H-4 of 14.09.2012 on the characterization of surface waters in Bulgaria (State Gazette, № 22. 5.03.2013); ' – the values above: AAV (Average annual value) and '' – MPC (maximum permissible concentration) according to Ordinance № 256 of 1.11.2010 for Standards on environmental quality for priority substances and for certain other pollutants (State Gazette №. 88, 9.11.2010).

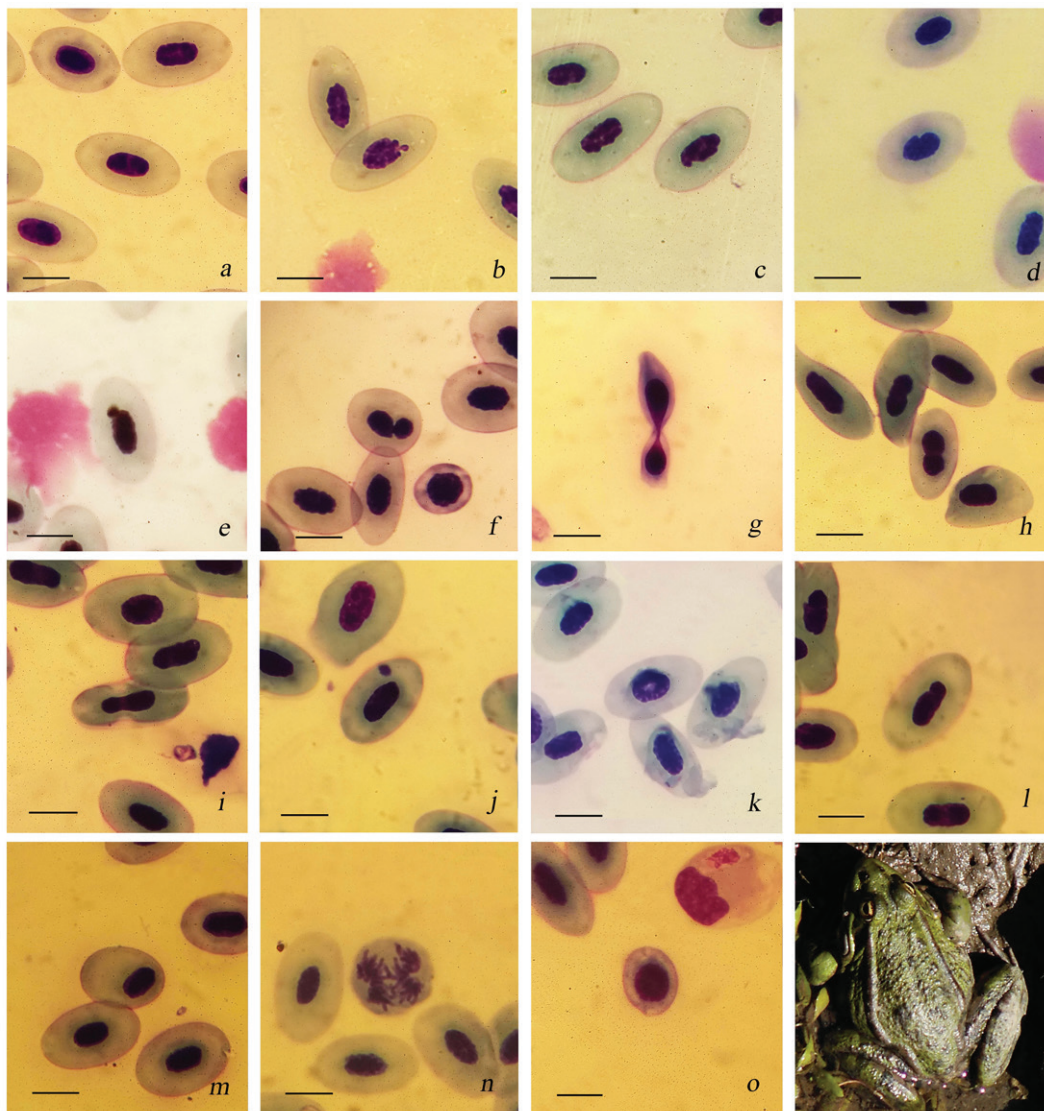


Fig. 1. Photomicrographs of representative nuclear aberrations of erythrocytes recorded in *P. ridibundus* individuals inhabiting on industrial area in southern Bulgaria. Legend: (a) normal; (b) lobed nucleus; (c) notched nucleus; (d) kidney shaped nucleus; (e) blebbed nucleus (sprouted nucleus); (f) binucleate (typical); (g) binucleate erythrocyte with plasma nuclear bridge; (h) binucleate (segmented nucleus); (i) binucleate (pear-shaped nucleus); (j) micronuclei; (k) vacuolated nucleus; (l) low cut nucleus; (m) oval cell with eccentrically placed nucleus; (n) erythrocyte undergoing cell division (mitotic erythrocyte); (o) immature erythrocytes; (p) female *P. ridibundus* from the polluted site. Scale bars = 10 μ m.

rocytes (BE – Fig. 1f, 1g, 1h and 1i), micronuclei (MN – Fig. 1j), vacuolated nucleus (VN – Fig. 1k), low-cut nucleus (LCN – Fig. 1l) and oval cell with eccentrically placed nucleus (EPN – Fig. 1m). The incidences of each of these abnormalities are presented in Table 2 and Fig. 1.

Four types of pathologies such as binucleate erythrocytes were classified: a typical binucleate erythrocyte (Fig. 1f), a binucleate erythrocyte with a plasma nuclear bridge (Fig. 1g), a segmented nucleus (Fig. 1h) and a pear-shaped nucleus (Fig. 1i).

The analyses of genotoxic effects and the frequency of erythrocyte nuclear abnormalities re-

vealed significant differences between frogs inhabiting the two sites: in animals of both sexes from PS, there was a significantly higher frequency of MN and the other eight types of ENAs ($p < 0.0001$) (Table 2). We recorded the highest frequency of encounter with the greatest number of cells registered and, respectively, the greatest number of animals in the group manifesting the pathology in five types of ENAs (MN, NN, BE, VN and LN) in frogs from PS (Fig. 2). The comparison between the frequencies of appearing mitotic erythrocytes (ME) and immature erythrocytes (IE) in the two populations also established significantly higher values in frogs

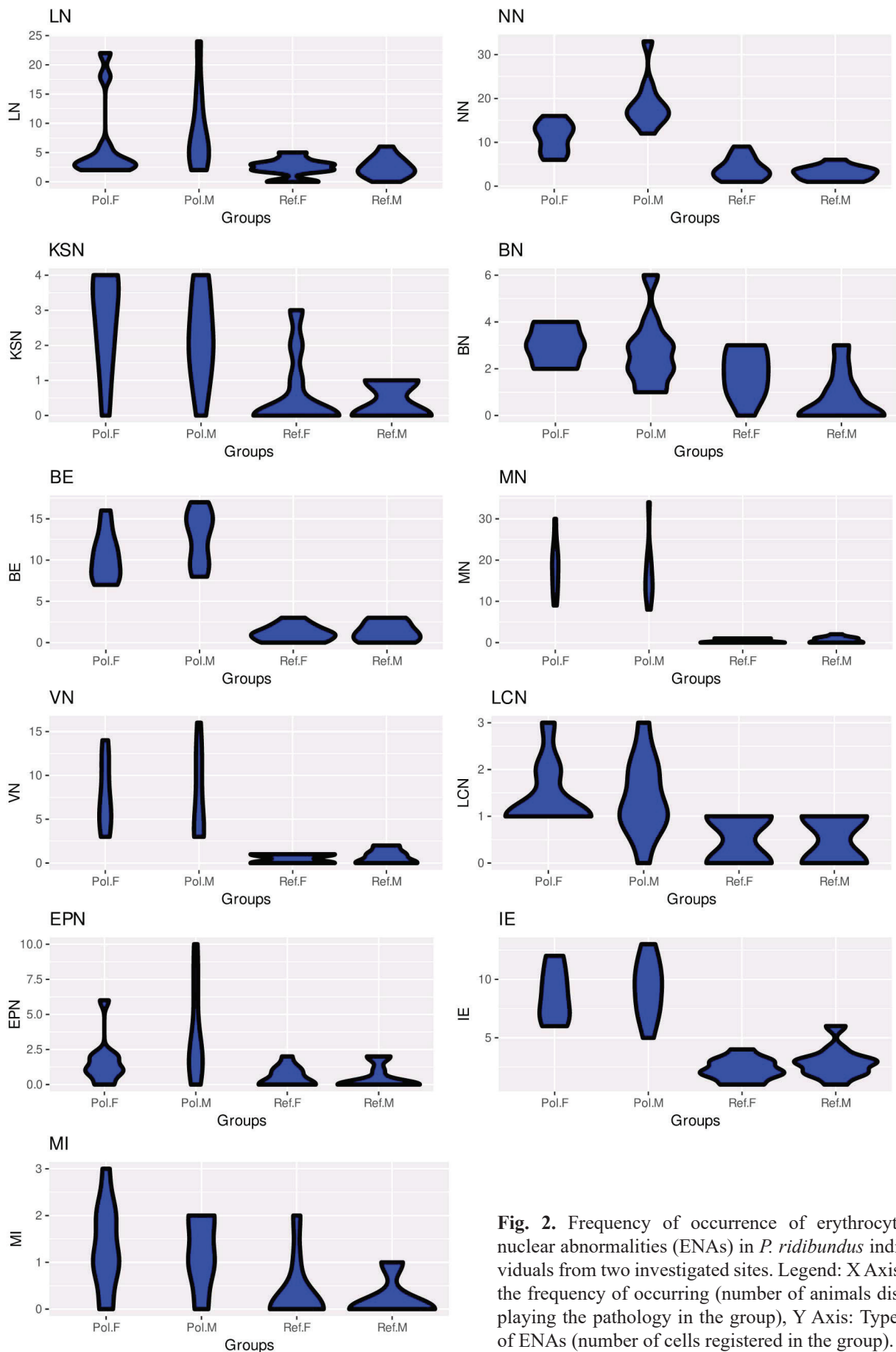


Fig. 2. Frequency of occurrence of erythrocyte nuclear abnormalities (ENAs) in *P. ridibundus* individuals from two investigated sites. Legend: X Axis: the frequency of occurring (number of animals displaying the pathology in the group), Y Axis: Types of ENAs (number of cells registered in the group).

Table 2. Nuclear abnormality assays in erythrocytes of *P. ridibundus* individuals from two investigated sites: Mean \pm SEM and Lower–Upper values. The asterisk (*) indicates statistically reliable differences when comparing frogs of the respective sexes ($\text{♀}/\text{♀}$ and $\text{♂}/\text{♂}$) of the two sites. Significance codes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Micronuclei and Erythrocyte nuclear abnormality (ENA)	Reference site		Polluted site		Kruskal-Wallis test	
	Female	Male	Female	Male	χ^2	p
Lobed nucleus (LN)	2.4 \pm 0.51 0–5	2.5 \pm 0.56 0–6	6.6* \pm 2.28 2–22	8.6** \pm 2.19 2–24	11.45	0.0009
Notched nucleus (NN)	4.0 \pm 0.83 1–9	3.0 \pm 0.49 1–6	11.2*** \pm 1.09 6–16	18.7*** \pm 1.85 12–33	30.758	0.0001
Kidney shaped nucleus (KSN)	0.6 \pm 0.34 0–3	0.4 \pm 0.16 0–1	2.7*** \pm 0.45 0–4	2.2*** \pm 0.42 0–4	18.036	0.0004
Blebbled nucleus (BN)	1.8 \pm 0.33 0–3	0.7 \pm 0.33 0–3	3.0* \pm 0.26 2–4	2.7** \pm 0.47 1–6	16.606	0.0008
Binucleate erythrocytes (BE)	1.1 \pm 0.31 0–3	1.3 \pm 0.37 0–3	10.4*** \pm 0.95 7–16	12.8*** \pm 1.02 8–17	30.281	0.0001
Micronuclei (MN)	0.4 \pm 0.16 0–1	0.6 \pm 0.22 0–2	18.0*** \pm 1.94 9–30	16.6*** \pm 2.31 8–34	30.404	0.0001
Vacuolated nucleus (VN)	0.5 \pm 0.17 0–1	0.7 \pm 0.26 0–2	7.7*** \pm 1.22 3–14	7.5*** \pm 1.54 3–16	30.121	0.0001
Low cut nucleus (LCN)	0.5 \pm 0.17 0–1	0.5 \pm 0.17 0–1	1.4** \pm 0.22 1–3	1.4** \pm 0.27 0–3	14.096	0.002
Oval cell with eccentrically placed nucleus (EPN)	0.5 \pm 0.22 0–2	0.5 \pm 0.27 0–2	1.6* \pm 0.54 0–6	3.5* \pm 1.06 0–10	13.31	0.004
Mitotic erythrocyte (ME)	0.3 \pm 0.21 0–2	0.2 \pm 0.13 0–1	1.3** \pm 0.30 0–3	1.2** \pm 0.25 0–2	14.317	0.002
Immature erythrocytes (IE)	2.3 \pm 0.30 1–4	2.9 \pm 0.43 1–6	8.7*** \pm 0.72 6–12	9.2*** \pm 0.79 5–13	29.43	0.0001

of the two sexes inhabiting PS ($p < 0.0001$) (Table 2, Fig. 2). The comparisons of MN and ENAs across sexes showed a statistically insignificant variation between individuals of both sexes inhabiting RS (Bonferroni post-hoc test, $p > 0.5$). There were no statistically significant differences in the counts of ENAs between sexes in the PS. The only exception was for NN, which had higher counts in females (Bonferroni post-hoc test, $p < 0.01$).

The Spearman rank correlation between levels of heavy metals bioaccumulation in frog tissues and erythrocyte nuclear abnormalities in their blood showed the following results: five positive correlations in females from the RS (Zn_L / EPN, $r = 0.44$, $p < 0.05$; Zn_L / KSN, $r = 0.71$, $p < 0.05$; Cu_L / NN, $r = 0.45$, $p < 0.05$; As_L / EPN, $r = 0.69$, $p < 0.01$ and Pb_L / EPN, $r = 0.68$, $p < 0.01$) and four positive (Se_M / MN, $r = 0.45$, $p < 0.05$; Pb_L / VN, $r = 0.63$, $p < 0.05$; As_M / KSN, $r = 0.66$, $p < 0.05$ and Se_L / VN, $r = 0.68$, $p < 0.05$) and one negative (Pb_M / KSN, $r = -0.82$, $p < 0.05$) correlations in male individuals from this site. For the frogs inhabiting the PS, the Spearman rank correlation results were as follows: two positive (Pb_M / BN, $r = 0.76$, $p < 0.05$ and Cu_L / LCN, $r = 0.53$, $p < 0.01$) and

seven negative (Cd_L / LN, $r = -0.37$, $p < 0.05$; Se_L / NN, $r = -0.72$, $p < 0.05$; Pb_L / EPN, $r = -0.75$, $p < 0.05$; Pb_M / BN; $r = -0.76$, $p < 0.05$; Se_M / LCN, $r = -0.61$, $p < 0.01$; As_M / VN, $r = -0.73$, $p < 0.01$ and Se_M / VN, $r = -0.80$, $p < 0.01$) correlations in the females and two positive (Se_M / KSN, $r = 0.54$, $p < 0.05$ and As_M / VN, $r = 0.72$, $p < 0.01$) and five negative (As_L / MN, $r = -0.29$, $p < 0.05$; Se_L / VN, $r = -0.32$, $p < 0.05$; Pb_L / NN, $r = -0.35$, $p < 0.05$; Se_M / VN, $r = -0.63$, $p < 0.05$ and As_L / LCN, $r = -0.86$, $p < 0.01$) correlations in male individuals.

Discussion

The micronucleus test in erythrocytes (SCHMID 1975) is widely applied in analyses *in situ* to evaluate the negative genotoxic effects at the cellular level in populations of anurans exposed to the chronic impact of various types of environmental pollutants (including the ones of anthropogenic origin), manifesting clastogenic and aneugenic properties. Because the nuclear anomalies of erythrocytes have a similar origin with the anomalies occurring in the micronuclei, over the last decades they are increas-

ingly acknowledged, in *in situ* analyses, as reliable biomarkers of anthropogenic stress (ŞIŞMAN et al. 2015, SAID et al. 2016, POLLO et al. 2017, CORDUK et al. 2018). The mechanisms responsible for the occurrence of ENAs in amphibians are not yet entirely understood. There are hypotheses that high frequencies of occurrence could be caused by the damaging impact of various xenobiotics on the synthesis of DNA or gene mutations in the structural constituents of nuclear envelope of exposed organisms which may result in nuclear anomalies (LAJMANOVICH et al. 2014). Other authors, like SHIMIZU et al. (1998) and CROTT et al. (2001), suppose that nuclear budding in interphase (which corresponds to blebbed and lobed nuclei) can be seen as a process for eliminating amplified genes from the nuclei and be a precursor of micronuclei. The damages in the genetic material can lead to a cascade of negative biological consequences decreasing the organism's chances to survive under stressful conditions. Their transfer to subsequent generations can lead to even greater ecological damage, threatening the survival of entire populations (LEE & STEINERT 2003).

Our results demonstrated that the frequencies of MN in the blood of frogs from the PS were over 20 times higher for animals of both sexes compared to frogs from the RS (Table 2). That unequivocally shows that the frogs from PS are exposed to strong stress factors that damage the genetic material in their chromosomes. Our data corroborate results reported by other authors, who register increased frequency of MN in populations of anurans from habitats polluted with industrial waste waters (LAJMANOVICH et al. 2014, ŞIŞMAN et al. 2015, POLLO et al. 2017). Other authors (RAGHUNATH et al. 2017, HEGDE et al. 2019) find high frequencies of MN in the blood of anurans from habitats with intense agricultural activities (use of pesticides and fertilizers) as compared to frogs from less disrupted habitats. The observed frequencies of all eight ENAs in the blood of frogs from PS established in the present research are significantly higher than those in the blood of frogs from RS. At that, the frequencies of NN, BE and VN in frogs of both sexes from PS are 6 to 15 times greater than those in the blood of frogs from RS (Table 2). Similar results are published for populations of anurans inhabiting polluted areas in Turkey (ŞIŞMAN et al. 2015, CORDUK et al. 2018), India (RAGHUNATH et al. 2017, HEGDE et al. 2019) and Argentina (LAJMANOVICH et al. 2014, POLLO et al. 2017). In the present study, we have established a significantly higher number of mitotic erythrocytes (ME) and immature erythrocytes (IE) in frogs from PS. The presence of mitotic erythroid cells in the

blood of animals that inhabit polluted conditions indicates a stimulation of erythropoiesis. Some authors (BARNI et al. 2007, POLLO et al. 2017) have speculated that the saturation of the peripheral circulation of adult anurans with ME and IE can be viewed as a short-term mechanism for increasing the oxygen carrying capacity of the blood. At the same time, the significant increase of IE can be seen as a reaction in answer to stress of pollutants present in the environment and is specifically connected to the reduction of oxygen dissolved in the water (POLLO et al. 2017).

All results regarding the genotoxic damage found in the blood of frogs from the PS confirm the fact that those animals suffer a strong ecological stress. In their habitat the concentration of toxicants (mainly nitrates, nitrites, ammonium and heavy metals) exceeds multiple times the norms acceptable for clean waters in the Republic of Bulgaria (Table 1). The data of bioaccumulation of heavy metals and metalloids in the tissues (Zn, Cd, Pb, Cu, As and Se in liver and muscle) of the tested frogs from both sites obtained in the study of ZHELEV et al. (2020) allowed us to look for associations between them and the currently presented data on the frequencies of MN and ENAs. Positive associations between the frequency of MN and ENAs in frogs and the presence of heavy metals in their habitat have been established earlier (ŞIŞMAN et al. 2015, CORDUK et al. 2018). The results of the performed Spearman rank correlation analysis in the present research, however, do not allow us to bind the higher frequency of MN and ENAs directly to heavy metals (in frogs from PS, negative correlations prevail). At the same time, however, we cannot categorically discard the role of heavy metals and metalloids accumulated in the tissues of the frogs from PS (positive correlations between Pb_M / BN, Cu_L / LCN, Se_M / KSN and As_M / VN) as a toxic agent stimulating the rise in MN and ENAs. Heavy metals act as catalysts in the oxidizing reactions of biological macromolecules, which can lead to oxidization damage to tissue (ERCAL et al. 2001), and oxidative stress is one of the mechanisms responsible for DNA synthesis impairment (PROKIC et al. 2017). It has been established that the industrial waste waters entering the habitat in PS also contain high doses of nutrients (ammonium nitrogen $\text{NH}_4^+\text{-N}$; nitrate nitrogen $\text{NO}_3^-\text{-N}$; nitrite nitrogen $\text{NO}_2^-\text{-N}$; and total nitrogen TN). The negative effects of nutrients on blood parameters in anurans have been reported in the literature (CAMARGO et al. 2005). Cumulative and synergistic interactions between the toxicants of different origins that would increase the negative effects on

frogs are also possible. On the other hand, the interactions of toxicants with environmental factors could modify their action. As an example, the toxicity levels of many pollutants depend primarily on pH, salinity, temperature, and interactions between different chemical compounds (CANLI & ATLI 2003). The optimal pH range for aquatic organisms is within the limits of 6.5–8, with anything above or below these values putting them under physiological stress (ADDY et al. 2004). For amphibians, pH values for a normal development are between 6.3 and 7.7 (GARCÍA & FONTÚRBEL 2003). In our study, pH in the water of PS has values above the optimal limits to aquatic organisms (Table 1). In the literature, there are data that increase of pH is generally associated with decreasing the solubility of many heavy metals (AVILA-PÉREZ et al. 1999). On the other hand, the high pH combined with high concentrations of ammonia and nitrates are toxic to aquatic organisms (CHAPMAN 1998). Based on the comments made above, we think that in the conditions of an *in situ* experiment as the present study it is very difficult to give categorical answers about the concrete causes of the genotoxic effects found in the blood of frogs from the PS. For all those questions to receive an answer, future research must be done, including in a laboratory-controlled environment. Still, the results obtained in the present study together with those reported in our previous work, namely a low body mass of the frogs from the PS, a weakened immunity and defensive powers of the organism (ZHELEV et al. 2020) clearly show that the health status of frogs from the PS is extremely deteriorated and the animals are in a critical state. The cause of those negative effects on the frog population from the biotope of the Chaya River is obvious – living in conditions of strong and chronic anthropogenic stress.

Conclusions

In this study, we established high frequencies of micronuclei and erythrocyte nuclear abnormalities in the blood of *P. ridibundus* individuals inhabiting the polluted site compared with frogs from the reference site. This is proof of a genotoxic damage in their cells. We suggest the negative genotoxic effects thus found in the erythrocytes of these frogs are the result of living in conditions of strong and chronic anthropogenic stress caused by industrial waste waters containing mixtures of high doses of toxicants. The results of this study confirm the usefulness of the erythrocytes MN and ENAs as a potent means of monitoring to discover genotoxic agents and the genotoxic damage they cause in aquatic frogs. On

the other hand, these types of genotoxic analyses make it possible for the biomonitoring assessments of the ecological state of the respective water ecosystems. Certainly those assessments cannot be an alternative to the physicochemical analyses of water but they can offer reliable and additional indirect information about the state of water bodies.

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