BIOCHEMICAL INVESTIGATIONS ON *RANA RIDIBUNDA* PALL. AND *RANA ESCULENTA* L.

CIPRIAN RADU MÎNDRESCU[1], GOGU GHIORGHIŢĂ[2], VLAD ARTENIE[3]

Key words: *Rana ridibunda, R. esculenta*, biochemical indices

Abstract: Our investigations pointed out the existence of a high variability of some biochemical indices in the hepatic, muscular and blood tissue at *Rana ridibunda* Pall. and *Rana esculenta* L. Higher amounts of sugars and transaminases were found in the tissues of *Rana esculenta* L. species, while *Rana ridibunda* Pall. was characterised by higher levels of fats (triglycerides and cholesterol). With some exceptions, the highest values of the analysed biochemical indices were found in blood. The electrophoresis of plasma blood proteins of the two species of green frogs showed the presence of 3 – 4 proteic fractions in blood. The albuminic fraction at *Rana esculenta* L. contains two sub-fractions, and this fact sustains the idea of a hybrid nature of this species.

INTRODUCTION

The amphibians have a complex vital cycle, which makes them influenced by a larger range of abiotic factors than other groups of animals. The amphibians are extremely sensitive to the climatic changes, which may interrupt their growth time, the hibernation period, the ability to find food (Donnely and Crump, 1998; Blaustein et al., 2001). The amphibians are key-elements in various ecosystems, thus the changes produced in their populations also affect the other species from the community (Donnely and Crump, 1998). Regarding all theses facts and that there is a global tendency of a decreasing biodiversity, including of the amphibians (approximately 200 species are in this situation, other 32 species are affected by extinction according to (Alford and Richards, 1999; Houlahan et al., 2000) programs were launched to supervise the amphibians, which use varied research methods and techniques to get information on their adaptation potential.

There are also numerous references on the metabolism of different compounds, on the influence of several environmental agents (some ions), on some biochemical parameters at the amphibian species (Matei-Vladescu, 1964; Şuteu and Pora, 1971; Alyousif, 1991). As the study of the plasmatic albumins represents a discriminating technique for the species from the *Rana esculenta* complex, the literature offers many data on this topic (Uzzell and Berger, 1975; Wijnands and VanGelder, 1976; Günther and Lübcke, 1979; Csata, 1998).

Our aim was to investigate the level of some biochemical indices in the hepatic and muscular tissue, and in the blood of two species of frogs: *Rana ridibunda* Pall. and *Rana esculenta* L.

MATERIAL AND METHODS

Ten green frogs were chosen to dose some biochemical compounds from the blood, 6 from the *Rana ridibunda* Pall. species and 4 from the *Rana esculenta* L. species. Finally, a sufficient quantity of blood was drawn for analysis, at three individuals from the first species and three from the second one. We also have done the electrophoresis of the proteins from the blood serum. The analyzed biochemical components were the same at the level of the hepatic and muscular tissue. In this respect cell homogenates were prepared of 0.5 g of tissue (for each), which was mortared with pounded glass up to the consistence of a paste. The final product was then brought to the state of a cell suspension, using 4.5 ml of physiological serum. The obtained homogenates (with a standard dilution of 1:10) were collected in centrifugal test tubes and centrifuged to a 4000 rot/min speed. The supernatants were flowed in dry test-tubes. These samples were processed in the Clinical and Microbiological Lab from the Municipal Hospital Dorohoi, using its technical means: the Cobas Mira automatic system for biochemical analyses and the typical reagents used by this equipment.

The analyzed blood was drawn from the abdominal vein, using a 4% sodium citrate solution as an anti-clotting solution.

The following biochemical indices were analyzed for each investigated individual: carbohydrates, proteins, triglycerides, cholesterol, urea, GOT (Glutamic Oxalacetic Transaminase), GPT (Glutamic Pyruvic Transaminase), creatinine, and amylase. Regarding the fact that the extraction of the carbohydrates from the liver or muscle was made with physiological serum, we may consider that the analysed components are soluble carbohydrates, (especially glucose, probably some fructose, as well). Together with the above mentioned analysis, the blood serum protein electrophoresis was accomplished as well for six male frogs (3 of *R. ridibunda* Pall. and 3 of *R. esculenta* L.). The interpretation of the results was done using the following statistic indices: the minimum and maximum values, the average and standard

deviation (stdev). The Student t-test was used to compare the average of each parameter for the two investigated species. The results are presented in the Tables 1 – 6.

To obtain the blood plasma for electrophoresis, the blood samples were centrifuged for 15 minutes at 4000 rot/min speed. The electrophoresis of the blood proteins was done on a sheet of cellulose acetate, using the BIOTEC Fischer Phaero Stab 0305 F electrophoresis kit. The obtained plasma was taken with a tiny pipette and introduced in the buckets of a special device. The strip of cellulose acetate was dipped in an ATX buffer solution (code 070961) for 2 – 3 minutes using some tweezers, then it was dried by pressing it between two sheets filter paper. The migration strip thus obtained was placed on the electrophoresis deck (after one end had been cut to control the order of the samples). The migration strip was placed in the start position with an applicator which was immersed for 5 seconds in the buckets with plasma. Afterwards it was applied on the cellulose strip from the electrophoresis deck (by a slight press for 5 seconds). This way, approximately 10 µl plasma was transferred on the migration strip in the initial position. After that, the electrophoresis deck was dipped in the ATX buffer solution (code 070965) so that the ends of the strip of cellulose acetate to be sunk into the solution.

The migration took 30 minutes, using a 220 V voltage and an electric current of 30 mA/bath. After that the migration time was over, the cellulose strip was introduced in a Ponceau colouring solution for 7 minutes. The decolouration of the samples was made through two successive baths, three minutes in a standard solution ATX (code 9353210). The samples were then subbmitted for at least 3 minutes to an operation of transparency (to eliminate any trace of colouring substance) using the Phaero-clear solution (code 353310). In order to dry, the strip of cellulose acetate was applied on a glass blade, which was introduced in a thermostat at 80 – 100 °C, for 30 – 45 minutes. After this, the strip sticks closely to the glass blade, thus the handling of the samples being easier. The results are presented in pictures 2 and 3.

All these experiments took place in the Clinical and Microbiological Lab from the Municipal Hospital in Dorohoi, using the Lab's programme for scanning and interpreting the samples: TurboScan (1.2.9) BIOTEC – Fischer GmbH. The processing of the data was made with a programme calibrated for human blood. The diagram for the protein fractions separated through the electrophoresis (albumins, globulins α_1, α_2, β and γ) was automatically drawn by the above mentioned programme.

RESULTS AND DISCUSSIONS

Comparing the results of the biochemical analyses, we may state that there are some quantitative differences, both due to the species and organs which were analysed. Thus, when compared to *Rana esculenta* L., *Rana ridibunda* Pall. shows in the hepatic tissue higher values of cholesterol (which is absent in *R. esculenta*), urea, amylase, triglycerides and creatinine, lower values of carbohydrates and GOT and similar values of GPT and proteins (Table 1). There are also differences between the two species regarding the minimum and maximum values of the analysed parameters. In the case of *Rana ridibunda* Pall., the biggest differences between the extreme values can be noticed with the triglycerides, carbohydrates, cholesterol and total proteins, and the smallest with the creatinine (Table 1).

Table 1 - The values of some biochemical parameters in the hepatic tissue of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* Pall.					*R. esculenta* L.				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	6	0	0	0	0	4	0,01	0,02	0,015	0,002
GPT (UE/g tissue)	5	0,20	0,28	0,23	0,01	4	0,19	0,22	0,21	0,006
Amylase (UE/g tissue)	6	0,25	0,47	0,38	0,04	4	0,11	0,41	0,24	0,06

Carbohydrates (mg% tissue)	6	690	1660	1043,3	151,60	4	850	1800	1332,5	197,41
Cholesterol (mg% tissue)	6	12	295	117,5	57,73	4	0	0	0	0
Triglycerides (mg% tissue)	6	194	1891	979,5	281,50	3	82	263	177,66	52,50
Proteins (mg% tissue)	6	3600	7000	4783,3	564,75	4	2900	10000	5075	1654,48
Urea (mg% tissue)	6	73	114	99,17	5,80	4	63	99	74	8,47
Creatinine (mg% tissue)	5	1,0	3,3	2,10	0,52	3	0,4	1,0	0,63	0,18

In the muscular tissue (Table 2), the species *Rana ridibunda* Pall. registers higher average values of the cholesterol, triglycerides and amylase, lower values for carbohydrates, proteins and close values of the urea and creatinine, when compared to *Rana esculenta* L. It should be noticed that the GOT activity in the muscular tissue is absent at the two investigated species. The cholesterol was absent in the hepatic and muscular tissue of *Rana esculenta* L. At the level of the muscular tissue, the analysed indices registered high individual variations. Thus, at *Rana ridibunda* Pall., GPT ranges between 0,02 and 0,101 UE/g, the quantity of carbohydrates is between 20 and 240 mg/100 g, triglycerides - between 36 and 244 mg/100 g. A similar situation was found at *Rana esculenta* L. for these parameters.

Table 2 - The values of some biochemical parameters in the muscular tissue of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* Pall.					*R. esculenta* L.				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	6	0	0	0	0	4	0	0	0	0
GPT (UE/g tissue)	6	0,02	0,10	0,055	0,01	4	0,04	0,12	0,084	0,02
Amylase (UE/g tissue)	6	0,09	0,16	0,111	0,01	4	0,05	0,11	0,08	0,01
Carbohydrates (mg% tissue)	6	20	240	106,66	38,44	4	110	190	142.5	17,97
Cholesterol (mg% tissue)	6	3	39	15,33	5,22	4	0	0	0	0
Triglycerides (mg% tissue)	6	36	244	128,5	35,34	4	35	172	96,25	28,47
Proteins (mg% tissue)	6	2000	4900	3483,3	431,6	4	2400	6000	3975	759,8
Urea (mg% tissue)	6	35	75	50,83	6,54	4	37	62	49	5,30
Creatinine (mg% tissue)	6	0,8	5,8	2,61	0,94	4	0,5	5,9	2,6	1,15

In blood**,** the most of the analysed indices (Table 3) had higher values than in the liver or muscle, and at the *Rana esculenta* L. species the values were higher than those of *Rana ridibunda* Pall. Only the GOT displayed a higher value in *Rana ridibunda* Pall., (an average of 222,66 UE/g) than in *Rana esculenta* L. (an average of 132,6 UE/g), and the urea had similar values at the two species. The analyses displayed high levels of amylases (an average of 375 UE/g at *R. ridibunda* and 415 UE/g at *R. esculenta*) and of urea (an average value of 138,57 mg/100 g tissue at *R. ridibunda* and 138,3 mg/100 g tissue at *R. esculenta*) in blood. There was an important individual variability of the analysed indices in the sanguine tissue as well. At *R. ridibunda*, the highest variability limits were evinced for GOT (between 86 and 457 UE/g) and GPT (between 20 and 93 UE/g). These variability limits are much more obvious at *R. esculenta* and they extend to almost all the investigated parameters (Table 3).

Table 3 - The values of some biochemical parameters in the blood of *Rana ridibunda* Pall. and *Rana esculenta* L.

Parameter	*R. ridibunda* (Pall.)					*R. esculenta* (L.)				
	obs. no.	min	max	average	standard error	obs. no.	min	max	average	standard error
GOT (UE/g tissue)	3	86	457	222,67	117,70	3	100	177	132,66	22,98
GPT (UE/g tissue)	3	20	93	56,67	21,07	3	34	121	64	28,51
Amylase (UE/g tissue)	3	375	375	375	0	3	213	807	415,33	195,87
Carbohydrates (mg% tissue)	3	38	48	43,33	2,90	3	23	161	74,66	43,44
Cholesterol (mg% tissue)	3	35,9	51	41,97	4,60	3	54,5	99,3	82,23	13,99
Triglycerides (mg% tissue)	3	9,4	13,1	11,17	1,07	3	4	19,5	13,6	4,84
Proteins (mg% tissue)	3	14,4	20	16,70	1,69	3	12,3	29	18,7	5,19
Urea (mg% tissue)	3	128	154,7	138,57	8,19	3	104	160,2	138,36	17,30
Creatinine (mg% tissue)	3	0,11	0,21	0,16	0,03	3	0,25	0,38	0,31	0,04

Comparing the values of the analysed indices in the investigated tissues**,** we noticed that the highest levels of GOT, GPT, amylases and urea are in blood, and the highest amounts of carbohydrates, triglycerides, cholesterol and proteins are found in the liver – for the species *Rana ridibunda* Pall. Excepting the carbohydrates, whose amount is higher in the hepatic tissue of *Rana esculenta* L., all the other parameters have maximum values in blood, (Table 1–3).

Following the information within the Tables 4–6 (which resulted after the Student t-test was applied to establish the main differences between the averages of the biochemical parameters), we may state that, for the hepatic tissue, urea has statistic significant value. For the

muscular tissue, any biochemical parameter is not statistically significant. In the case of blood, only creatinine is statistically significant.

Table 4 -Statistical values of the analyzed parameters in the hepatic tissue of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	0	0,015	-	-
GPT	0,23	0,21	2,364	0,157
Amylase	0,38	0,24	2,306	0,083
Carbohydrates	1043,33	1332,50	2,306	0,272
Cholesterol	117,50	0	-	-
Triglycerides	979,50	177,66	2,364	0,093
Proteins	4783,33	5075	2,306	0,849
Urea	99,17	74	2,306	0,034
Creatinine	2,10	0,63	2,446	0,081

Table 5 - Statistical values of the analyzed parameters in the muscular tissue of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	0	0	-	-
GPT	0,055	0,084	2,306	0,207
Amylase	0,111	0,08	2,306	0,108
Carbohydrates	106,66	142,5	2,306	0,494
Cholesterol	15,33	0	-	-
Triglycerides	128,5	96,25	2,306	0,533
Proteins	3483,33	3975	2,306	0,559
Urea	50,83	49	2,306	0,847
Creatinine	2,61	2,6	2,306	0,991

Table 6 - Statistical values of the analyzed parameters in the blood of *R. ridibunda* and *R. esculenta*

Biochemical parameters	Statistical parameters			
	Averages		t (calculated)	p (two tail)
	R.ridibunda	*R.esculenta*		
GOT	222,67	132,66	2,776	0,494
GPT	56,67	64	2,776	0,846
Amylase	375	415,33	2,776	0,846
Carbohydrates	43,33	74,66	2,776	0,511
Cholesterol	41,97	82,23	2,776	0,052

Triglycerides	11,17	13,6	2,776	0,649
Proteins	16,70	18,7	2,776	0,728
Urea	138,57	138,36	2,776	0,992
Creatinine	0,16	0,31	2,776	0,034

The specialist's references on the hybrid nature of the *Rana esculenta* L. species are based on the study of serical albumins, that display varied migration speeds during the electrophoretic analysis of the proteins from the blood plasma at the two species of green frogs. Thus, Uzzell and Berger (1975), Wijnands and Van Gelder (1976), Günther and Lübcke 91979) obtained electrophoregrams in which three types of migration bands were present (figure 1): the rapid migration (A) corresponds to *Rana lessonae* Cam., the slow one (B) to *Rana ridibunda* Pall., and the one in between the two other (AB), corresponds to the hybrid species *Rana esculenta* L. These results were confirmed by Csata (1998) in Covasna county investigations.

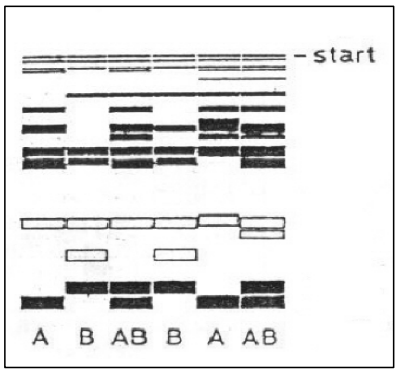

Fig. 1 - Serical albumins at green frogs (Wijnands and Van Gelder, 1976)

Reading the graphs from the electrophoretical analysis of serical proteins for the two species (figures 2**,** 3), one may notice 3 or 4 obvious peaks (each representing a proteic fraction). It may be noticed as well that, while the graph for *Rana ridibunda* Pall. has two distinct peaks in the area of albumins, the graph for *Rana esculenta* L. shows three peaks, which suggests the electrophoretic separation of a supplementary protein fraction at this species. This result sustains the specialists' opinion on the hybrid nature of *Rana esculenta* L. species.

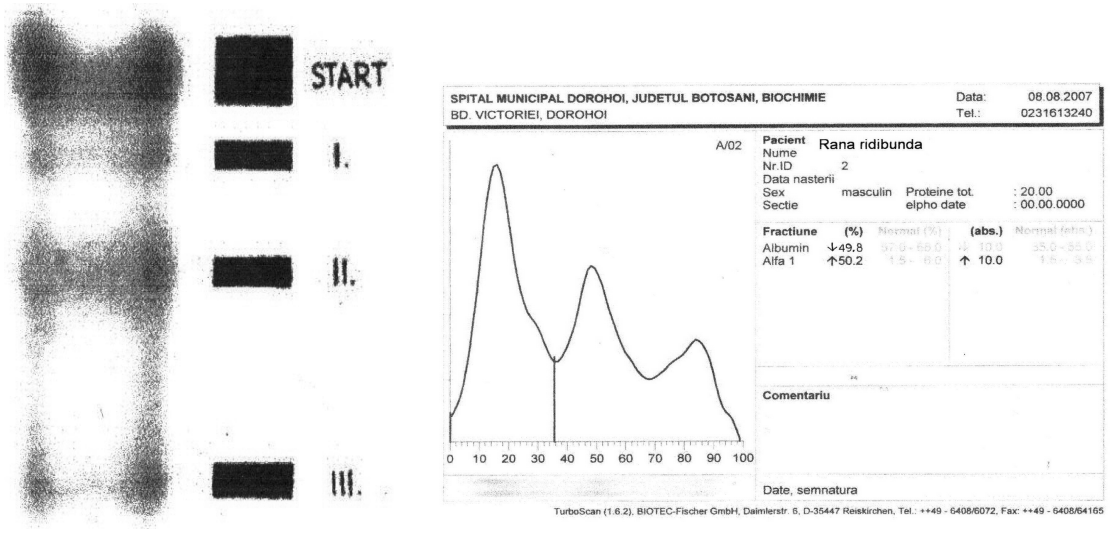

Figure 2 - The electrophoresis of serical proteins at *Rana ridibunda* (Pall.)

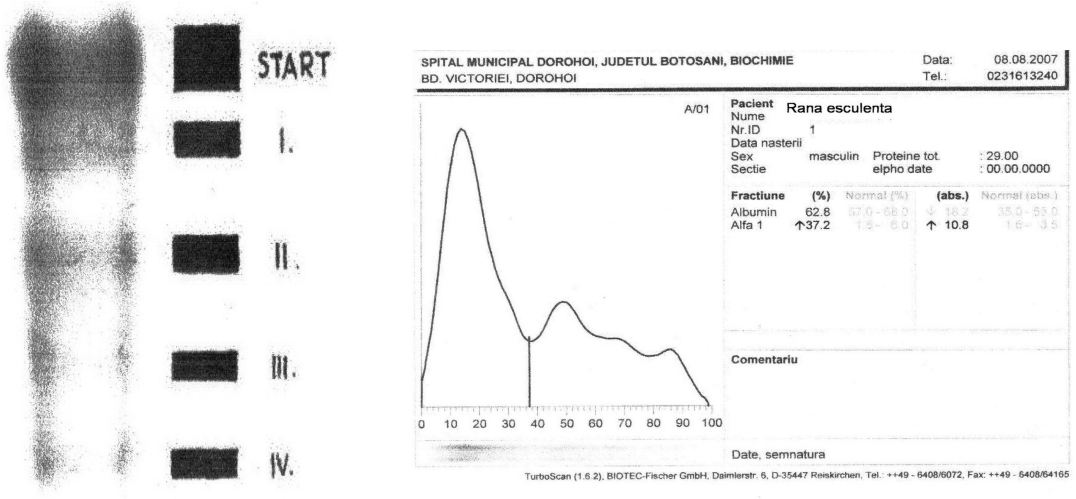

Figure 3 - The electrophoresis of serical proteins at *Rana esculenta* (L.)

CONCLUSIONS

The study of some biochemical indices in the hepatic, muscular and blood tissue at *Rana ridibunda* Pall. and *Rana esculenta* L. pointed out the existence of a high variability, depending on the species, tissue, and even on the investigated individual;

In the liver of *Rana ridibunda* Pall. is found more urea than in the *Rana esculenta* L. liver while the level of creatinine in blood of *Rana ridibunda* Pall. species is lower than in blood of *Rana esculenta* L. species;

The electrophoretical analysis of the blood plasma proteins of the two species of green frogs showed the presence of 3 to 4 proteic fractions. The albuminic fraction of *Rana esculenta* L. contains two sub-fractions, a fact that sustains the idea of a hybrid nature of this species.

REFERENCES

Alford R.A., Richards S.J., 1999. *Global amphibian declines: A problem in applied ecology*. Annual Review of Ecology and Systematics, 30, 133 – 165.

Alyousif M., 1991. *A study on cholesterol content in some tissues of the saudi frog: Rana ridibunda*. Comparative Biochemistry and Physiology, part A: Physiology, 10, 133 – 134.

Blaustein A. R., Belden L., Olson D., Green D., Root T. Kiesecker J., 2001. *Amphibian Breeding and Climate Change*. Conservation Biology, 15, 1804 – 1809.

Csata Z., 1998. *Studiu serologic şi morfologic asupra formelor aparţinând complexului Rana esculenta*. Muzeul Naţional Secuiesc, Sf.Gheorghe, I, 111 – 140.

Donnelly M. A., Crump M. L., 1998. *Potential effects of climate change on two neotropical amphibian assemblages*. Climate change, 39, 541 – 561

Günther R., Lübcke S.,1979. *Serological studies in water frogs from the GDR, Bulgaria and the Soviet Union*. Mitteilungen aus dem zoologischen Museum in Berlin, 55, 1, 225-229.

Houlahan J. E., Findley C. S., Schmidy B. R., Meyer A.H., Kuzmin, S. L., 2000. *Quantitative evidence for global amphibian declines*. Nature, 404, 752 – 755.

Matei-Vlădescu C., 1964. *Cercetări asupra reglării metabolismului glucidic la amfibieni. Acţiunea insulinei*. Stud. Cerc. Biol., Ser. Zool. Bucureşti, 16, 5, 369 – 498.

Şuteu D., Pora E., 1971. *Influenţa K^+ şi Ca^{++} din mediul extern asupra evoluţiei unor parametri biochimici la Rana esculenta*. Stud. Cerc. Biol., Ser. Zool. Bucureşti, 23, 2, 117 – 126.
Uzzell T., Berger L., 1975. *Electrophoretic phenotypes of Rana ridibunda, Rana lessonae and their hybridogenetic associate, Rana esculenta*. Proceeding of the Academy of Natural Science of Philadelphia, 127, 2, 13 – 24.
Wijnands H. E. J., VanGelder, J. J., 1976. *Biometrical and serological evidence for the occurence of three phenotypes of green frogs (Rana esculenta complex) in the Netherlands*. J. Zool., 26, 414 – 424.

[1] National College "Grigore Ghica" Dorohoi, Botoşani county
[2] University of Bacău, Academy of Romanian Scientists
[3] University "Al. I. Cuza" Iaşi, Romania