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ORIGINAL PAPEER

The variability of some characters within local populations of amphibians in Dorohoi area, Botoşani county

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Abstract.
To investigate the phenotypic variability, in response to the interaction of environmental factors on gene pool, was used as biological material individuals belonging to Rana esculenta complex [(Rana ridibunda (Pall.) and Rana esculenta (L.) hybrid] collected from two separate water basins, located in Dorohoi municipality, offering different living conditions. Investigations aimed to highlight morphometric, chromatic, cytogenetic and biochemical variability, both in the same population and in different populations. The obtained data were statistically processed and interpreted. It was found a significant variability at interspecific, intraspecific and even individual level.

Keywords: *Rana ridibunda*, *Rana esculenta*, cytogenetics, morphometry, chromatic polymorphism, biochemical indices.

1. Introduction

Starting from the concept of selection action at the population level, we have to keep in mind that the original material on which selection acts is variability, essentially ensured by mutation and genetic recombination. Selection at the individual level, acts on phenotypes, while being advantaged or disadvantaged genotypes that determine the manifestation of these phenotypes.

Understanding the mechanisms by which populations evolve, as a result of permanent confrontation between gene pool and the concrete conditions of the environment, represents an important and topical concern of specialists.

Aspects of amphibians variability are captured in papers which presents their different adaptation to different environmental conditions, which bring new arguments regarding the ability of populations to answer the selective pressure of the environment.

Relatively recent studies have revealed the mechanisms of population coming to counteract the extreme effects of the environment. Thus, frost tolerance is an adaptive response that allows species to survive and employs biochemical and physiological mechanisms genetically regulated. Storey and Storey (1984) have shown that in winter, frogs accumulate large amounts of glycogen in the liver, which will serve as a source of frost protection. Lee et al. (1990) have shown that

the mechanism of cryoprotection is also achieved through dehydration and rearrangement of molecules in tissues, knowing that the major problem of frost is represented by the mechanical damage of the vascular system. During autumn, the liver loses up to 50% from the normal amount of water and skeletal muscles, between 20 and 30%. Thus, amphibian freeze tolerance was achieved by changing some phenotypic features which determined the adaptation through dehydration and increase the ability to store large amounts of glycogen and metabolize glucose during frost (Churchill and Storey, 1993).

One way to separate the genetic differences of those caused by environment was analyzed through mutual transfer of populations. Such experiments, performed by Berveni (1982), Plytycz *et al*. (1983), Conover and Schultz (1995), Miaud and Merila (2000), Laugen *et al*. (2002), Laurilla *et al*. (2002), followed in a comparative way the manifestation of individuals in new conditions of life to those left in the populations of origin. The studies were focused on major stages in the life cycle of amphibians because they represent important targets on which natural selection acts in different habitats. Experiments have shown a strong inductive effect of the environment on larval development even if they brought some evidence of direct involvement of genetic factor. Berveni (1982) has demonstrated the involvement of the environment at a rate of 80% in terms of larval period, by transfer of individuals from low to high altitude population.

Outstanding issues regarding the large adaptive plasticity of amphibians are reported also in studies pursuing chromatic variability and evolution under the pressure imposed by the relationship prey - predator. A phylogenetic hypothesis for the tandem evolution of chromatics and toxicity, based on genetic analysis, was made by Summers and Clough (2001) in the family *Dendrobatidae*. Although the study does not guarantee the existence of a positive correlation between coloration and toxicity, however, indicate a link between the toxicity and the evolution of aposematic color brightness. The selective role of the predator on phenotypic variability was tested at an endemic species of Guiana, with a large chromatic variability (Noonan and Gaucher, 2006). In this situation, the color brightness not only plays a aposematic role in dealing with predators, but also a role of sexual attractant. By introducing into the environment some colored patterns, alongside aposematic normal form, there was observed a high incidence of bird attack made on the new phenotypes compared to the normal one (yellow). For example, the blue phenotype has a three times higher risk of being attacked by predators than the local phenotype, which confirms the purifying effect of the predator on population, which is thereby maintained under pressure (Noonan and Gaucher, 2006).

Regarding the action of aquatic environment on amphibian, the studies carried out in recent years sheds new light on the adaptive potential of populations in terms of pollutants factors variation. Eutrophication, associated with physical and

biological changes in the aquatic environment is a potential source of stress for aquatic organisms. Johansson *et al*. (2001) studies regarding the degree of tolerance to nitrates of the species *Rana temporaria*, indicated that the northern population of Scandinavia is less tolerant to nitrates than the southern. This observation coincides with the picture provided by the research aimed at water chemistry in Scandinavia that shows a decrease in nitrate concentration from south to north. Nitrates cause a decrease in growth rate and affects northern larval metamorphosis (Johansson *et al*., 2001), suggesting a genetic differentiation of populations regarding the nitrate tolerance depending on the region of origin.

Given the global decline of biodiversity, research is moving towards monitoring programs involving the use of various methods and techniques to form a clearer picture of the species adaptive potential, in terms of climate change more obvious. If we consider the climate changes as a result of indirect action of man, and his deliberate intervention on ecosystems in general, we can understand the enormous pressure faced by populations in their environment.

In the same area of concern, in this paper we proposed to realize a synthesis of our contributions to the knowledge of green frogs biodiversity, by assessing morphometric, chromatic, cytogenetic and biochemical variability in *Rana ridibunda* (Pall.) and *Rana esculenta* (L.), using adult individuals collected from Dorohoi municipality (Botosani County). Some of the results obtained in these investigations there were published in previous papers (Mîndrescu and Ghiorghiţă, 2008, 2011, 2012; Mîndrescu et al., 2009).

2. Material and methods

To achieve the objectives we chose as biological material the green frog species *Rana ridibunda* (Pall.) and *Rana esculenta* (L.), inhabiting two ponds near Dorohoi City, Balţii Pond and Gheorghiu Lake. From the first pond there were collected 74 amphibians, from which 49 of the species *Rana ridibunda* (Pall.), (32 males and 17 females) and 25 of the species *Rana esculenta* (L.), (21 males and 4 females), and from Gheorghiu Lake a total of 100 individuals, 78 of the species *Rana ridibunda* (Pall.), (48 males and 30 females) and 22 of the species *Rana esculenta* (L.), (17 males and 5 females).

The capturing of the individuals was performed using a ”ciorpac”. Very good results were obtained hiding under water the ”ciorpac´s” ring and luring individuals with a colorful float, set at the end of a pole line. For the transport of individuals we used plastic containers in which was added a small amount of water from the natural environment, in order to provide optimal conditions for the transport. In addition, perforated drums caps to ensure a good ventilation.

Except the individuals used in cytogenetic investigations and biochemical analyzes who were sacrificed, the individuals used in the study of morphological characters were released in their natural environment after the observations and

measurements. The individuals were marked before release in order not to be repeated investigations into the recapture. As tagging technique was used the method with armbands (Elmberg, 1989, Rice and Taylor, 1993 quoted by Cogalniceanu, 1997).

2.1. Morphometric investigation method

Biometric recording was made using a digital caliper Mannesmann (Brüder Mannesmann Werkzenge GmbH, Germany) with an accuracy of 0.01 mm. The individuals subjected to observations and analyzes belonged to adult forms. In this respect, we used as a criterion in the samples formation the body length of individuals according to biometric data from RPR Fauna Amphibia, Vol XIV, Fascicle 1 (1960). Thus, the sample of the species *Rana ridibunda* (Pall.) included individuals with body length between 62 - (76.4) - 94 mm, and the sample of the species *Rana esculenta* (L.) included individuals in which body length ranged from 54 - (65) - 76 mm.

For biometric analysis were performed the following measurements: interpalpebral distance (Sp.p.), eye length (L.o.), head width (Lt.c.), eardrum length (L.tymp.), head length (L.c.), body length (L.), forelimb length (L.m.a.), femur length (F.), cannon bone length (T.), tarsal joint length (L.tars.) metatarsal tubercle length (C.int.), first finger length (D.p.).

Morphometric study results are presented in Tables 1-4 and Figures 1-2.

2.2. The method used in the analysis of chromatic variability

According to the methodology proposed by Ishcenko (1978) and undertaken by Nicoara (2004), dorsal and ventral morph of amphibian *Rana esculenta*-complex are:

1. *Maculata* (M) - is characterized by the presence of dark spots with a diameter of 2-3 and 6-7 mm, their number and arrangement typically ranges;
2. *Hemimaculata* (hm) - the same dark spots as previous morph, but their number is smaller, usually 2-6;
3. *Burnsi* (B) - at specimens of this morph total or partial absence of spots;
4. *Punctata* (P) - the back has intense pigmentation, with small points;
5. *Hemipunctata* (hp) - the pigmentation of this morph is less pronounced compared to previous morph. The number of points is smaller, namely dispersion is greater;
6. *Striata* (S) - for this morph is characteristic the presence of a dorso-medial green strips. In principle, it can not be an independent morph, it forms combinations with other morphs.

For the ventral part the pigmentation variability determines two main morphs:

1. *Albicollis-albiventris* (AC/AV) - no pigment;
2. *Nigricollis-nigriventris* (NC/NV) - total and uniform pigmentation, both oropharyngeal and abdominal region;

To these two ventral morphs variants may exist:
1. *Nigricollis-albiventris* (NC/AV) - pigmentation presence only in the region of sacs;
2. *Albicollis-nigriventris* (CA/NV) - pigmentation only in the abdomen.

For the analysis of chromatic polymorphism in green frogs collected from the two ponds, there were used the images shot with Panasonic Lumix digital camera (DMC - LS 3) with a resolution of 5 MP. We have made dorsal and ventral photos of captured specimens.

The results of these observations are presented in Tables 5-8 and Figures 3-6.

2.3. Cytogenetic investigation method

After testing several working techniques to highlight mitotic chromosomes, there were obtained very good results using the technique Spurway and Callan (1960). We brought the following changes of this method: for pretreatment, we used a 0.2% colchicine solution, injected into the dorsal lymph sacs (0.1 ml/10 g); after at least 8 hours the individual was sacrificed and the organs (testicles) were taken. For treatment, good results were obtained using 0.075M diluted saline for 2 hours at room temperature and fixation was performed in ethanol-acetic acid (3:1); the duration of treatment can reach up to 24 hours in fresh fixative, kept in the refrigerator.

The microscopic preparations were performed by *squash* technique, with 2% acetic carmine solution for 20 min.

The microscopic observation were done at binocular microscope series 119, code 50119026, manufactured by Auxilab SL Beriáin (Navarra) Spain, 2004 with 10X oculars (field 18 mm) and objectives 4X, 10X, 40X and 100X.

The determination of morphological types of chromosomes was based on the methodology proposed by Levan *et al*., (1964), and measurements were performed using DCM 130 camera attached to microscopic tube and ScopePhoto 2.0 application.

Through measurements of 10 different metaphases we realized the karyotypes and idiogrames of the species.

The results of the cytogenetical investigations are presented in Tables 9-12 and Figures 7-12.

2.4. Methods for investigating biochemical variability

Analysis of biochemical components were performed on cell homogenate obtained from different tissues (taken from different individuals) and in blood. Cell homogenates were made from liver and muscle tissue, both being important deposits of organic substances, having an intense metabolic activity. All samples were collected in the same time slot (in the morning). The biological samples

collected were preserved by freezing until the next day, when the tests were carried out.

The cell homogenates consisted in 0.5 g of tissue, which was crushed with glass mortar until had a paste consistency. The product obtained was brought to the stage of cell suspensions using 4.5 ml saline. The homogenate was collected with a 1:10 dilution in centrifuge tubes and subjected to centrifugation at 4000rpm for 15 minutes. From each collected sample were performed two tests.

In blood´s case, centrifugation was performed at 4000rpm for 10 minutes to separate plasma from figurative elements. From each vial with plasma were performed two samples that were analyzed separately. Both in cell homogenates and in blood plasma were dosed the following compounds: cholesterol, carbohydrates, triglycerides, total protein, urea and creatinine.

Quantitative determination of these compounds were performed using Cobas Mira automatic biochemical analysis within the Clinical Microbiology Laboratory of Municipal Hospital Dorohoi.

The results of our biochemeical investigations are presented in Tables 20-26.

3. Results and Discussion

3.1. Morphometric variability in green frogs

3.1.1. Morphometric variability in green frogs from Balţii Pond

Analysis of the 49 individuals of *Rana ridibunda* (Pall.) from Balţii Pond showed a reduced variability for eye length, head width, eardrum length, head length, body length, forelimb length, femur length, cannon bone length, tarsus and first finger length. For internal metatarsal tubercle length the variability is medium, and the interpalpebral distance is medium to low. In the case of *Rana esculenta* (L.) individuals from Balţii Pond was observed a medium variability for eardrum and internal metatarsal tubercle length, a medium to low variability for interpalpebral distance and head length, the other investigated parameters having low variability. The results are presented in Table 1.

Table 1 - Values of morphological parameters (in mm) in green frogs from Balţii Pond

Biometric parameter	Statistical parameters	*Rana ridibunda*	*Rana esculenta*
Interpalpebral distance (Sp.p.)	Minimum	2.8	2.3
	Maximum	4.5	3.5
	Media	3.414	3.116
	Median	3.4	3.1
	Standard deviation	0.366	0.296
	Standard error	0.052	0.059
	Coefficient of variation	10.72	9.49
Eye length (L.o.)	Minimum	7.4	6.4
	Maximum	11.0	9.0
	Media	8.955	7.676
	Median	8,9	7,6
	Standard deviation	0.770	0.628
	Standard error	0.110	0.125
	Coefficient of variation	8.59	8.18
Head width (Lt.c.)	Minimum	2.,8	19.8
	Maximum	35.4	26.7
	Media	28.518	23.964
	Median	28.3	24.1
	Standard deviation	2.709	1.698
	Standard error	0.387	0.339
	Coefficient of variation	9.49	7.08
Eardrum length (L.tymp.)	Minimum	4.5	4.0
	Maximum	6.6	6.4
	Media	5.628	5.176
	Median	5.6	3.3
	Standard deviation	0.433	0.596
	The standard error	0.049	0.119
	Coefficient of variation	7.62	11.51
Head length (L.c.)	Minimum	23.6	19.1
	Maximum	37.0	30.6
	Media	28.891	25.816
	Median	28.8	25.8
	Standard deviation	2.799	2.730
	Standard error	0.399	0.546
	Coefficient of variation	9.68	10.57
Body length (L.)	Minimum	62.6	56.5
	Maximum	91.7	72.2
	Media	75.932	66.824
	Median	76.5	67.6
	Standard deviation	6.108	4.010
	The standard error	0.872	0.802

	Coefficient of variation	8.04	6.00
Forelimb length (L.m.a.)	Minimum	16.5	15.0
	Maximum	24.2	21.4
	Media	21.030	18.376
	Median	21.3	18.6
	Standard deviation	1.815	1.615
	Standard error	0.259	0.323
	Coefficient of variation	8.63	8.78
Femur length (F.)	Minimum	31.0	26.9
	Maximum	47.3	38.7
	Media	37.102	31.904
	Median	37.0	31.8
	Standard deviation	3.421	2.933
	Standard error	0.488	0.586
	Coefficient of variation	9.22	9.19
Cannon bone length (T.)	Minimum	30.8	24.8
	Maximum	43.7	33.6
	Media	36.320	28.988
	Median	36.5	29.0
	Standard deviation	3.042	2.106
	Standard error	0.434	0.421
	Coefficient of variation	8.37	7.26
Tarsus length (L.tars.)	Minimum	14.9	14.3
	Maximum	21.7	18.4
	Media	18.426	16.120
	Median	18.2	16.2
	Standard deviation	1.758	1.320
	Standard error	0.251	0.264
	Coefficient of variation	9.54	8.18
Metatarsal tubercle length (C.int.)	Minimum	3.0	2.8
	Maximum	4.7	5.1
	Media	3.971	4.304
	Median	4.0	4.5
	Standard deviation	0.495	0.587
	Standard error	0.070	0.117
	Coefficient of variation	12.46	13.63
First finger length (D.p.)	Minimum	12.0	11.3
	Maximum	16.9	15.0
	Media	14.620	13.304
	Median	14.5	13.3
	Standard deviation	1.169	1.055
	Standard error	0.167	0.211
	Coefficient of variation	7.99	7.92

3.1.2. Morphometric variability in green frogs from Gheorghiu Lake

Regarding the species *Rana ridibunda* (Pall.) of Gheorghiu Lake, the data obtained from measurements performed on the 78 individuals investigated, showed a low variability for eye length, head length and forelimb length and a average variability for the other biometric parameters. For the species *Rana esculenta* (L.) from Gheorghiu Lake, biometric test results on the 22 individuals analyzed, showed that only the eye length has a medium to low variability, the other parameters having a low variability (Table 2).

Table 2 - Values of morphological parameters (in mm) in green frogs from Gheorghiu Lake

Biometric parameter	Statistical parameters	*Rana ridibunda*	*Rana esculenta*
Interpalpebral distance (Sp.p.)	Minimum	2.5	2.6
	Maximum	4.1	3.9
	Media	3.278	3.136
	Median	3.2	3.10
	Standard deviation	0.361	0.303
	Standard error	0.040	0.064
	Coefficient of variation	11.01	9.66
Eye length (L.o.)	Minimum	6.6	6.4
	Maximum	10.8	8.8
	Media	8.698	7.381
	Median	8.8	7.30
	Standard deviation	0.872	0.742
	Standard error	0.098	0.158
	Coefficient of variation	10.02	10.05
Head width (Lt.c.)	Minimum	21.3	19.8
	Maximum	33.7	25.8
	Media	26.060	22.681
	Median	26.25	23.05
	Standard deviation	2.782	1.709
	Standard error	0.315	0.364
	Coefficient of variation	10.67	7.53
Eardrum length (L.tymp.)	Minimum	4.1	4.4
	Maximum	6.9	6.1
	Media	5.225	5.159
	Median	5.2	5.050
	Standard deviation	0.603	0.488
	The standard error	0.068	0.104
	Coefficient of variation	11.54	9.45
Head length (L.c.)	Minimum	22.6	21.5
	Maximum	33.5	28.9
	Media	26.355	24.236
	Median	26.3	24.50
	Standard deviation	2.312	1.984
	Standard error	0.261	0.423
	Coefficient of variation	8.77	8.18
	Minimum	61.0	58.3

Body length (L.)	Maximum	95.8	75.6
	Media	71.110	64.763
	Median	70.65	64.60
	Standard deviation	7.262	4.671
	Standard error	0.822	0.995
	Coefficient of variation	10.21	7.21
Forelimb length (L.m.a.)	Minimum	16.1	15.6
	Maximum	23.7	20.2
	Media	19.929	17.859
	Median	19.95	18.25
	Standard deviation	1.785	1.251
	Standard error	0.202	0.266
	Coefficient of variation	8.95	7.00
Femur length (F.)	Minimum	24.5	20.7
	Maximum	47.6	35.2
	Media	33.144	29.250
	Median	33.45	29.15
	Standard deviation	4.303	2.695
	Standard error	0.487	0.574
	Coefficient of variation	12.98	9.21
Cannon bone length (T.)	Minimum	26.4	25.0
	Maximum	45.8	34.6
	Media	32.962	27.727
	Median	33.1	27.65
	Standard deviation	3.869	2.125
	Standard error	0.438	0.453
	Coefficient of variation	11.73	7.66
Tarsus length (L.tars.)	Minimum	12.9	12.8
	Maximum	21.7	17.7
	Media	16.883	15.004
	Median	16.8	14.95
	Standard deviation	1.790	1.071
	Standard error	0.202	0.228
	Coefficient of variation	10.60	7.13
Metatarsal tubercle length (C.int.)	Minimum	2.9	3.6
	Maximum	5.2	4.7
	Media	3.679	4.603
	Median	3.7	4.00
	Standard deviation	0.444	0.315
	Standard error	0.050	0.067
	Coefficient of variation	12.06	6.84
First finger length (D.p.)	Minimum	9.4	11.3
	Maximum	16.6	14.4
	Media	13.428	12.659
	Median	13.5	12.65
	Standard deviation	1.550	0.861
	Standard error	0.175	0.183
	Coefficient of variation	11.54	6.80

3.1.3. Comparative analysis of green frogs populations in the two ponds

To achieve a more accurate interpretation of the influence of concrete conditions of life on green frogs populations, it was used the statistical analysis of biometric data, comparing the averages of the investigated parameters. The results obtained for the populations of *Rana ridibunda* (Pall.) and *Rana esculenta* (L.) are presented in Tables 3 and 4.

Table 3 – Results of statistical analysis of biometric parameters in populations of *Rana ridibunda* (Pall.) (significance level $\alpha = 0.05$)

Biometric parameter	Location	Average	No. obs.	F Test			t Test		
				Stat. F.	P.	Critical F.	Stat. t.	P.	Critical t.
Sp.p.	Balţii	3,414	49	1.028	0.448	1.520	2.052	0.021	1.657
	Gheorghiu	3,278	78						
L.o.	Balţii	8,955	49	0.779	0.177	0.641	1.684	0.047	1.657
	Gheorghiu	8,698	78						
Lt.c.	Balţii	28,518	49	0.948	0.427	0.641	4.895	$1.4x10^{-6}$	1.657
	Gheorghiu	26,060	78						
L.tymp.	Balţii	5,628	49	0.515	0.007	0.641	4.368	$1.3x10^{-5}$	1.657
	Gheorghiu	5,225	78						
L.c.	Balţii	28,891	49	1.465	0.066	1.520	5.542	$8.4x10^{-8}$	1.657
	Gheorghiu	26,355	78						
L.	Balţii	75,932	49	0.707	0.099	0.641	3.866	$8.8x10^{-5}$	1.657
	Gheorghiu	71,110	78						
L.ma.	Balţii	21,030	49	1.034	0.440	1.520	3.361	0.0005	1.657
	Gheorghiu	19,929	78						
F.	Balţii	37,102	49	0.632	0.044	0.641	5.733	$3.8x10^{-8}$	1.657
	Gheorghiu	33,144	78						
T.	Balţii	36,320	49	0.618	0.037	0.641	5.152	$4.8x10^{-7}$	1.657
	Gheorghiu	32,962	78						
L.tars.	Balţii	18,426	49	0.964	0.453	0.641	4.760	$2.6x10^{-6}$	1.657
	Gheorghiu	16,883	78						
C.int.	Balţii	3,971	49	1.239	0.198	1.520	3.444	0.0003	1.657
	Gheorghiu	3,679	78						
D.p.	Balţii	14,620	49	0.569	0.018	0.641	4.920	$1.3x10^{-6}$	1.657
	Gheorghiu	13,428	78						

Table 4 – Results of statistical analysis of biometric parameters in populations of *Rana esculenta* (L.) (significance level α = 0.05)

Biometric parameter	Location	Average	No. obs.	F Test			t Test		
				Stat F.	P.	Critical F.	Stat t.	P.	Critical t.
Sp.p.	Balţii	3,116	25	0.957	0.456	0.496	-0.23	0.408	1.679
	Gheorghiu	3,136	22						
L.o.	Balţii	7,676	25	0.715	0.213	0.496	1.471	0.074	1.679
	Gheorghiu	7,381	22						
Lt.c.	Balţii	23,964	25	0.987	0.484	0.496	2.574	0.006	1.679
	Gheorghiu	22,681	22						
L.tymp.	Balţii	5,176	25	1.488	0.180	2.054	0.105	0.458	1.679
	Gheorghiu	5,159	22						
L.c.	Balţii	25,816	25	1.893	0.071	2.054	2.241	0.014	1.679
	Gheorghiu	24,236	22						
L.	Balţii	66,824	25	0.736	0.234	0.496	1.627	0.065	1.679
	Gheorghiu	64,763	22						
L.ma.	Balţii	18,376	25	1.667	0.120	2.054	1.213	0.115	1.679
	Gheorghiu	17,859	22						
F.	Balţii	31,904	25	1.183	0.350	2.054	3.213	0.001	1.679
	Gheorghiu	29,250	22						
T.	Balţii	28,988	25	0.982	0.479	0.496	2.038	0.023	1.679
	Gheorghiu	27,727	22						
L.tars.	Balţii	16,120	25	1.519	0.168	2.054	3.152	0.001	1.679
	Gheorghiu	15,004	22						
C.int.	Balţii	4,304	25	3.469	0.002	2.054	1.774	0.041	1.679
	Gheorghiu	4,063	22						
D.p.	Balţii	13,304	25	1.499	0.175	2.054	2.274	0.013	1.679
	Gheorghiu	12,659	22						

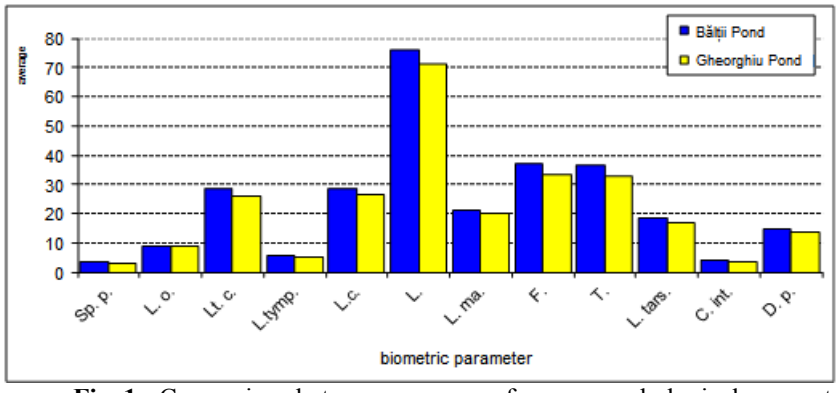

Fig. 1 - Comparison between averages of some morphological parameters in *Rana ridibunda* (Pall.) populations

Sp.p. = interpalpebral distance; L.o. = eye length; Lt.c. = head width; L.tymp. = eardrum length, L.c. = head length, L. = body length, L.m.a.= forelimb length; F. = femur length; T. = cannon bone length; L.tars. = tarsus length; C.int. = metatarsal tubercle length, D.p. = first finger length

Comparative analysis of *Rana ridibunda* (Pall.) populations from the two ponds, by applying Fisher-Snedecor test shows equal dispersions (variances) for the following biometric parameters values: interpalpebral distance, eye length, head width, head length, body length, forelimb length, tarsus and internal metatarsal tubercle. For all these parameters was applied the homoscedastic t-Test to compare averages. Starting from the null hypothesis of parameters averages equal in the two samples, the Student tests applied with a significance level of 0.05, indicates probability values below the threshold chosen for all parameters analyzed. Under these conditions, the tests are statistically significant, which requires the acceptance of the alternative hypothesis (H_1). Thus, one can say that all the averages are different in the two samples. Moreover, the conclusion of t-Tests application is that all parameters have higher average in Balţii Pond, with an error of 0.05 (Fig. 1).

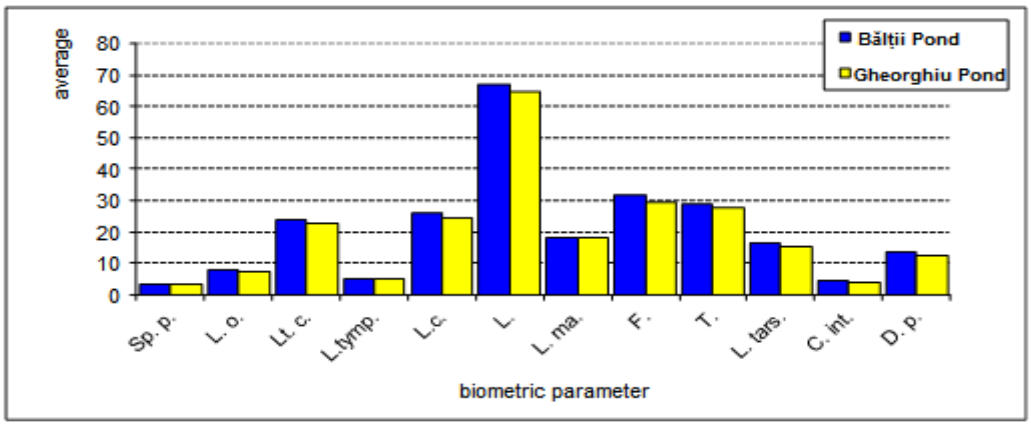

Fig. 2 - Comparison between averages of some morphological parameters in *Rana esculenta* (L.) populations

Sp.p. = interpalpebral distance; L.o. = eye length; Lt.c. = head width; L.tymp. = eardrum length, L.c. = head length, L. = body length, L.m.a.= forelimb length; F. = femur length; T. = cannon bone length; L.tars. = tarsus length; C.int. = metatarsal tubercle length, D.p. = first finger length

Comparative analysis of *Rana esculenta* (L.) populations in the two ponds indicates an equal dispersion of values in the investigated samples for most biometric parameters, except the internal metatarsal tubercle length. For the latter, F test, applied with a significance level of 0.05 is significant (P = 0.002), thereby determining the acceptance of alternative hypothesis. Thus, the comparison of biometric parameters averages in samples from the two ponds was performed using heteroscedastic t-Test for internal metatarsal tubercle length and homoscedastic t-Test for the other parameters with equal variances. Starting from the assumption of equal averages for all parameters in the samples, t-Test confirmed this hypothesis (H_0) only for interpalpebral distance, eye length, eardrum length, body and forelimb length. For the other biometric parameters, t-

Test is statistically significant, which means that the null hypothesis is rejected, the averages are different. It can be said that, in *Rana esculenta* (L.) the population from Balţii Pond, the average values of the parameters with significant differences are greater than those of Gheorghiu Lake population (Fig. 2).

3.2. Aspects of chromatic variability in green frogs

Within the sample represented by individuals of *Rana ridibunda* (Pall.) from Balţii Pond, we noted the presence of 3 main dorsal morphs (*maculata*, *hemimaculata*, *striata*) and 2 combined morphs (*striata-maculata* and *striata-hemimaculata*). Regarding the ventral chromatic, *Rana ridibunda* (Pall.) population displays all possible morphs. Regarding the dorso-ventral chromatic combinations in *Rana ridibunda* (Pall.) population, was observed the expression of 15 different phenotypes (Table 5, Fig. 3).

Table 5 - Frequency of dorso-ventral chromatic combinations in *Rana ridibunda* (Pall.)

Dorso-ventral morph	Number of individuals	Frequency
Maculata+albicollis-albiventris (M+AA)	5	10.20%
Maculata+albicollis-nigriventris (M+AN)	4	8.16%
Maculata+nigricollis-nigriventris (M+NN)	1	2.04%
Hemimaculata+albicollis-albiventris (hm+AA)	6	12.24%
Hemimaculata+albicollis-nigriventris (hm+AN)	3	6.12%
Hemimaculata+nigricollis-nigriventris (hm+NN)	1	2.04%
Striata+albicollis-nigriventris (S+AN)	1	2.04%
Striata+nigricollis-nigriventris (S+NN)	1	2.04%
Striata-maculata+albicollis-albiventris (S-M+AA)	5	10.20%
Striata-maculata+albicollis-nigriventris (S-M+AN)	1	2.04%
Striata-maculata+nigricollis-nigriventris (S-M+NN)	3	6.12%
Striata-hemimaculata+albicollis-albiventris (S-hm+AA)	7	14.28%
Striata-hemimaculata+albicollis-nigriventris (S-hm+AN)	6	12.24%
Striata-hemimaculata+nigricollis-albiventris (S-hm+NA)	1	2.04%
Striata-hemimaculata+nigricollis-nigriventris (S-hm+NN)	4	8.16%

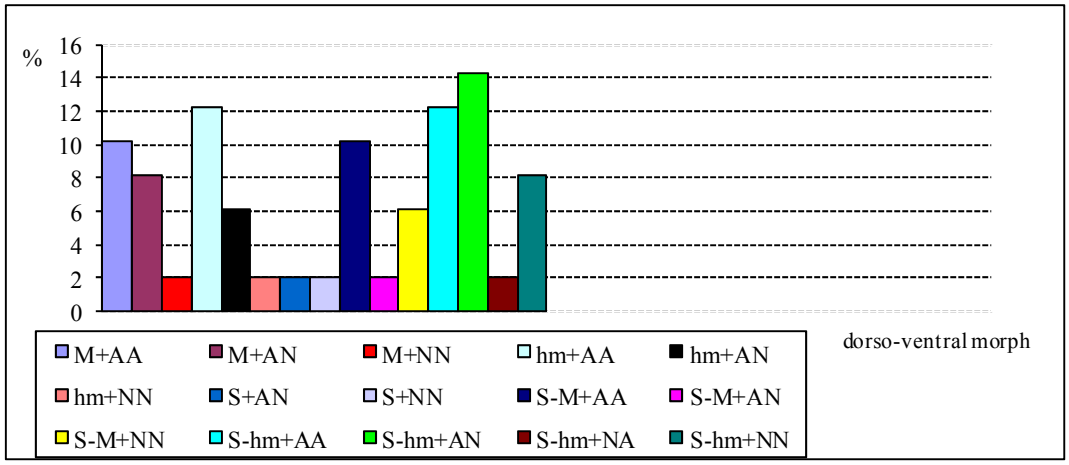

Fig. 3 - Frequency of dorso-ventral chromatic combinations in *R. ridibunda* (Pall.)

At individuals of the species *Rana esculenta* (L.) from Balţii Pond was found the fenotipization of of 3 base dorsal morphs (*hemimaculata*, *burnsi* and *striata*) and a single dorsal complex morph (*striata-hemimaculata*) and ventral 2 morphs are present: *albicollis-albiventris* and *albicollis-nigriventris*. Regarding the dorso-ventral chromatic combinations reported for the species *Rana esculenta* (L.), it was observed the presence of 6 morphological types, (Table 6, Figure 4).

Table 6 - Frequency of dorso-ventral chromatic combinations in *Rana esculenta* (L.)

Dorso-ventral morph	Number of individuals	Frequency
Hemimaculata+albicollis-albiventris	5	22.72%
Hemimaculata+albicollis-nigriventris	1	4.54%
Burnsi+albicollis-albiventris	2	9.09%
Striata+albicollis-albiventris	7	31.81%
Striata+albicollis-nigriventris	1	4.54%
Striata-hemimaculata+albicollis-albiventris	6	27.27%

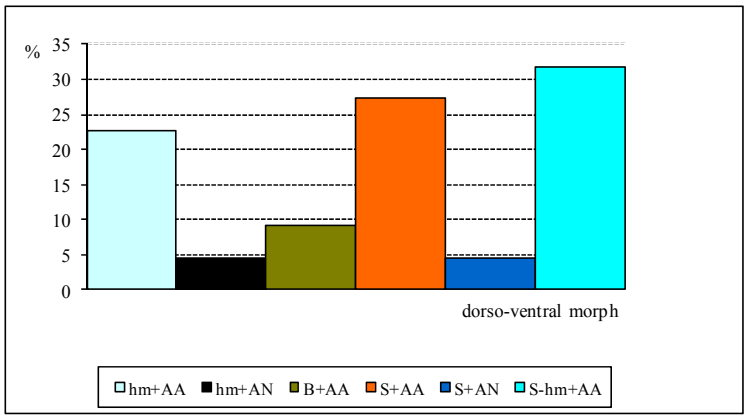

Fig. 4 - Frequency of dorso-ventral chromatic combinations in *Rana esculenta* (L.)

Regarding dorsal chromatic variability in the sample of frogs from the species *Rana ridibunda* (Pall.) of Gheorghiu Lake, it was established the manifestation of 7 distinct morphs, of which 5 are basic morphs and 2 are complex morphs. Regarding the ventral chromatic variability in *Rana ridibunda* (Pall.) population from Gheorghiu Lake there were identified all 4 morphs.

In the entire population of *Rana ridibunda* (Pall.) there are expressed 18 distinct dorso-ventral phenotypes (Table 7, Fig. 5).

Table 7 - Frequency of dorso-ventral chromatic combinations in *Rana ridibunda* (Pall.) from Gheorghiu Lake

Chromatic combination	Number of individuals	Frequency
Maculata+albicollis-albiventris (M+AA)	9	11.53%
Maculata+albicollis-nigriventris (M+AN)	1	1.28%
Hemimaculata+albicollis-albiventris (hm+AA)	18	23.07%
Hemimaculata+albicollis-nigriventris (hm+AN)	2	2.56%
Hemimaculata+nigricollis-nigriventris (hm+NN)	1	1.28%
Hemipunctata+albicollis-albiventris (hp+AA)	1	1.28%
Burnsi+albicollis-albiventris (B+AA)	2	2.56%
Striata+albicollis-albiventris (S+AA)	3	3.84%
Striata+albicollis-nigriventris (S+AN)	4	5.12%
Striata+nigricollis-nigriventris (S+NA)	1	1.28%
Striata+nigricollis-nigriventris (S+NN)	1	1.28%
Striata-maculata+albicollis-albiventris (S-M+AA)	9	11.53%
Striata-maculata+albicollis-nigriventris (S-M+AN)	3	3.84%
Striata-maculata+nigricollis-albiventris (S-M+NA)	2	2.56%
Striata-maculata+nigricollis-nigriventris (S-M+NN)	1	1.28%
Striata-hemimaculata+albicollis-albiventris (S-hm+AA)	17	21.79%
Striata-hemimaculata+albicollis-nigriventris (S-hm+AN)	2	2.56%
Striata-hemimaculata+nigricollis-albiventris (S-hm+NA)	1	1.28%

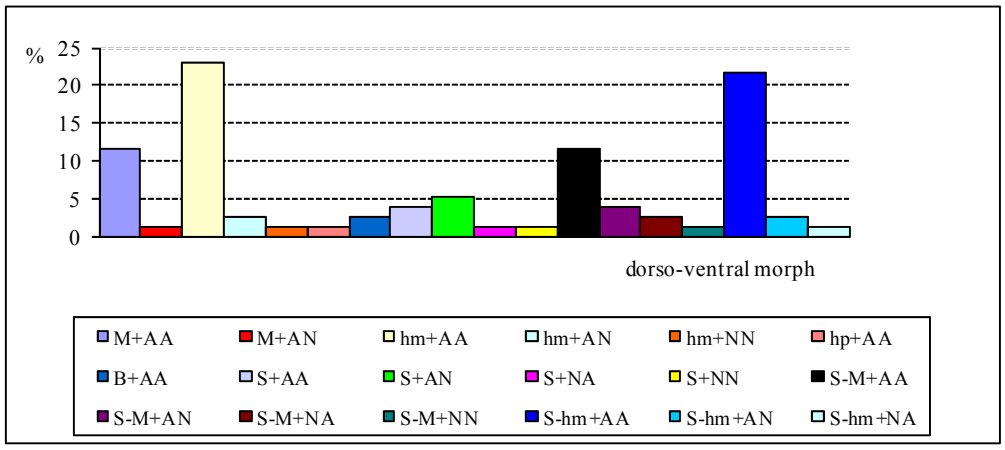

Fig. 5 – The frequency of dorso-ventral chromatic combinations in *R. ridibunda* (Pall.) from Gheorghiu Lake

Analyzing the dorsal chromatic at individuals of the species *Rana esculenta* (L.) from Gheorghiu Lake, we observed the fenotipization of 6 morphs of which, 5 are basic (*maculata*, *hemimaculata*, *hemipunctata*, *burnsi* and *striata*) and one complex (*striata-hemipunctata*). Ventral, it was recorded the fenotipization of only 2 distinct morphs: *albicollis-albiventris* and *nigricollis-nigriventris*. Regarding the dorso-ventral chromatic combinations observed in *Rana esculenta* (L.) population from Gheorghiu Lake, there is a small number of them (Table 8, Fig. 6).

Table 8 - Frequency of dorso-ventral chromatic combinations in *R. esculenta* (L.) from Gheorghiu Lake

Chromatic combination	Number of individuals	Frequency
Maculata+nigricollis-nigriventris (M+NN)	1	4.54%
Hemimaculata+albicollis-albiventris (hm+AA)	1	4.54%
Hemipunctata+albicollis-albiventris	10	45.45%
Burnsi+albicollis-albiventris (B+AA)	2	9.09%
Striata+albicollis-albiventris (S+AA)	3	13.63%
Striata-hemipunctata+albicollis-albiventris (S-hm+AA)	5	22.72%

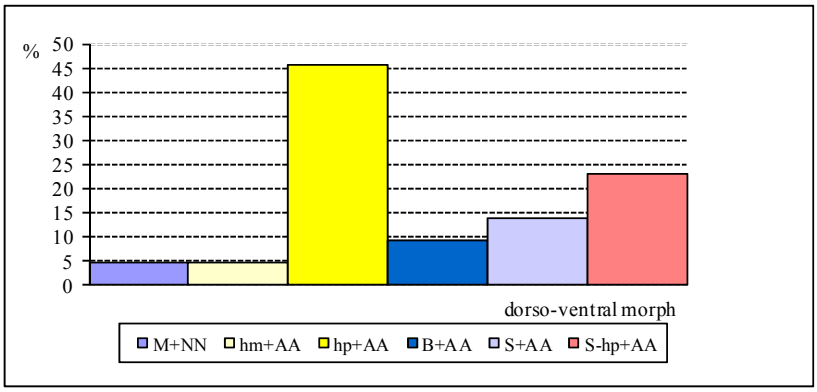

Fig. 6 - Frequency of dorso-ventral chromatic combinations in *R. esculenta* (L.) from Gheorghiu Lake

3.3. The results of cytogenetic investigations

3.3.1. Karyotype and idiograme in *Rana ridibunda* (Pall.)

To accomplish the karyotype and idiograme for *Rana ridibunda* (Pall.) species were used 3 male individuals collected from Gheorghiu Lake. The best metaphases were obtained from processing testicles. After making the microscopic preparations were selected from the entire biological material a total of 10 metaphases, in which the chromosoms were well-spread. For each chromosome were determined separately the following parameters: length of the long arm (q), length of the short arm (p) and total length (L). These measurements were made on both chromatids and then was made the average. We calculated the arm ratio (q/p) and centromeric index (p/L x 100). Based on these measurements and calculations were identified the morphological pairs of chromosomes and their grouping in descending order of size, to achieve *Rana ridibunda* (Pall.) karyotype (Fig. 7, 8).

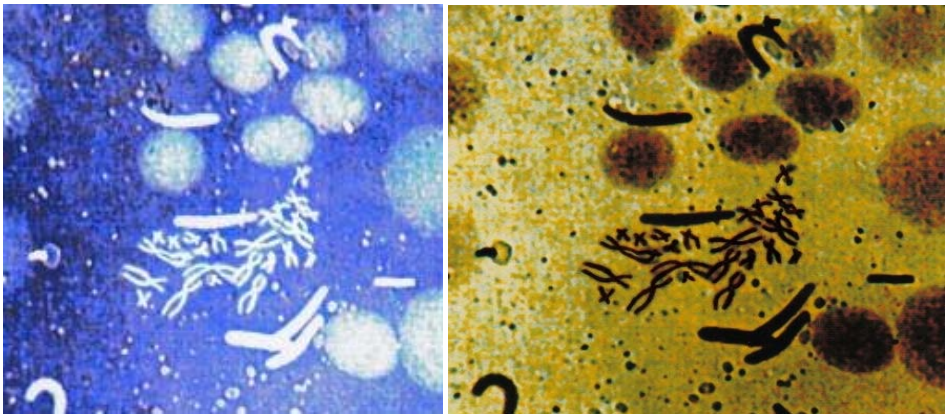

Fig. 7 – Metaphase used in determining *Rana ridibunda* karyotype

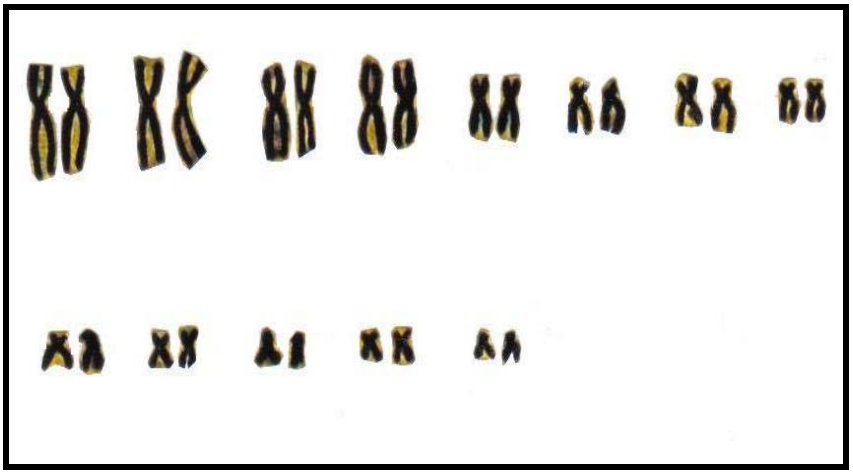

Fig. 8 - *Rana ridibunda* (Pall.) karyotype

Our investigations showed that the diploid number of somatic chromosomes in *Rana ridibunda* (Pall.) species is 26, which confirms the results obtained by other authors: Raicu and Georm㠃neanu (1977), Schmid (1982), Koref-Santibanez and Gunther (1980), Miura (1995), Miura *et al*. (1997), Al-Shehri and Al-Saleh (2005). As did other authors (Raicu and Georm㠃neanu, 1977 Koref-Santibanez and Gunther, 1980; Miura, 1995), *R. ridibunda* (Pall.) chromosomes were classified into two groups: the first group comprises 5 pairs of higher size, and the second, eight pairs of small chromosomes.

For each of the 13 pairs of chromosomes we were determined the minimum, maximum, average and standard error. Based on the average values of the anlyzed parameters (presented in Table 9), it was achieved the idiograme of *Rana ridibunda* Pall. (Fig. 9).

Table 9 - Values of some statistical indicators of metaphase chromosomes in *Rana ridibunda* (Pall.)

	Statistical parameter	Pairs of chromosomes I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XII
Long arm (μm)	Minimum	8.30	6.92	5.67	5.85	4.12	3.70	2.85	2.33	2.70	2.23	2.30	1.62	1.63
	Maximum	9.42	10.35	8.13	7.93	6.03	4.57	5.51	3.83	3.97	3.38	3.96	2.76	3.20
	Average	8.86	8.68	6.49	6.99	5.20	4.18	3.88	3.23	3.37	2.83	2.95	2.16	2.23
	Standard error	0.11	0.46	0.21	0.23	0.23	0.08	0.27	0.13	0.12	0.13	0.16	0.11	0.14
Short arm (μm)	Minimum	4.53	5.16	4.60	3.36	2.47	1.31	1.20	1.33	1.05	1.00	0.45	0.80	0.28
	Maximum	8.33	6.71	5.55	4.88	5.40	2.51	2.73	2.51	1.66	2.01	1.01	1.48	0.72
	Average	6.47	5.97	5.06	4.10	4.08	1.93	1.89	1.84	1.35	1.44	0.75	1.12	0.52
	Standard error	0.50	0.16	0.11	0.15	0.37	0.12	0.16	0.12	0.05	0.10	0.05	0.06	0.04
Overall length (μm)	Minimum	12.13	12.08	10.53	10.65	6.61	5.36	4.69	4.65	4.06	3.25	2.93	2.50	2.11
	Maximum	17.76	17.07	13.70	12.01	11.38	6.70	6.71	5.85	5.52	5.40	5.22	4.25	3.66
	Average	15.28	14.66	11.61	11.21	9.29	6.08	5.77	5.18	4.74	4.29	3.76	3.30	2.76
	Standard error	0.64	0.62	0.30	0.14	0.60	0.16	0.19	0.14	0.15	0.22	0.21	0.17	0.14
	Minimum	1.12	1.29	1.21	1.29	1.10	1.74	1.18	1.32	2.01	1.67	2.88	1.59	3.08

Report arms (μm)	Maximum	1.96	1.60	1.44	2.13	1.66	2.91	2.99	2.47	3.30	2.60	6.37	2.57	6.98
	Average	1.51	1.44	1.27	1.75	1.33	2.27	2.07	1.87	2.50	2.00	3.96	1.94	4.26
	Standard error	0.10	0.04	0.02	0.10	0.07	0.14	0.22	0.14	0.12	0.08	0.32	0.09	0.45
Index centro-meric (μm)	Minimum	35.32	38.41	41.02	31.89	37.49	24.44	25.04	28.75	23.21	27.69	13.55	27.93	11.15
	Maximum	46.86	43.50	45.60	43.49	47.50	36.37	45.69	42.91	32.92	37.37	25.74	38.74	24.40
	Average	41.61	41.00	43.82	36.69	43.20	30.87	34.33	35.46	28.76	33.52	20.83	34.32	20.12
	Standard error	1.69	0.69	0.46	1.42	1.41	1.53	2.70	1.77	0.95	0.94	1.17	1.00	1.48
Type		m	m	m	sm	m	sm	sm	sm	sm	sm	st	sm	st

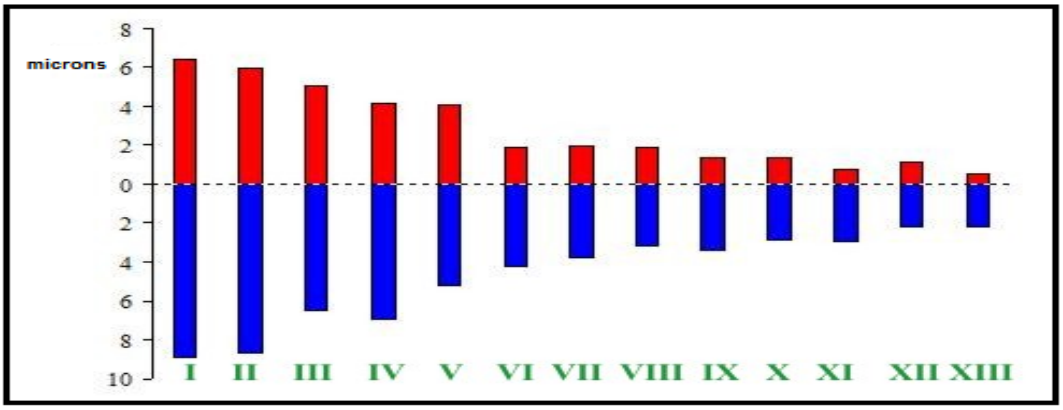

Fig. 9 –Idiograme of the species *Rana ridibunda* (Pall.)

Based on the data obtained, the 13 pairs of homologous chromosomes were classified into 3 morphological groups. Thus, 4 of the pairs are metacentric (pairs 1, 2, 3 and 5), 7 pairs are submetacentric (pairs 4, 6, 7, 8, 9, 10 and 12) and 2 pairs are subtelocentric (pairs 11 and 13). As a percentage, 30.76% of the karyotype specific chromosomes in *R. ridibunda* is composed of metacentric chromosomes, 53.84% of chromosomes are submetacentric and 15.38% are subtelocentric. Our observations are consistent with other observations of this kind from the profile literature. Thus, Mëszaros and Bartos (1978) have shown in the species *Rana ridibunda* karyotype (Pall.) 6 pairs of metacentric chromosomes (1, 4, 5, 6, 7, 11), 5 pairs of submetacentric chromosomes (2, 3, 10, 12 and 13) and 2 pairs of subtelocentric chromosomes (8 and 9). Suryadna (2003) has differentiated at this species 4 pairs of metacentric chromosomes (1, 5, 6 and 7), 7 pairs of submetacentric (2, 3, 4, 10, 11, 12 and 13) and 2 pairs of subtelocentric (8 and 9). But there are other ways in the literature for the classification of chromosomes in the karyotype for the species *R. ridibunda* (Pall.). Thus, Gang *et al*. (1992) divided the karyotype of this species from Xiangjiang region into two morphological groups: 7 pairs of metacentric chromosomes (1, 2, 4, 5, 6, 7 and 11) and 6 pairs of submetacentric chromosomes (3, 8, 9, 10 , 12, 13). Similar proceeded Al-Shehri and Al-Saleh (2005) in a study conducted in Saudi Arabia: 8 metacentric chromosomes (1-7) and 6 submetacentric (8-13). Koref-Santibanez (1980) established a karyotype of this species consisted of 6 pairs of metacentric

chromosomes (1, 4, 5, 6, 7 and 11), 4 pairs of submetacentric (3, 10, 12 and 13), the second pair was labeled as metacentric or submetacentric, and pairs 8 and 9 as submetacentric or subtelocentric.

Average size of metaphase chromosomes from *R. ridibunda* (Pall.) ranged between 15.25 μm (pair 1) and 2.75 μm (pair 13). In Table 10 we present the relative values of metaphase chromosome length compared with published data in other papers.

Table 10 - Relative length of metaphase chromosomes in *Rana ridibunda* (Pall.)

Pair of chromosomes	Author, year			
	Koref-Santibanez, 1797	Wei *et al*., 1992	Suryadna, 2003	Personal data
1	16.69	15.60	16.7	15.25
2	13.72	12.63	13.4	14.66
3	12.41	11.79	12.2	11.61
4	11.79	11.32	11.5	11.07
5	9.74	9.75	9.7	9.29
6	5.80	5.99	5.9	6.08
7	5.01	5.60	5.3	5.76
8	4.93	5.19	4.9	5.18
9	4.44	4.82	4.8	4.74
10	4.54	4.68	4.4	4.22
11	4.12	4.40	4.2	3.76
12	3.57	3.95	3.8	3.30
13	3.00	3.58	3.1	2.75

3.3.2. The study of karyotype and idiograme in *Rana esculenta* (L.)

Karyotype of the species *Rana esculenta* (L.) was conducted using microscopic preparations from the testes of three individuals collected from Gheorghiu Lake. The working protocol followed for the karyotype and idiograme preparation was identical to that used for the species *R. ridibunda* (Pall.).

Microscopic observations indicated the presence in *Rana esculenta* (L.) karyotype of a number of 26 chromosomes (Fig. 10, 11), which confirms the results obtained by other authors (Mëszaros and Bartos, 1978; Koref-Santibanez, 1980; Suryadna, 2003).

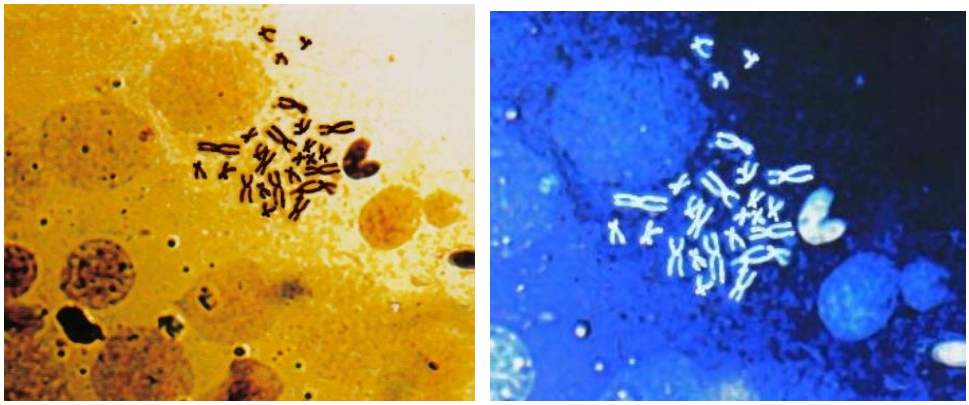

Fig. 10 - Metaphase used to achieve *Rana esculenta* L. karyotype (positive and negative)

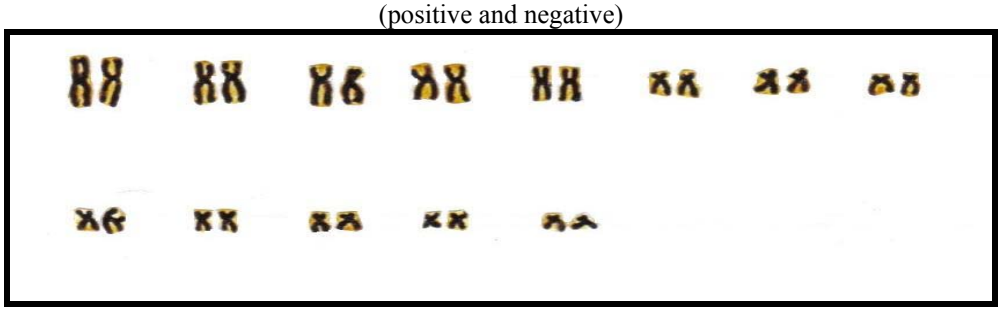

Fig. 11 - *Rana esculenta* (L.) karyotype

We determined the minimum, maximum, average and standard error for each parameter investigated in the 13 pairs of homologous chromosomes from the 10 metaphases selected for *R. esculenta* (L.). Based on the values of arms ratio and centromeric index, the karyotype chromosomes were classified by morphological types. The results are presented in Table 11. Based on the data in the table, it was made the idiograme of *R. esculenta* (L.) species (Fig. 12).

Table 11 - Statistical analysis of some parameters of metaphase chromosomes in *Rana esculenta* (L.)

	Statistical parameter	Pairs of chromosomes												
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XII
Long arm (μm)	Minimum	5.88	5.06	5.75	4.57	3.43	3.19	2.51	2.92	1.69	1.61	1.66	1.16	1.60
	Maximum	8.75	7.35	8.00	6.75	5.36	5.03	4.13	4.30	3.00	2.67	3.05	2.14	2.88
	Average	7.00	6.03	6.46	5.48	4.38	3.97	2.89	3.52	2.25	2.10	2.26	1.54	2.10
	Standard error	0.33	0.30	0.36	0.29	0.22	0.23	0.24	0.15	0.15	0.12	0.16	0.11	0.15
Short arm (μm)	Minimum	3.69	2.21	1.85	2.08	1.15	1.37	0.96	0.61	1.33	1.38	0.59	0.79	0.29
	Maximum	6.61	4.48	3.43	4.17	3.52	2.07	2.53	2.53	2.22	2.51	1.20	1.77	0.67
	Average	4,81	3,17	2,45	2,94	2,13	1,68	1,89	0,99	1,72	1,86	0,79	1,68	0,38
	Standard error	0.32	0.30	0.21	0.28	0.32	0.06	0.13	0.17	0.09	0.12	0.06	0.11	0.04

Overall length (μm)	Minimum	9.52	7.28	7.10	6.65	4.58	4.56	4.14	3.74	3.02	3.00	2.25	1.96	1.90
	Maximum	15.37	11.84	11.44	10.93	8.89	7.11	5.58	5.45	5.22	5.19	4.67	3.92	3.55
	Average	11.80	9.22	8.89	8.41	6.55	5.66	4.81	4.52	3.98	3.94	3.14	2.71	2.48
	Standard error	0.68	0.60	0.58	0.57	0.54	0.29	0.15	0.19	0.24	0.24	0.27	0.23	0.19
Report arms (μm)	Minimum	1.32	1.64	2.33	1.61	1.52	2.04	1.15	2.88	1.23	1.06	2.53	1.20	4.29
	Maximum	1.60	2.31	2.98	2.19	2.97	2.89	1.54	6.13	1.45	1.16	3.58	1.47	6.85
	Average	1.47	1.97	2.68	1.92	2.35	2.35	1.36	4.47	1.29	1.11	2.86	1.34	5.64
	Standard error	0.03	0.08	0.07	0.07	0.21	0.08	0.04	0.28	0.02	0.01	0.09	0.31	0.26
Index centro-meric (μm)	Minimum	38.76	30.35	25.10	32.01	25.13	25.79	39.59	14.18	40.71	46.09	21.80	40.44	12.73
	Maximum	43.01	37.84	30.03	38.21	39.60	32.83	46.42	25.73	44.76	48.52	28.26	45.25	18.87
	Average	40.50	33.88	27.31	34.35	31.08	30.01	42.33	18.80	43.42	47.29	26.01	42.67	15.28
	Standard error	0.46	0.97	0.57	0.79	2.17	0.69	0.78	1.00	0.47	0.31	0.55	0.56	0.62
Type		m	sm	sm	sm	sm	sm	m	st	m	m	sm	m	st

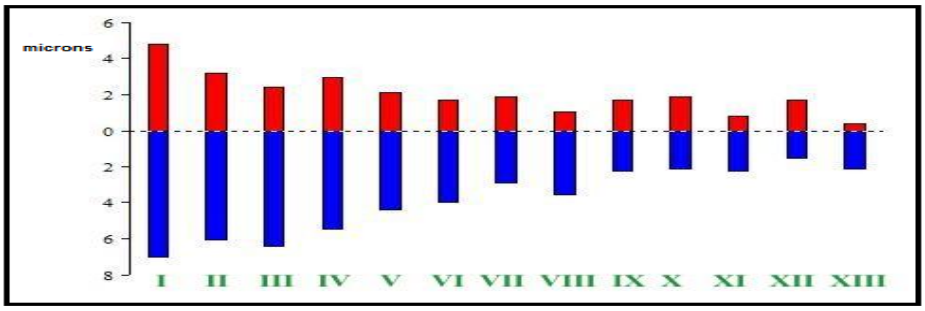

Fig. 12 – Idiograme of the species *Rana esculenta* (L.)

Of the 13 pairs of homologous chromosomes from the karyotype of *Rana esculenta* (L.) species, 5 pairs are metacentric (1, 7, 9, 10 and 12), 6 pairs are submetacentric (2, 3, 4, 5, 6 and 11) and 2 pairs are subtelocentric (8 and 13). Thus, in *Rana esculenta* (L.) karyotype, submetacentric chromosomes represent 46.15%, metacentric chromosomes 38.46%, and subtelocentric 15.38%.

Relative length of metaphase chromosomes for the species *Rana esculenta* (L.) ranges between 11.80 μm (pair 1) and 2.48 μm (pair 13). Comparing the relative values of the total length of metaphase chromosomes obtained by us with the data of other authors, our values are lower for all chromosomes of the karyotype (Table 12).

Table 12 - Relative length of metaphase chromosomes in *Rana esculenta* (L.)

Pair of chromosomes	Author, year			
	Mëszaros and Bartos, 1978	Koref-Santibanez, 1979	Suryadna, 2003	Personal data
1	18.14	16.18	15.5	11.80
2	13.85	13.38	12.6	9.22
3	12.77	12.41	11.9	8.89
4	11.58	11.61	11.2	8.41
5	9.46	9.55	9.6	6.55
6	5.73	6.03	6.3	5.66
7	4.76	5.19	5.7	4.81
8	4.99	4.97	5.5	4.52
9	4.24	4.58	5.3	3.98
10	4.11	4.56	4.7	3.94
11	3.97	4.32	4.5	3.14
12	3.63	3.88	4.0	2.71
13	3.14	3.38	3.3	2.48

Analyzing karyotypes of the two species of green frogs we have found that all chromosomes are homologous and that there is no pair of chromosomes morphological differentiated (heterosomal). Although, in amphibians, it is considered that sexual determinism is due to some masculinized and feminized genes - in interaction and in relation with the environment specific conditions (especially temperature). There are also information that accredit the idea of heterosomal existence in these species. It seems that all amphibians possess one of the mechanisms of genetic determinism of gender specific to vertebrates (Cîrlan and Creangă, 2001). Although much less prevalent than in fish, the hermaphroditism can be also met in amphibians, especially in *Anura* order, the family *Bufonidae*, but also in the family *Ranidae*. In the family of *Bufonidae*, hermaphroditism is not functional, while some species of the family *Ranidae*, such as *Rana temporaria* (L.), the hermaphroditism is a natural sexual status. Hermaphrodites are functional, they can reproduce by self-fertilization or may act as males (Cîrlan and Creangă, 2001). Moreover, in terms of gender genetic determinism in amphibians there are many observations in the specialized literature, of which we will mention a few.

In *Rana esculenta* (L.) there were found individuals in which sexual differentiation occurs in the early stages of ontogenesis, and individuals with late male sexual differentiation, in which case a large number of males were female in early ontogenetic stages, male differentiation being acquired later (Băra and Cîmpeanu, 2003). In the process of conversion from female to male in *Rana esculenta* (L.),the individuals go through a stage of hermaphroditism.

Hillis and Green (1990), quoted by Cîrlan and Creangă (2001), considered that the evolution of sex chromosomes in amphibians was repeated several times, starting with the base model - represented by ZW system. The oldest state appears to be heterogametic female and after the system XY has been developed. A case of reversion from the chromosomal complement XY to ZW was described in *Rana rugosa* (Ogata *et al*., 2003). Heterogametic change involved hiring a different set of autosomes to produce sex chromosomes.

Both in *Anura* and *Urodele* there were remarked the heterogametic males (XY) and heterogametic females (ZW), which leads to the idea of not being able to generalize a particular type of sexual determinism in amphibians. Moreover, the *Rana rugosa* females are XX in some populations and in the other populations have ZW genotype (Miura *et al*., 1998). Research conducted in Japan by Ogata *et al*. (2003) showed that at *Rana rugosa* species exist two types of gender chromosomal determinism (XX/XY and ZZ/ZW) separated in two local populations, indicating a common origin by hybridization with two other forms, both having heterogametic males but homomorphic chromosomes.

The presence of heteromorphic heterosomes was reported by Al-Shehri and Al-Saleh (2005) in a study performed in populations of *Rana ridibunda* (Pall.) of Saudi Arabia. According to this study, in the male karyotype the second pair of chromosomes is represented by the XY heterosomes, and in the female karyotype by XX; the difference between X and Y is given by the greater length of X with about 4 μm.

Lately, however, the experts consider that amphibians possess homomorphic heterosomes that can not be detected by conventional staining methods. Thus, of all species of the *Rana* and *Bufo* genera – in which there were discovered sexual chromosomes type XX/XY or ZZ/ZW, the vast majority have homomorphic chromosomes, (Yang, 2004). Similarly, in a study performed in Iran by Fakharzadeh *et al*. (2009) there were detected the presence of sex chromosomes in *Rana ridibunda* (Pall.), which confirms somehow the view of the existence of a sexual determinism due to the homomorphic chromosome.

The impossibility to differentiate sex chromosomes through classical methods determined the identification of some more selective methods. Thus, it was developed the fluorescent banding technique by incorporating in the cell's DNA 5-bromodeoxyuridine (BrdU), which allows the visualization of replication in S phase of the cell cycle. This technique enables precise identification of all

chromosomes based on the manner of replication (Schempp and Schmid, 1981). Using this method, it was found that the homomorphic chromosomes of the fourth pair from *Rana esculenta* (L.) are involved in gender determinism. In males, one of the chromosomes of the fourth pair has a region with very late replication, compared to the other chromosome in the pair. In females, both chromosomes from the fourth pair replicate synchronous. Thus, although the chromosomes are homomorphic and can not be detected cytologically, they can be considered type XY in males and type XX in females (Schempp and Schmid, 1981; Yang, 2004).

3.4. Variability of some biochemical characters in green frogs

3.4.1. The study of some biochemical compounds in green frogs from Balţii Pond

For the determination of the biochemical constituents in *Rana ridibunda* (Pall.) from Balţii Pond there were used 10 individuals. As we already showed, all analyzed compounds were identified in both liver and muscle cell homogenates and in the blood plasma.

Comparing the results from the two types of cell homogenates, we see that, excepting creatinine, in the liver we recorded higher values of the analyzed indicators. The statistical study showed significant differences between averages (Table 13).

Table 13 – Results of t-Test applied to the values of some biochemical indices of cell homogenates from *R. ridibunda*

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average mg/100g wet tissue	Liver	211.95	1833.5	66.8	9265	145.05	0.865
	Muscle	158.1	60	35.2	4740	18.1	1.94
Statistical t		2.010	27.003	2.148	5.935	6.275	-4.151
P		0.032	$3.1x10^{-10}$	0.026	$4.9x10^{-5}$	$7.2x10^{-5}$	0.0003
Critical t		1.770	1.833	1.782	1.795	1.833	1.734
α		0.05	0.05	0.05	0.05	0.05	0.05

From the data presented in Table 14, obtained from the analysis of the same biochemical components from blood plasma, we observed a greater dispersion of the data, in the case of carbohydrates and cholesterol, while triglycerides and creatinine are characterized by a reduced quantitative variability. For carbohydrates, the range of variation is between 28 mg/dl and 88 mg/dl, with a mean of 52±6.63 mg/dL, and for cholesterol limits are between 14.4 mg/dl and 54 mg/dl, with a mean of 29.02±4.74 mg/dl. Triglyceride levels ranged from 2.8 mg/dl and 6.5 mg/dl, with a mean of 3.94±0.41 mg/dl. The narrow range of variation has been detected for creatinine, which has a mean value of 0.22±0.02 mg/dl.

Table 14 – Content of some organic compounds in *R. ridibunda* plasma (Balţii Pond)

	Biochemical constituents (mg/dl)						
		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Statistical parameters	Minimum	14.4	28	2.8	13.9	3.1	0.17
	Maximum	54	88	6.5	24.7	13.3	0.34
	Average	29.02	52	3.943	17.4	6.87	0.216
	Median	22.85	50	3.6	16.6	6.15	0.195
	Standard deviation	14.984	20.986	1.289	3.200	3.331	0.050
	Standard error	4.738	6.636	0.407	1.012	1.053	0.016

The sample for the biochemical analysis in *Rana esculenta* (L.) from Balţii Pond consisted of 10 individuals. The results showed that the average values of the investigated indices varied depending on the analyzed tissue.

According to t-Test, only for cholesterol the averages are statistically equal in the two cell homogenate. For triglycerides and creatinine the averages are higher in muscle, while for the other analyzed compounds, the averages are higher in liver (Table 15).

Table 15 – Results of t-Test for compounds dosed in cell homogenates in *R. esculenta*

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average mg/100g wet tissue	Liver	111.3	2208.7	69.4	6475	306.2	0.775
	Muscle	109.7	27.5	88.2	3337.5	88.37	1.3
Statistical t		0.220	6.477	-4.079	7.121	10.378	-2.659
P		0.419	0.003	0.003	0.002	$2.3x10^{-5}$	0.018
Critical t		2.353	2.353	1.943	2.353	1.943	1.943
α		0.05	0.05	0.05	0.05	0.05	0.05

In *R. esculenta* (L.) plasma, the mean values were: for cholesterol - 29.67 mg/dl, for carbohydrates - 110.25 mg/dl, for triglycerides - 21.17 mg/dl, for proteins - 13.32 mgdl, for urea - 15.40 mg/dl and for creatinine 0.2 mg/dl (Table 16).

Table 16 - Values of some biochemical compounds in *R. esculenta* plasma (Balţii Pond)

	Biochemical constituents (mg/dl)						
		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Statistical parameters	Minimum	26.1	102	10.8	12.9	14.9	0.1
	Maximum	34.1	121	38.5	13.6	15.9	0.3
	Average	29.675	110.75	21.175	13.325	15.4	0.2
	Median	29.25	110	17.7	13.4	15.4	0.2
	Standard deviation	3.392	7.973	12.121	0.340	0.439	0.008
	Standard error	1.696	3.986	6.060	0.170	0.219	0.040

We performed a comparative study based on the averages of biochemical constituents for every tissue and every species. We used the statistical method of the Student test and analysis of variance (Fisher-Snedecor test or F test). The results of this comparative study are presented, separately for each tissue, in Tables 17-19.

Table 17 – Results of statistical tests for liver biochemical compounds on the two species of frogs from Balţii Pond

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average	*R.ridibunda*	211.95	1833.5	66.8	9625	145.05	0.865
	R.esculenta	111.37	2208.7	69.37	6475	306.25	0.775
Statistical F		0.036	10.673	0.019	0.127	0.299	0.152
P-F test		0.009	0.002	0.003	0.058	0.174	0.074
Critical F		0.113	3.862	0.113	0.113	0.113	0.113
Statistical t		-3.961	1.093	0.185	-2.447	4.691	-0.254
P-t test		0.001	0.176	0.428	0.015	0.0002	0.401
Critical t		1.812	2.353	1.812	1.782	1.782	1.782
α		0.05	0.05	0.05	0.05	0.05	0.05

Table 18 – Results of statistical tests for muscle biochemical compounds in the two species of frogs from Balţii Pond

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average	*R.ridibunda*	158.1	60	35.2	4740	18.1	1.94
	R. esculenta	109.75	24.47	88.25	3337.5	88.37	1.3
Statistical F		0.002	0.006	0.155	0.003	208.95	0.397
P-F test		0.0002	0.0008	0.076	0.0002	$1.2x10^{-8}$	0.241
Critical F		0.113	0.113	0.113	0.113	3.862	0.113
Statistical t		-4.287	-4.052	5.649	-5.202	6.044	-2.512
P- t test		0.001	0.001	$5.3x10^{-5}$	0.0002	0.004	0.013
Critical t		1.833	1.833	1.782	1.833	2.353	1.782
α		0.05	0.05	0.05	0.05	0.05	0.05

Table 19 – Results of statistical tests for plasma compounds in the two species of frogs from Balţii Pond

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average	*R. ridibunda*	29.02	52	3.94	17.4	6.87	0.216
	R. esculenta	29.67	110.75	21.17	13.32	15.4	0.2
Statistical F		0.051	0.144	88.292	0.011	0.017	2.581
P-F test		0.016	0.069	$5.3x10^{-7}$	0.001	0.003	0.118
Critical F		0.113	0.113	3.862	0.113	0.113	3.862
Statistical t		0.130	5.336	2.836	-3.970	7.927	-0.450
P- t test		0.449	$8,8x10^{-5}$	0.032	0.001	$6.3x10^{-6}$	0.330
Critical t		1.795	1.782	2.353	1.833	1.812	1.782
α		0.05	0.05	0.05	0.05	0.05	0.05

According to the above, we can say that in *R. ridibunda* liver (Pall.) the quantity of cholesterol is higher than in the liver of the species *R. esculenta* (L.). Also, the protein level from *R. ridibunda* (Pall.) liver is higher than the level of liver protein for *R. esculenta* (L.) species, according to the results offered by t-Test. Although we could say that cholesterol and proteins are characteristic for the liver tissue at the species *R. ridibunda* (Pall.), the analysis of the average of urea in the two species showed a statistically significant difference in favor of the species *R. esculenta* (L.). According to the results of the comparative applied tests for the muscle homogenate, we found statistically significant differences for all six analyzed biochemical constituents. The result of these tests confirm higher averages for cholesterol, carbohydrates, protein and creatinine in *R. ridibunda* (Pall.), and higher averages for triglyceride and urea in *R. esculenta* (L.).

Significant differences between the averages appear also in the plasma for carbohydrates, triglycerides, proteins and urea. Thus, glycemia, uremia and triglyceride levels are higher in *R. esculenta* (L.) compared with *R. ridibunda* (Pall.), while proteinemia is higher in *R. ridibunda* (Pall.).

3.4.2. The study of some biochemical compounds in green frogs from Gheorghiu Lake

Biochemical analyzes were performed on 8 individuals of *R. ridibunda* (Pall.). The tests also targeted the dosing of the same biochemical constituents in liver, muscle and plasma cell homogenates.

In the liver homogenate from *R. ridibunda* (Pall.) stands the elevated values for carbohydrates (1345.63 mg/100g wet tissue), and in muscle homogenates are missing the triglycerides and were found large variations of protein and cholesterol, but lower variations of creatinine. The largest variability limits were recorded for the proteins in the muscle homogenates, of 4000 and respectively 6000 mg/100 g wet tissue.

The comparative analysis of liver and muscle homogenates from *R. ridibunda* (Pall.) indicated mean values statistically equal only for cholesterol. Carbohydrates, protein and urea have higher mean values in the liver tissue, and creatinine in muscle (Table 20).

Table 20 – Results of t-Test applied to compounds dosed in cell homogenates to *R. ridibunda*

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea
Average mg/100g wet tissue	Liver	125.5	1345.6	7075	242.9	0.312
	Muscle	153.3	73.12	5193	31.8	2.1
Statistical t			14.836	3.155	12.990	-10.417
P			$2.1x10^{-7}$	0.003	$1.8x10^{-6}$	$2.8x10^{-8}$
Critical t			1.859	1.761	1.894	1.761
α			0.05	0.05	0.05	0.05

The biochemical analysis performed in the plasma of individuals within *Rana ridibunda* (Pall.) species, revealed the existence of all six investigated constituents (Table 21).

Table 21 - Values of some biochemical compounds in *R. ridibunda* plasma (Gheorghiu Lake)

	Biochemical constituents (mg/dl)						
		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Statistical parameters	Minimum	25.9	39	5	21.2	2.1	0.2
	Maximum	78.1	117	10.9	39.6	6.5	0.65
	Average	58.475	86.125	7.587	29.475	4.075	0.388
	Median	61.85	91.5	7.15	30.1	3.7	0.375
	Standard deviation	16.891	30.324	2.016	6.791	1.333	0.135
	Standard error	5.972	10.721	0.713	2.401	0.471	0.048

According to the results obtained from the plasma analyzes, the biochemical constituents with the largest variability limits are carbohydrates and cholesterol. For carbohydrates, the upper limit determined in serum is 117 mg/dl, and the lower 39 mg/dl, with a mean of 86.13±10.72 mg/dl. For cholesterol, the maximum was 78.1 mg/dl, and the minimum 25.9 mg/dl with a mean of 58.48±5.97 mg/dl. The narrower ranges of variability, and hence the low dispersion around the average, were recorded for plasma urea and creatinine: 6.5 mg/dl and 2.1 mg/dl, with a mean of 4.08±0.47 mg/dl for urea, and respectively 0.65 mg/dl and 0.2 mg/dl, with a mean of 0.39±0.05 mg/dl for creatinine.

In the samples of *R. esculenta* (L.) from Gheorghiu Lake, the analyzes performed in liver homogenates indicated the highest level for protein, with a mean value of 5275 mg/100g wet tissue, and the lowest for creatinine, 0.337 mg/100g wet tissue. In the case of muscle cell homogenate was found the absence of triglycerides and a high protein level.

Comparing the results of analyzes performed in cell homogenates from *Rana esculenta* (L.) we observed differences in the content of triglycerides. While in the liver they are present in all individuals, in muscle they are absent. For the other compounds, the comparative analysis indicated average values close for protein, higher average values for carbohydrates and urea in the liver and higher mean values for cholesterol and creatinine in muscle (Table 22).

Table 22 – Results of t-Test applied to biochemical compounds in cell homogenates of *R. esculenta* (L.)

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea
Average mg/100g wet tissue	Liver	89.875	710	5275	213.6	0.337
	Muscle	160.875	42.5	5312	47	1.65
Statistical t		-4.064	3.848	-0.057	7.131	-2.980
P		0.007	0.015	0.477	0.0001	0.020
Critical t		2.131	2.353	1.943	1.943	2.131
α		0.05	0.05	0.05	0.05	0.05

In the plasma *of Rana esculenta* (L.) individuals collected from Gheorghiu Lake, the analyses indicated the presence of all investigated biochemical constituents (Table 23).

Table 23 - Values of biochemical compounds in the plasma of *R. esculenta*, (Gheorghiu Lake)

	Biochemical constituents (mg/dl)						
		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Statistical parameters	Minimum	27	96	13	16	4,5	0.24
	Maximum	78.2	127	64.6	38.4	6.1	0.83
	Average	40.625	110	27.1	23.675	5.45	0.447
	Median	38.65	108.5	19.4	20.15	5.6	0.36
	Standard deviation	25.071	13.098	25.034	10.033	0.692	0.264
	Standard error	12.535	6.544	12.517	5.016	0.347	0.132

The data in Table 23 show that the limit of dispersion for cholesterol values in blood plasma ranged between 78.2 mg/dl and 27 mg/dl (with an average of 40.63±12.54 mg/dl), and for triglyceride, from 64.6 mg/dl to 13 mg/dl (with a mean of 27.1±12.52 mg/dl). Urea and creatinine had values who varied in lower limits: 6.1 - 4.5 mg/dl urea (an average of 5.45±0.35 mg/dl) and 0.83 - 0.24 mg/dl for creatinine (an average of 0.45±0.13 mg/dl).

To compare biochemically the two species of frogs from Gheorghiu Lake it was applied the Fisher-Snedecor test, in order to determine the variances. Based on this and the t-Test we compared the averages of the samples. The results are shown in Tables 24 - 26.

Table 24 – Results of statistical tests for liver biochemical compounds on the two species of frogs from Gheorghiu Lake

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average	*R. ridibunda*	125.5	1345.62	158.687	7075	242.93	0.625
	R. esculenta	89.875	710	104.125	5275	213.62	0.337
Statistical F		0.058	2.130	0.009	0.372	0.535	0.865
P-F test		0.020	0.184	0.001	0.224	0.327	0.454
Critical F		0.112	4.346	0.112	0.112	0.112	0.107
Statistical t		-2.264	-3.785	-1.390	-2.300	-1.131	-1.528
P- t test		0.026	0.001	0.103	0.022	0.142	0.088
Critical t		1.859	1.812	1.894	1.812	1.812	1.943
α		0.05	0.05	0.05	0.05	0.05	0.05

Table 25 – Results of statistical tests of muscle biochemical compounds in two species of frogs from Gheorghiu Lake

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea
Average	*R. ridibunda*	153.312	73.125	5193.75	31.875	2.1
	R. esculenta	160.875	42.5	5312.5	47	1.65
Statistical F		0.378	0.284	1.117	32.286	7.765
P-F test		0.228	0.164	0.404	0.0001	0.012
Critical F		0.112	0.112	4.346	4.346	4.346
Statistical t		0.252	-1.081	0.208	0.917	-1.035
P-t test		0.403	0.152	0.419	0.213	0.188
Critical t		1.812	1.812	1.812	2.353	2.353
α		0.05	0.05	0.05	0.05	0.05

Table 26 – Results of statistical tests of plasma compounds in two species of frogs from Gheorghiu Lake

Statistical parameters		cholesterol	carbohydrates	triglyceride	protein	urea	creatinine
Average	*R. ridibunda*	58.47	86.12	7.58	29.47	4.075	0.388
	R. esculenta	40.625	110	27.1	23.675	5.45	0.447
Statistical F		2.203	0.186	154.096	2.182	0.271	3.784
P-F test		0.175	0.097	$9.3x10^{-7}$	0.178	0.155	0.066
Critical F		4.346	0.112	4.346	4.346	0.112	4.346
Statistical t		-1.479	1.478	1.556	-1.198	1.904	0.521
P- t test		0.084	0.084	0.108	0.129	0.043	0.306
Critical t		1.812	1.812	2.353	1.812	1.812	1.812
α		0.05	0.05	0.05	0.05	0.05	0.05

The values recorded in the liver homogenates for the two green frog species show statistically significant differences for cholesterol, carbohydrates and protein. Thus, the averages of the three compounds are higher in *Rana ridibunda* (Pall.) comparative with *Rana esculenta* (L), with an error of 0.05.

In the case of muscle homogenates, although the mean values of analyzed biochemical compounds are different, they are not statistically significant. Therefore, for the studied species, are no differences regarding the content of cholesterol, carbohydrates, protein, urea and creatinine in muscle.

In plasma, our investigations indicated significant differences only on urea. Thus, the applied F-test showed equal variances in both species samples and the homoscedastic t-Test confirms a higher average in *Rana esculenta* (L.) compared with *Rana ridibunda* (Pall.).

Conclusions

The study of some morphological, cytogenetic, physiological and biochemical characters in two species of green frogs, *Rana ridibunda* (Pall.) and *Rana esculenta* (L.) from two populations (ponds) located near Dorohoi town (Botosani County) showed a significant variability of these characters depending on species, habitat, analyzed indices etc, as follows:

1. Individuals of both species of green frogs and ponds showed significant variability of the morphological characters investigated. The variability of these characters was higher in the population of *Rana ridibunda* (Pall.) from Gheorghiu Lake in comparison with the same species population of Balţii Pond. The analyzed biometric indices recorded higher averages in *R. ridibunda* than in *R. esculenta*;

2. The chromatic intra-populational variability was evident in both aquatic basins, and more intense in Gheorghiu Lake. The chromatic polymorphism is more pronounced in *Rana ridibunda* (Pall.) than in *Rana esculenta* (L.) in both basins. Our observations showed a tendency of fenotipization for the ventral morph completely depigmented (*albicollis-albiventris*);

3. In both species of frogs we identified a total number of chromosomes 2n = 26 (NF = 52). For the species *Rana ridibunda* (Pall.) the chromosomal formula comprised 4m + 7sm + 2st, and for the species *Rana esculenta* (L.) included 5m + 6sm + 2st. The total relative length of metaphase chromosomes was higher in *Rana ridibunda* (Pall.) than in *Rana esculenta* (L) for pairs 1, 2, 3, 4, 5, 7, 8, 9, 11, 12, and was not significantly different for pairs 6, 10 and 13;

4. The gender determinism is achieved in both species of chromosomes that can not be identified morphologically by classical methods of staining (homomorphic chromosomes);

5. At the species *Rana ridibunda* (Pall.), the analysis of some biochemical compounds indicated the presence of cholesterol, carbohydrates, triglycerides, proteins, urea and creatinine both in liver and muscle tissues cell homogenates, as well as blood plasma. Triglycerides were absent in muscle homogenates of both species in Gheorghiu Lake;

6. The content of biochemical compounds analyzed in both species was much lower in blood plasma than in liver and muscle tissues. The level of biochemical parameters was different depending on habitat: the populations of *Rana ridibunda* (Pall.) of the two ponds were distinguished by the values of all biochemical parameters analyzed in plasma, and the populations of *Rana esculenta* (L.) were distinguished by the values of these parameters in the liver tissue.

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