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Blood Composition Changes in *Rana ridibunda* (Anura, Amphibia) from an Area of Highly Developed Chemical Industry

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Abstract: The status of some basic quantitative and qualitative indices of blood (number of erythrocytes, haemoglobin contents, haematocrit, colour index, erythrocyte sedimentation speed) has been studied in *Rana ridibunda*, derived from an area of a highly developed chemical industry. The data is compared to our previous studies (ZHELEV *et al.* 2002, ZHELEV *et al.* 2003 - in press). Differences from the frogs, derived from the other two compared regions have been reported.

Key words: erythrocytes, *Rana ridibunda*, haemoglobin contents, haematocrit value, erythrocyte sedimentation speed, South Bulgaria

Introduction

It is well known that the blood-creating organs of animals are highly sensitive to different influences upon the organism, a reflection of which are the changes in the blood composition.

Due to this reason it is of great importance for the ecological-physiological studies to be determined how and due to what changes in the blood parameters the organism responds to the decreased contents of oxygen in environment, its smaller accessibility or its increased need (SCHWARZ *et al.* 1968).

Incompleteness exists in the literature with regard to data characterizing internal species changeability of the haematological indices in amphibians, although different authors have studied some quantitative and qualitative blood parameters for separate species of amphibians (GULLIVER 1875, ALDER, HUBER 1923, BARKROFT 1924, SMITH 1925, WINTROBE 1933, ARVY 1947 KORJUEV 1949, 1960, 1964, HARRIS 1963, FOXON 1964, HUTCHISON 1965, ROUF 1969, TACHEV 1975, ZHUKOVA 1978, 1987, ZHELEV *et al.* 2001).

The determination of the norm for the different haematological indices in Anura is not yet developed in Bulgaria. The aim of the present work is the study and determination of some differences of quantitative and qualitative indices of the blood (number of erythrocytes, haemoglobin contents, haematocrit, colour index, erythrocyte sedimentation speed) in *Rana ridibunda* PALLAS, 1771, derived from an area of a highly developed

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chemical industry. Due to the lack of established standards and in order to achieve the aim stated, we use data from our previous research, carried out in a relatively unpolluted area (the town of Harmanli) and from an area with highly developed energetics ("Maritsa-Iztok-1" Thermoelectrical Power Station) near the town of Galabovo (ZHELEV *et al.* 2002, ZHELEV *et al.* 2003 - in press) for comparison.

Material and Methods

For the study 30 frogs were used from the species *Rana ridibunda* of both sexes with sizes 7-13 cm. The animals are caught in May 2002 in ponds located between the two industrial collectors of "Neochim" JSC, near Dimitrovgrad.

Biogeographically the study area belongs to the Middle Bulgarian region, with transitional continental climate with altitude of 100 m (GRUEV 1994).

Dimitrovgrad is one of the centres of heavy industry in Bulgaria. The biggest polluting factories are: "Neochim" - JSC (chemical factory), "Maritsa - 3" Thermoelectrical Power Station; "Vulkan" (cement factory). The biggest part of contaminants in the region belongs to nitrogen oxides (NO_x), hydrogen sulphide (H_2S), ammonia (NH_3) and sulphur dioxide (SO_2), (Table 1) (bulletins for the status of atmospheric air for Dimitrovgrad for 1997, 1998, 1999, 2000, 2001).

According to the Ministry of Environment and Waters, the Regional Inspection of Environment Protection - Haskovo data, the water in the the Maritsa River in the area of the studied ponds meets the requirements for 3rd category (physical-chemical analysis of surface water sample from the Maritsa River (3 km after "Neochim"- JSC, for 1999, 2000, 2001) (Table 2).

The study was carried out after the catching of animals and their keeping at 2° C for 24 h and "the rest equal conditions", with blood taken from the heart according to confirmed clinical methods (PAVLOV 1989).

Table 1. Data of the atmospheric air condition in the area of the town of Dimitrovgrad for the period 1997-2001.

Pollutants	SO_2	H_2S	NH_4	NO
Limit admissible concentration, mg/m^3	0.15	0.001	0.20	0.16
Max single sample, mg/m^3	1.56	0.70	1.20	1.03
Number of samples of limit admissible concentration, mg/m^3	448	4494	1419	7528

Table 2. Data of the polluting substances in the Maritsa River by the town of Dimitrovgrad for the period 1999-2001.

Pollutants	NO_2	Phosphates	Phenols	Oil products
Norms for 3rd category, mg/l	0.06	2.00	0.10	0.50
Max single sample, mg/l	6.16	7.27	0.15	1.50
Number of analyses exceeding the 3rd category	10	4	1	4

The number of erythrocytes is determined according to the chamber method of Wierord, using Burker chamber.

Contents of haemoglobin is determined according to the cyan-haemoglobin method with chemical agent of Drabkin at λ = 540 nm wavelength.

Haematocrit value is determined according to the centrifugal method with heparinated glass capillaries, at 5 000 turns per 5 min., with reading of the height of erythrocyte column.

Colour index (CI), average contents of haemoglobin in one erythrocyte (MCH), average concentration of haemoglobin in erythrocyte cells (MCHC), average volume of erythrocyte (MCV) are calculated according to standard formulas.

Speed sedimentation of erythrocytes (SSE) is studied according to the micro method of Panchenko, with reading of the results after 1h and 2 h after the loading of sample along the length of the plasmatic column.

The following mathematical indices are used: mean value - , standard error of mean value - m, coefficient of variation - Vs (Table 3, 4, 5).

A linear correlation was made between the number of erythrocytes and SSE for the 1st and the 2nd hour, with a view to determination of a relation between them. The following indices have been calculated: coefficient of correlation - r, standard aberration of the coefficient - Sr, coefficient of determination - r^2 and the ratios - statistical significance of correlation and - coefficient of regression (Table 6).

The results were compared to data from our previous studies, carried out with the

Table 3. Results of the comparative variation- statistical analysis of haematological parameters of animals of Harmanli and Dimitrovgrad regions.

Haematological parameters		Harmanli			Dimitrovgrad			Value of t-criterium
		$\bar{x}$	m	V_s	$\bar{x}$	m	V_s	
Number of Er., thousands/ml		297.40	21.22	39.13	426.70	24.83	31.83	0.26
Hb, g/l		20.10	2.20	10.95	23.00	1.20	28.74	8.08
CI		0.41	0.02	33.17	0.15	0.01	47.26	13.63
Haematocrit		0.27	0.02	44.44	0.23	0.01	31.82	3.18
SSE, mm	1 h	13.00	0.55	23.07	10.83	0.66	33.92	10.60
	2 h	21.80	0.61	15.46	21.13	1.12	29.26	4.02

Table 4. Results of the comparative variation- statistical analysis of haematological parameters of animals of "Maritsa Iztok-1" Power Station and Dimitrovgrad regions.

Haematological parameters		Thermoelectrical Power Station "Maritsa Iztok - 1"			Dimitrovgrad			Value of t-criterium
		x	m	V_s	x	m	V_s	
Number of Er., thousands/ml		437.10	29.27	36.70	426.70	24.83	31.83	0.26
Hb, g/l		38.70	1.51	21.45	23.00	1.20	28.74	8.08
CI		0.45	0.02	21.11	0.15	0.01	47.26	13.63
Haematocrit		0.30	0.02	30.00	0.23	0.01	31.82	3.18
SSE, mm	1 h	21.00	0.70	18.30	10.83	0.66	33.92	10.60
	2 h	26.12	0.53	11.22	21.13	1.12	29.26	4.02

same methods, with the same number of individuals, during the same season (ZHELEV *et al.* 2001, ZHELEV *et al.* 2003 - in press), by using Student-Fisher t-test.

In order to extend the analyticity of the study on the basis of the data used in the comparison and the data received for the animals from Dimitrovgrad region, three additional indices have been estimated: MCH, MCHC and MCV.

The differences obtained by the comparisons are accepted as strongly significant for values of t=3 (SEPETLIEV 1972).

Table 5. Results of the comparative variation- statistical analysis of the following parameters: MCH, MCHC, MCV of animals from the three regions.

Parameter	Harmanli			Dimitrovgrad			Value of t-criterium
	$\bar{x}$	m	V_s	$\bar{x}$	m	V_s	
MCH(pg)	14.10	1.32	51.49	6.09	0.48	43.13	2.73
MCHC(g/l)	142.07	15.74	60.70	99.12	5.21	28.88	2.59
MCV(fl)	145.30	16.58	62.52	62.33	4.26	37.52	4.85
	Thermoelectrical Power Station "Maritsa Iztok - 1"			Dimitrovgrad			
MCH(pg)	11.30	0.84	32.12	6.09	0.48	43.13	5.39
MCHC(g/l)	135.38	7.58	30.68	99.12	5.21	28.88	3.94
MCV(fl)	105.07	0.60	30.11	62.33	4.26	37.52	9.93
	Thermoelectrical Power Station "Maritsa Iztok – 1"			Harmanli			
MCH(pg)	11.30	0.84	32.12	14.10	1.32	51.49	1.79
MCHC(g/l)	135.38	7.58	30.68	142.07	15.74	60.70	0.38
MCV(fl)	105.07	0.60	30.11	145.30	16.58	62.52	2.42

Table 6. Results of the linear correlation analysis between erythrocyte sedimentation speed and erythrocyte count of animals from the region of Dimitrovgrad.

Er – SSE	r	Sr	$\frac{r}{S_r}$	r2	$r.\frac{S_y}{S_x}$
1h	0.36	0.02	18.40	21.50	9.62
2h	0.41	0.02	23.80	26.33	16.66

Results

The results from the studied haematological parameters obtained for the animals from Dimitrovgrad region and their comparison with data from ponds of "Maritsa-Iztok - 1" Thermoelectrical Power Station and the town of Harmanli show (Table 3, 4, 5, 6):

1. The number of erythrocytes is 426.77 thousand/ml. This value is significantly higher than in the area of Harmanly (t = 3.96).

2. The value of the contents of haemoglobin (23.00 g/l) is close to the one given for the frogs from the biotope around Harmanli (20.10 g/l) and it is significantly lower than the value for the specimens from the region of "Maritsa-Iztok - 1" Thermoelectrical Power Station (38.70 g/l).

3. The colour index (0.15) is significantly lower than the one obtained for the frogs from the other two comparative biotopes.

4. It has been found statistically significant reduction in the value of haematocrit (0.23) by comparison with the one for the animals from the region of "Maritsa-Iztok - 1" Thermoelectrical Power Station (0.27).

5. Regarding SSE we establish a statistically considerable difference for both periods of the study with the frogs from the biotope around "Maritsa-Iztok - 1" Thermoelectrical Power Station.

6. Parameters MCH, MCHC, MCV for the frogs from the region of Dimitrovgrad are with the lowest values in comparison with the ones for the animals from the other two regions. The differences between the above three indices and the ones for the frogs from "Maritsa-Iztok - 1" Thermoelectrical Power Station are statistically significant, as well as the ones regarding MCV in the specimens from the biotope around Harmanli.

7. The linear correlation analysis reveals that SSE is not influenced by the number of erythrocyte cells for the animals from the region of Dimitrovgrad.

Discussion

The comparison of the obtained results shows stimulated erythropoiesis for the animals from Dimitrovgrad region compared to those inhabiting the relatively clean biotope around Harmanli. Higher values in the number of erythrocyte cells in the specimens from both industrial regions ("Maritsa-Iztok - 1" Thermoelectrical Power Station and the town of Dimitrovgrad) can be accepted as an adaptive reaction of the studied species *Rana ridibunda* living in conditions of oxygen insufficiency, caused by the anthropogenic influence on their living places. Regarding the index number of erythrocytes in frogs from Dimitrovgrad region, the obtained value is close to the one reported by ZHUKOVA (1987) for *Rana ridibunda* inhabiting ponds with pesticide contamination (450.0 thousand/ml) and to the one of KOSAREVA, VASIUKOV (1976) for the same species from ponds around chemical factories (550.0 thousand/ml).

The number of erythrocytes in frogs from the biotope around industrial non-contaminated region of Harmanli (297.4 thousand/ml) is close to those indicated in the literature for other relatively clean regions: (380.0 thousand/ml) for *Rana ridibunda* from the region of Plovdiv (TACHEV *et al.* 1975) and (322.0 - 440.0 thousand/ml) for the same species from the region of Krasnodar in Russia (ZHUKOVA 1987). Taking into account that Anura appeared as far back as the Jurassic period and remained almost unchanged until nowadays and the anthropogenic factor has been known hardly for several decades, we presume that there is an absence of a specially built-up protective mechanism in these animals in response to the oppressive influence of the atmospheric pollutants. Because of this we consider that the frogs from Dimitrovgrad region, as well as the ones from the biotope near the "Maritsa-Iztok 1" Thermoelectrical Power Station react to the oxygen insufficiency in the atmospheric air, caused by the pollutants, in the same way as they would react if they are placed in conditions of "oxygen shortage" in their natural living places. ZHUKOVA (1978) has established stimulated erythropoiesis (491.1 - 558.5 thousand/ml) for specimens from the *Rana ridibunda* species in conditions of oxygen insufficiency during anabiosis.

Low values of the indices of haemoglobin contents, colour index (CI), haematocrit value, MCH, MCHC and MCV, given for the animals of Dimitrovgrad region as well as the carried out comparative analysis with the other two groups of animals give us a reason to affirm that the frogs from the biotope around Dimitrovgrad have the smallest

haemoglobin charge, both in the separate cells and in the total erythrocyte mass.

The results from the mathematical processing of the data show that the intimate mechanisms of regulation in the biosynthesis of haemoglobin for the frogs from Dimitrovgrad region are more strongly influenced than the ones in animals from the other industrial region - "Maritsa-Iztok - 1" Thermoelectrical Power Station. The reliable differences in haemoglobin values, CI, MCV and MCHC between them form a basis for such statement. The significantly lower haematocrit values for the animals from the region of Dimitrovgrad in comparison to those from the region of "Maritsa-Iztok - 1" Thermoelectrical Power Station, in our opinion can be explained with the form of their erythrocyte cells. The data for stimulated erythropoiesis for both groups of frogs in comparison with those from the region of Harmanli support the same statement.

We should notice that in the literature the comparative data for the present studied haematological parameters for amphibians are scanty and unsystematic. Data for haemoglobin contents and CI for *Rana ridibunda* from a region in Russia, presented by ZHUKOVA (1987) have higher values than our results.

The mathematical processing of the data for the SSE index proves that cell factors do not influence this parameter and we presume that for the erythrocyte cells of frogs from Dimitrovgrad region SSE is determined by the factors of plasma nature: change in Z potentials of erythrocyte membranes, change in the values of albumin-globulin coefficient, the establishment of which is a subject of more detailed biochemical analysis.

The values of the coefficient of variation for the haematological indices, number of Er., CI, haemoglobin contents, haematocrit and SSE within the limits of 28.74 - 47.26% for the animals from the region of Dimitrovgrad, show greater variation in comparison with the frogs from the regions "Maritsa-Iztok - 1" Thermoelectrical Power Station and Harmanli, where the values of Vs for the same indices are respectively 11.22 - 36.70% and 10.95 - 44.44%.

Regarding parameters such as MCH, MCHC and MCV, the values of the coefficient of variation for the animals from the region of Dimitrovgrad (28.88 - 43.13%) are closer to those for the frogs from the region of "Maritsa-Iztok - 1" Thermoelectrical Power Station (30.11 - 32.12%). The most variable group in relation to these indices is the group of animals from the region of Harmanli (51.49 - 62.52).

Conclusions

1. Stimulated erythropoiesis was established with the animals from Dimitrovgrad region in comparison with those from the relatively clean biotope of Harmanli.

2. The lowest values of CI, haematocrit, MCH, MCHC and MCV belong to the frogs from Dimitrovgrad region compared to those for the frogs from the other two regions, which confirms that in the separate erythrocyte as well as in the whole erythrocyte mass the contents of haemoglobin is of the smallest value.

3. Statistically reliable differences were providet regarding SSE for the 1st and the 2nd hour, for the frogs of "Maritsa-Iztok - 1" biotope, as the values by the animals from Dimitrovgrad region are lower for both hours.

4. The linear correlation analysis between SSE and the number of erythrocytes for the animals from Dimitrovgrad region excludes the cell factor, and confirms the role of plasmatic factors.

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Изменения в състава на кръвта у *Rana ridibunda* (Anura, Amphibia) от район с развита химическа промишленост

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(Резюме)

В настоящата работа е извършено изследване на някои качествени и количествени хематологични показатели (брой на еритроцитите, хемоглобиново съдържание, хематокрит, индекс на оцветяване, скорост на утаяване на еритроцитите) при *Rana ridibunda* PALLAS, 1771 от район с развита химическа индустрия. Данните са сравнени с наши предишни изследвания - ZHELEV *et al.* 2002, ZHELEV *et al.* 2003 (in press). Установени са различия в сравнение с жабите от другите два съпоставими района.