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A Study of Some Metric Parameters of the Erythrocytes in *Rana ridibunda* (Amphibia: Anura) Derived from an Area of Highly Developed Chemical Industry

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Abstract: Some metric parameters of the erythrocyte (big cell diameter (D-cell), small cell diameter (d-cell), big nuclear diameter (D-nucleus) and small nuclear diameter (d-nucleus)) in the blood of *Rana ridibunda*, inhabiting highly developed chemical industry area, were established. The results were compared with the data from our previous work, carried out in a relatively unpolluted area and another industrial area with different kind of pollution. Considerable variations in cell and nuclear parameters were detected.

Key words: red blood cells, frogs, cell diameters, nuclear diameters, Prise-Jones curves

Introduction

Erythrocytes in the blood are a physiologically regulated system of cells the composition of which has to be heterogeneous and this heterogeneity has to be subordinated to certain physiologically based regularities (Gitelson, Terskov 1960).

Various authors have studied the haematology of amphibians (Korzhuev 1960, Hutchison, Szarski 1965, Zhukova 1978, 1987), but a lot of them concentrate on counting blood cells of different amphibian species. There are, however, studies concerning the size of blood cells of various amphibians (Wintrobe 1933, Szarski, Czopek 1966, Günther 1977, Kuramoto 1981, Arikan 1990, Hawkey *et al*. 1991, Atatür *et al*. 1998, 1999), but the ones carried out in Bulgaria are relatively rare (Tachev *et al*. 1975, Boyadzhieva *et al.* 2001, Zhelev *et al*. 2001, Zhelev *et al*. 2002a, 2002b).

The town of Dimitrovgrad is one of the several regions with highly developed chemical industry in Bulgaria. The biggest polluting factories in the region are: "Neohim" (chemical factory); Steam Power Station "Maritsa-3"; "Vulkan" (cement factory). The pollutants that frequently exceed the limit of admissible concentration

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in the atmosphere are nitrogen dioxide (NO_2), hydrogen sulphide (H_2S), ammonia (NH_4) and sulphuric dioxide (SO_2) (Bulletin for the state of the atmospheric air of the town of Dimitrovgrad 1997, 1998, 1999, 2000, 2001, 2002) (Table 1).

Table 1. Data of the atmospheric air condition in the area of the town of Dimitrovgrad for the period 1997-2002.

Value \ Year	1997	1998	1999	2000	2001	2002
Hydrogen sulphide (H_2S)						
Average annual value	0.0002	0.0001	0.0001	0.0001	0.0001	0.0008
Max single sample, mg/m^3	0.2470	0.1483	0.0540	0.0342	0.0431	0.0056
Percentage above limit admissible concentration	0.58	0.06	0.06	0.03	0	0
Ammonia (NH_4)						
Average annual value	0.0617	0.0117	0.0171	0.0072	0.0130	0.0570
Max single sample, mg/m^3	1.3305	0.3102	0.2376	0.1150	0.2350	0.3410
Percentage above limit admissible concentration	6.5	1.4	0.41	0	0.2	0
Sulphur dioxide (SO_2)						
Average annual value	0.0465	0.0308	0.0494	62.83	34.73	25.2
Max single sample, mg/m^3	1.5690	0.6952	0.7790	823.3	421.2	379.0
Percentage above limit admissible concentration	1.1	2.2	2.5	1.2	0.2	0
Nitrogen dioxides (NO_2)						
Average annual value	0.0150	0.0177	0.0125	12.9732	13.47	15.8
Max single sample, mg/m^3	0.1580	0.1522	0.0883	45	53.2	63.1
Percentage above limit admissible concentration	0	0.22	0	0	0	0

According to the data of the Ministry of Environment and Waters, and the Regional Inspection of Environmental Protection - Haskovo, the water in Maritsa River in the zone of the studied biotope meets the requirements for 3rd category (Physical-chemical analysis of surface water sample from Maritsa River (3 km after "Neohim" - JSC) 1997, 1998, 1999, 2000, 2001, 2002). According to the physical-chemical analysis the pollutants that have the largest number of samples exceeding the 3rd category are phosphates, oil products, ammonia (NH_4) and nitrogen oxides (NO_x) (Table 2).

In our previous work (Boyadzhieva *et al*. 2001, Zhelev *et al.* 2002b), carried out in the relatively unpolluted area (the town of Harmanli) and in another industrial area with different kind of pollution (sulfuric oxides) (Steam Power Station "Maritsa-Iztok-1", near the town of Galabovo), we established considerable differences in the metric parameters of the erythrocytes of *Rana ridibunda* Pallas, 1771. The present work is their continuation and its aim is the establishing of the metric parameters of erythrocyte in *Rana ridibunda* inhabiting the area of the town of Dimitrovgrad and their comparison with the data from the other two localities.

Table 2. Data of the polluting substances in Maritsa River by the town of Dimitrovgrad for the period 1999-2002.

Value \ Year	1997	1998	1999	2000	2001	2002
Nitrogen oxides (NO_x)						
Average annual value	0.08	0.10	0.48	0.08	0.05	0.06
Max single sample, mg/l	0.17	0.11	5.10	0.31	0.11	0.12
Norm for the III category, mg/l	0.06	0.06	0.06	0.06	0.06	0.06
Ammonia (NH_4)						
Average annual value	2.63	2.63	2.46	3.29	2.30	2.59
Max single sample, mg/l	5.70	5.30	5.20	8.82	9.20	5.25
Norm for the III category, mg/l	5.0	5.0	5.0	5.0	5.0	5.0
Phosphates						
Average annual value	1.12	1.30	1.60	1.20	1.39	1.32
Max single sample, mg/l	2.40	2.60	2.67	2.42	2.89	2.89
Norm for the III category, mg/l	2.0	2.0	2.0	2.0	2.0	2.0
Oil products						
Average annual value	2.46	0.79	0.55	0.43	0.58	0.66
Max single sample, mg/l	8.40	2.50	1.60	0.80	0.90	1.90
Norm for the III category, mg/l	0.5	0.5	0.5	0.5	0.5	0.5

Material and Methods

Thirty adult frogs (body length - 7-13 cm) of the species *Rana ridibunda* from both sexes have been used in this study. The animals were caught in May 2002 in water ponds situated between two industrial collectors of "Neohim" flowing in Maritsa River near the town of Dimitrovgrad with altitude of 100 m.

The captured frogs were tempered for 24 hours at 2°C and "other equal conditions". The necessary blood samples were obtained by cardiac puncture. The erythrocyte measurements were determined microscopically on blood smears stained according to Romanovski-Gimsa by means of ocular micrometer.

Four parameters are measured from 100 cells of each smear: big cell diameter (D-cell), small cell diameter (d-cell), big nuclear diameter (D-nucleus) and small nuclear diameter (d-nucleus). For each individual the percentage ratio of the number of the cell towards their size for each parameter is represented by the Prise-Jones curves distributed and analyzed according to our typification (Zhelev *et al*. 2002a), similar to the graphical methods for describing the metric parameters of erythrocytes (erythrograms) used by Gitelson, Teraskov (1959, 1960), Goldberg (1960).

The results have been compared to the data from our previous studies, carried out with the same methods, with the same number of individuals (using 3000 erythrocyte), during the same season (Boyadghieva *et al*. 2001, Zhelev *et al.* 2002b), by using Student-Fisher t-test.

The differences obtained by the comparisons are accepted as strongly significant for values of $t \geq 3$ (Sepetliev 1972).

Results

The final results from the measuring and the statistical processing of 3000 erythrocytes of *Rana ridibunda* inhabiting the area of the town of Dimitrovgrad and their comparison to the relative number of cells from the other two biotopes (Harmanli and SPS "Maritsa-Iztok-1") (Table 3,4) are:

Table 3. Results from the comparative variation-statistical analysis of metric erythrocyte parameters of the frogs, inhabiting the town of Harmanli and Dimitrovgrad.

Haematological parameters	Harmanli		Dimitrovgrad		Value of t-criterium
	$\overline{x}$ ± m	σ	$\overline{x}$ ± m	σ	
D-cell	24.18±0.04	2.10	24.81±0.03	1.74	12.60
d-cell	14.71±0.03	1.44	14.62±0.02	1.08	2.49
D-nucleus	9.96±0.02	1.21	8.89±0.02	1.22	37.81
d-nucleus	6.19±0.02	1.32	5.30±0.01	0.59	39.73

Table 4. Results from the comparative variation-statistical analysis of metric erythrocyte parameters of the frogs inhabiting the area of SPS "Maritsa-Iztok-1" and the town of Dimitrovgrad.

Haematological parameters	SPS "Maritsa- Iztok-1"		Dimitrovgrad		Value of t-criterium
	$\overline{x}$ ± m	σ	$\overline{x}$ ± m	σ	
D-cell	23.75±0.04	2.32	24.81±0.03	1.74	21.20
d-cell	14.18±0.03	1.45	14.62±0.02	1.08	12.19
D-nucleus	9.71±0.02	1.04	8.89±0.02	1.22	28.98
d-nucleus	5.75±0.02	0.97	5.30±0.01	0.59	15.90

1. In reference to the big cell parameter (D-cell) we report the biggest value of the mark (24.18 μm) and in the two compared situations the difference is statistically significant.

2. In reference to the small diameter of the cell (d-cell), the erythrocytes of the animals inhabiting the area of the town of Dimitrtovgrad manifest an intermediate value of the parameter (14.62 μm) compared to the animals inhabiting the biotopes near the town of Harmanli (14.71 μm) and SPS "Maritsa–Iztok-1" (14.18 μm). We have established a close to statistically significant result from the first comparison, whereas from the second one it is statistically significant.

3. The biggest diameter of the nucleus (D-nucleus) is with the smallest average value of the parameter (8.89 μm) compared to that in the animals of the other two biotopes. The differences are statistically significant.

4. The small diameter of the nucleus (d-nucleus) of those individuals, which inhabit the area of the town of Dimitrovgrad, manifests the lowest value of the parameter (5.30 μm). The difference from the two comparisons is significant.

The analysis of the Prise-Jones curves about those 30 individuals that inhabit the area of the town of Dimitrovgrad regarding the four investigated metric parameters determines (Table 5) the following:

Table 5. Percentage distribution of the Prise-Jones curves of the frogs inhabiting the three compared areas.

Type of curves	Harmanli				SPS “Maritsa-Iztok-1”				Dimitrovgrad			
	D-cell	d-cell	D-nucleus	d-nucleus	D-cell	d-cell	D-nucleus	d-nucleus	D-cell	d-cell	D-nucleus	d-nucleus
One-peak	53.30%	100.00%	90.00%	60.00%	16.60%	70.00%	96.60%	46.60%	76.30%	76.70%	60.70%	63.30%
Two-peak	46.60%			3.30%	40.00%	30.00%		3.30%	10.00%			
Three-peak	6.60%				40.00%							
Four-peak					3.30%							
Parabolic								6.70%		6.70%	13.30%	13.30%
Descending			6.70%	33.30%			50.00%	3.30%		13.30%	6.70%	6.70%
Ascending			3.30%	3.30%		3.30%		3.30%		3.30%	13.30%	6.70%

1. In reference to the parameter D-cell we described five different types of curves and their percentage distribution is as follows: one-peak curves (76.3%); two-peak curves (10.0%); parabolic curves (6.7%); descending curves (3.3%); ascending curves (3.3%).

The one-peak curves have a size of the base about 20-33 μm and maximums close to 25 μm. The two-peak curves have a size of the base about 24-30 μm and maximums close to 25-30 μm and 23 μm. The size of the base of parabolic curves varies in the limits of 21-27 μm. The described ascending and descending curves have sizes of the bases as follows: 25-30 μm and 21-25 μm. In descending curves the biggest percentage of cells is detected close to 25 μm, whereas in ascending – 24 μm.

The parameter D-cell is characterized with the smallest individual variations compared to those of the rest two groups of animals, which is supported by the higher percentage of one-peak curves (76.3%).

2. In reference to the parameter d-cell we determined four types of curves: one-peak curves (76,7%), descending curves (13.3%), parabolic curves (6,7%), ascending curves (3.3%).

The one-peak curves have a size of the base about 12-20 μm and a maximum close to 14 and 15 μm. The parabolic curves are with a base 14-16 μm wide. The size of the base in descending curves varies from 14 to 20 μm. The biggest percentage of cells is reported close to the value 15 μm. The only ascending curve is with size of the base 13-15 μm and maximum close to 14 μm.

The reported variations of the individual values of the parameter d-cell (76.3 one-peak curves) are closer to those seen in the animals inhabiting the area of SPS ”Maritsa-Iztok-1” (70.0% of one-peak curves) compared to the stable presence of (100%) one-peak curves for the frogs inhabiting the area of the town of Dimitrovgrad. The results show similar changes (reduction) of the small cellular diameter in the erythrocytes of the animals from two industrial areas.

3. In reference to the parameter D-nucleus there are four types of curves: one-peak curves

(60.7%), parabolic and ascending curves (both 13.3%) and descending curves (6.7%).

The one-peak curves have a size of the base about 6-17 μm and maximums close to 9 and 10 μm. The size of the parabolic curves is about 7-12 μm. The ascending curves are with size of the base 6-11 μm and the highest percentage of cells is close to the values 9-10 μm. The two descending curves are with sizes of the bases 8-11 μm. The biggest percentage of cells is reported close to the values 7 and 9 μm.

For the parameter D-nucleus we report the highest heterogeneity in reference to the individual Prise-Jones curves compared to the animals from the other two regions. The results illustrate the reduction of the parameter for the animals from the town of Dimitrovgrad.

4. In reference to the parameter d-nucleus we have established four types of curves: one-peak curves (63.3%), parabolic curves (13.3%), descending and ascending curves (both 6.7%).

The curves with one peak have a size of the base 4-10 μm and maximums close to the value 5-6 μm. The size of the base of the parabolic curves is 4-6 μm. The descending curves are with the size of base 5-9 μm, the ascending one 4-6 μm and both curves have the biggest percentage of cells close to the value 5 μm.

The percentage distribution of the Prise-Jones curves of the parameter d-nucleus is close to that for the frogs inhabiting the rest two biotopes. This shows a stable presence of erythrocytes with a value near the average (5.30), which is the lowest in comparison to the rest two groups of animals.

Discussion

The erythrocytes of the studied specimens *Rana ridibunda* inhabiting the area of the town of Dimitrovgrad have a typical ellipsoidal shape. They are slightly elongated (higher values for the D-cell) and they have smaller nuclei compared to the erythrocytes of the frogs inhabiting the biotope near the town of Harmanli and SPS "Maritsa–Iztok-1". The results from our metric study are supported with the analysis of the graphic method of the Prise-Jones curves.

Atatür *et al.* (1998, 1999) determined differences in the metric parameters of erythrocytes from several species of Urodela and Anura in Turkey and they looked for the reasons in the different environmental conditions of the biotopes. Other authors (Haden 1940, Haris 1963) pointed out that various environmental factors are effective on the size of erythrocytes. Furthermore, Kruger, Parhomenko (1960) and Kosareva, Vasiukov (1976) even stressed that the anthropogenic influence affects the morphology of the red blood cells in amphibians.

According to Petkov (2001) pollutants such as nitrogen oxides and ammonia have methemoglobin-forming influence. Boytler (1981) stressed out that the process of methemoglobin formation is accelerated by some oxidants originating from aniline and nitrites (the latter are present as pollutants in the area of the town of Dimitrovgrad). According to the same author the heaping of methemoglobin in the human erythrocytes leads to a reduction of their size. Similar correlation is established by Gitelson, Gonziakova (1960).

We suppose that there is a correlation between the amounts of accumulated methemoglobin and the change of the erythrocytes size in amphibians, too, but its determination requires the organization of a more detailed future biochemical study.

In our previous paper (Zhelev *el al*. 2002b) we reported that the established reduction of the metric cellular and nuclear erythrocyte parameters in the frogs inhabiting the area of SPS "Maritsa-Iztok-1" compared to those from the area of the town of Harmanli may be due to the hypoxemic hypoxia caused by the industrial pollutants (mainly sulphur oxides and H_2S). Despite our expectations such reduction in the cellular parameters (D-cell, d-cell^2) was not detected in the animals inhabiting the area of the town of Dimitrovgrad. There was, however, a clear reduction of the nuclear size (D-nucleus, d-nucleus). Since the geographical and the environmental characteristics of SPS "Maritsa-Iztok-1"at the town of Dimitrovgrad are very similar, in our opinion it is possible that the reason for these differences can be found in the different kind of pollution in the region (mainly nitrogen oxides and ammonia).

Various authors, by different approach and methods, substantiate the possibilities for using amphibians for the purposes of biomonitoring (Piastolova *et al*. 1981, Bugaeva 1983, Piastolova, Vershinin 1989, Vershinin 1990, Vershinin, Tereshin 1996). The determination of such tendency in the erythrocyte behaviour (similar to the one we establish in Dimitrovgrad and SPS "Maritsa-Iztok-1") in the blood of amphibians in condition of industrial pollution could give an opportunity for using it as cytocryteria in the bioindication. From this point of view the possibilities for using the quantative and qualitative parameters of the blood in amphibians for the purposes of impact biomonitoring undoubtedly hold great interest.

Conclusions

1**.** The erythrocytes of frogs inhabiting the area of the town of Dimitrovgrad have an ellipsoidal shape, they are with bigger size of the cell and lower metric nuclear parameters compared to the animals inhabiting the area of SPS "Maritsa-Iztok-1" and the town of Harmanli.

2. The analysis of the Prise-Jones curves show the smallest individual variations of the parameters D-cell, d-cell, d-nucleus and high heterogeneity of the mark D-nucleus in the animals inhabiting the area of SPS "Maritsa-Iztok-1" and the town of Harmanli.

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Проучване състоянието на някои метрични параметри на еритроцитите у *Rana ridibunda* (Amphibia: Anura) от район с развита химическа промишленост

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(Резюме)

В настоящата работа е осъществено проучване на някои метрични параметри на еритроцитите (голям клетъчен параметър: D-кл.; малък клетъчен параметър: d-кл.; голям ядрен диаметър: D-ядро; малък ядрен диаметър: d-ядро) у представители на *Rana ridibunda,* обитаващи район със силно развита химическа промишленост. Резултатите са сравнени с наши предишни проучвания, проведени в относително незамърсен район и в друг индустриален район с различен тип замърсяване. Установени бяха значителни разлики в параметрите на клетката и ядрото.