Bulgarian Journal of Agricultural Science, 20 (No 5) 2014, 1202-1210
Agricultural Academy

CHANGES IN THE BASIC MORPHOPHYSIOLOGICAL PARAMETERS IN THE POPULATIONS OF *PELOPHYLAX RIDIBUNDUS* (AMPHIBIA: RANIDAE) FROM ANTHROPOGENICALLY POLLUTED BIOTOPES IN SOUTHERN BULGARIA. PART 1

Zh. M. ZHELEV[1*], G. S. POPGEORGIEV[2] and N. H. MEHTEROV[3]
[1]*University of Plovdiv "Paisii Hilendarski", Faculty of Biology, Department of Human Anatomy and Physiology, BG – 4000 Plovdiv, Bulgaria*
[2] *Bulgarian Society for the Protection of Birds (BSPB), BG – 4000 Plovdiv, Bulgaria*
[3] *Medical University, Department of Biology, BG – 4000 Plovdiv, Bulgaria*

Abstract

ZHELEV, Zh. M., G. S. POPGEORGIEV and N. H. MEHTEROV, 2014. Changes in the basic morphophysiological parameters in the populations of *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in southern Bulgaria. Part 1. *Bulg. J. Agric. Sci.,* 20: 1202-1210

This study was conducted with the purpose to assess the condition of some vital organs (heart, spleen, kidney and gonads) of *Pelophylax ridibundus* populations that live in conditions of anthropogenic pollution in biotopes in southern Bulgaria, through analysing their relative weights: cardiosomatic index (CSI), spleenosomatic index (SSI), renosomatic index (RSI) and gonadosomatic index (GSI). Populations from three biotopes were investigated: a relatively clean biotope (the control) and two polluted biotopes (with domestic sewage pollution and heavy metal pollution). We found that in both sexes, in conditions of pollution, the relative weight of the heart, spleen and kidney increased. In the population from biotope with heavy metal pollution, the relative weight of gonads was bigger.

Key words: Anthropogenic pollution, *Pelophylax ridibundus*, cardiosomatic index, spleenosomatic index, renosomatic index, gonadosomatic index

Introduction

Currently, environmental pollution is one of the global environmental problems occurring in all over the world. Today, the sources of pollution of anthropogenic origin, such as household waste, oil spills, fertilizers and pesticides that are used in farming, and wastewater from industrial plants cause many ecological disasters. They cause not only deterioration of habitats, but also massive reduction of the number of biota and even the extinction of entire species. This is particularly true for amphibians – a highly vulnerable group, whose populations have declined all over the world in the end of last and the beginning of this century (Garey and Bryant, 1995; Blaustein et al., 2003; Stuart et al., 2004). Nevertheless, among the representatives of Anura there are species which can adapt surprisingly well to anthropogenically transformed environment, they reproduce successfully and are abundant. This enables research that help not only to uncover the mechanisms of their survival, but it also offers opportunities for biomonitoring analyzes for assessing the extent of anthropogenic risk and environmental damage. The marsh frog *Pelophylax ridibundus* Pallas, 1771 (Speybroeck et al., 2010) is such a species, of big range in Eurasia, with high number and density of population, and in many countries (including Bulgaria: 41°14′-44°13′ N and 22°21′-28°36′ E, south-eastern part of the area) it is not under a special regime of conservation. From the complex European green frogs (*Pelophylax* kl. *esculentus* Linnaeus, 1758), the marsh frog *P. ridibundus* is a widely spread species in Bulgaria and it inhabits throughout the country; *P. esculentus* Linnaeus, 1758 can be found in the north – along the Danube, while *P. lessonae* Camerano, 1882, despite its presumable presence, has not been reliably documented (Biserkov et al., 2007; Stoyanov

---

Corresponding author: zhivko-m@uni-plovdiv.bg

et al., 2011). This amphibian species is very attached to the water basin (rarely leaves it and usually spend their lives not far from the breeding place), they can undergo high levels of urbanization (Vershinin, 2007, 2008) and pollution (Vogiatzis et al., 1998; Misyura and Marchenkovskaya, 2001; Misyura et al., 2008; Peskova, 2001; Zhelev, 2007, 2012a), which makes it work as a very good bioindicator (Loumbourdis and Vogiatzis, 2002; Papadimittriou and Loumbourdis, 2003; Korzh et al., 2012; Zhelev et al., 2013a, 2014).

One of the objective and sufficiently reliable methods for assessing the impact of anthropogenic pollution on the populations of amphibians is the method of morpho-physiologic indicators, proposed in the 1960s (Schwartz et al., 1968). As far as the method requires animal killing, it imposes certain restrictions, but today it is up to date and used in the countries, where there are representatives of amphibians, allowing its practical application.

The purpose of this study was to assess the condition of basic organs of *P. ridibundus* (heart, spleen, kidney and gonads), through analyzing their relative weight, by calculating the respective indices) of the populations that inhabit biotopes in southern Bulgaria with different levels and different kinds of anthropogenic pollution.

The research work is part of a large study conducted in the period 2009-2012 in the biotopes along the two most polluted river ecosystems in Bulgaria: the rivers Sazliyka and Topolnitsa. It also monitored changing the integral indicator for developmental stability – fluctuating asymmetry (Zhelev et al., 2012), as well as the changes in basic quantitative and qualitative haematological parameters (Zhelev 2012b; Zhelev et al., 2013b) in *P. ridibundus* populations.

Materials and Methods

The area of investigation

The samples for our study were collected in the spring of 2012 in three biotopes (for convenience labelled as 1, 2 and 3) located along the courses of two rivers in Southern Bulgaria: Sazliyka River in its top part near the village of Rakitnitsa – biotope 1, in its middle part in the region of the town of Radnevo – biotope 2 and Topolnitsa River near the village of Poibrene, after the flowing of Medetska River into the Topolnitsa water reservoir – biotope 3 (Figure 1).

Data from physicochemical analysis done in the water ecosystems

Average annual data about the water basins for 2012, from the reports of the Basin Directorate for water management – East Aegean Region – Plovdiv, Ministry of the Environment and Waters (http://www.bg-ibr.org) are presented in Table 1. Physicochemical monitoring is based on 21 indicators monitored. Annual averages of physicochemical analyses for the period 2009-2011 in each of the biotopes are presented in Zhelev et al. (2013b).

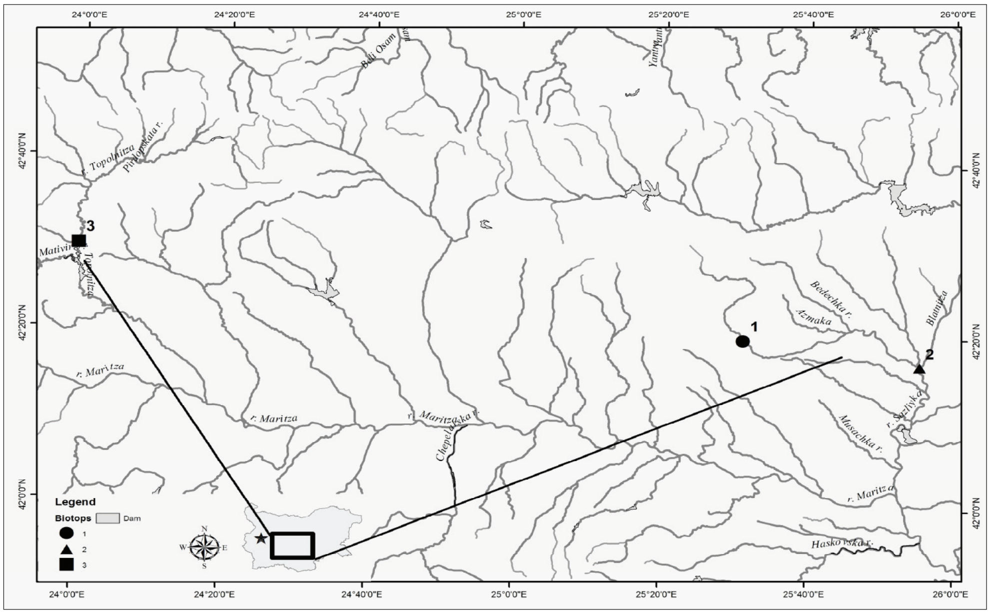

Fig. 1. Geographical location of the water ecosystems. Biotopes:
1 – Sazliyka River below the village of Rakitnitsa; 2 – Sazliyka River below the town of Radnevo;
3 –Topolnitsa River below the village of Poibrene

Table 1

Recent data on the biotopes at the time of the study (physicochemical analysis – surface water sample). The river Sazliyka below the village of Rakitnitsa – biotope 1 and below the town of Radnevo – biotope 2; The river Topolnitsa below the village of Poibrene – biotope 3)

Parameters	Units SI	Order №7/8.8.1986 categories			Biotopes 2012		
		I	II	III	1	2	3
pH	pH units	6.5-8.5	6.0-8.5	6.0-9.0	8.2	7.7	8
Temperature	°C	to 3° middle of the season			15.1	14.9	11.1
Insoluble substances	mg/dm^3	30	50	100	5	42.2	6.62
Electroconductivity	μS/cm	700	1300	1600	867	613	602
Dissolved oxygen	mgO$_2$/dm^3	6	4	2	7.23	5.3	5.55
Oxygenation	%	75	40	20	85.3	58.2	53
Biological oxygen demand. five days - BOD$_5$	mgO$_2$/dm^3	5	15	25	3.53	17.2*	3.3
Chemical oxygen demand - COD	mgO$_2$/dm^3	25	70	100	5.4	5.8	6.2
Nitrate ammonium N-NH$_4$	mg/dm^3	0.1	2	5	0.16	4.4*	0.02
Nitrate nitrogen N- NO$_3$	mg/dm^3	5	20	10	1.1	1.2	0.2
Nitrite nitrogen N-NO$_2$	mg/dm^3	0.002	0.04	0.06	0.002	0.2**	0.001
Orthophosphates P	mg/dm^3	0.2	1	2	0.022	0.433	0.302
Total nitrogen	mg/dm^3	1	5	10	1.95	5.3*	0.543
Total phosphorus - as P	mg/dm^3	0.4	2	3	0.28	0.42	0.32
Sulphates (SO$_4^{2-}$)	mg/dm^3	200	300	400	24.32	54.8	212
Iron - total (Fe)	mg/dm^3	SKOS – 0.1			< 0.001	< 0.001	0.20"
Manganese (Mn)	mg/dm^3	SKOS – 0.05					0.33"
Copper (Cu)	mg/dm^3	SKOS – 0.022					0.031"
Arsenic (As)	mg/dm^3	SKOS – 0.25					0.003
Lead (Pb)	mg/dm^3	SKOS – 0.0072					0.015"
Nickel (Ni)	mg/dm^3	SKOS – 0.02					0.09"

* – above TLV for category II, ** – above TLV for category III, ″ – above SKOS (assessment index) – very poor condition

There is no data about the anthropogenic pollution in Biotope 1 (all 21 indicators are within the standards for Category I (clean) and Category II (slightly polluted) water basins, in accordance with Order №7/8.8.1986 (State Gazette, № 96. 12. 12. 1986); in our study it is viewed as control. The other two biotopes 2 and 3 are polluted. In the biotope 2, the main pollution is of sewage-domestic type, and the biotope 3 is polluted with heavy metals.

Subject of study and methods for analyses

The subject of our study was the marsh frog *P. ridibundus*. *P. ridibundus* is listed in Appendix 4 of the Bulgarian Biodiversity Act (Prom. SG. 77, August 9th 2002). According to Article 42, Article 41 and Appendix 2 for Article 41 of the same law (permit regular catch and use a species of food including in possession of a valid ticket for hunting-fishing for the current year), there are no capture permits issued for *P. ridibundus* if in use for scientific research. Catching of animals we did once, at the beginning of the breeding period (early spring) as follows: in the evening, after sunset, in each of the biotopes we illuminated a spot of several square meters with 2 Petromax lanterns placed on the shore. Then in the illuminated area we caught adult individuals (at roughly measuring SVL > 60.0 mm) in the water (using a fish net) and on land, along the bank. The catch was selective in terms of quantity and sex – aiming for a roughly equal sample in each of the three biotopes. We separated them by sex, basing on secondary sexual characteristics: resonator bubbles in the corners of the mouth and "marital corns" on the first finger in male individuals. We caught a total of 202 animals distributed as follows: Biotope 1 – 75 individuals [39♂, 36♀ (15.04.2012)]; Biotope 2 – 65 individuals [31♂, 34♀ (19.04.2012)]; Biotope 3 – 62 individuals [31♂, 31♀ (3.05.2012)]. One day after the catch the animals were transported in buckets of water (to reduce stress) to the laboratory.

The Ethics Board for Experimental Animals, Faculty of Biology at Plovdiv University, approved the animal handing and laboratory methodology. According to the ethical standards for handling of animals (Steven et al., 2004), the live frogs had been aneasthesed with ether (according to Stetter, 2001). We used a calliper to measure the Snout-Vent Length (SVL) with an accuracy of 0.1 mm (Bannikov et al., 1977) and we weighed the animal – body weight (BW). After the animals had been euthanized by dipping them into 0.5% MS-222 solution, we removed the organs, drained (drying) them with filter paper and weighed them on electronic weighing scales with an accuracy of 0.001g: heart weight (HW), spleen weight (SW), kidney weight (KW) and gonads weight (GW). Using the formula: organ weight x 100/body weight, we calculated the following indices: cardiosomatic index (CSI), spleenosomatic index (SSI), renosomatic index (RSI), gonadosomatic index (GSI): ovarian somatic index (OSI) and testis somatic index (TSI).

Statistical procedures

Mathematical data processing was performed using standard statistical procedures with STATISTIKA, Release 7.0 software (Statistika, 2004). The normalcy in the distribution and homogeneity of variance of the examined morphophysiological parameters was checked by using a Shapiro-Wilk test (Shapiro et al., 1968) that indicated normal distribution: $p>0.05$. The statistical reliability of the differences in values of morphophysiological parameters received for individuals of both sexes in the biotopes compared was proved with a one-way ANOVA. LSD test was used as a post-hoc test. Results with $p<0.05$ [$\alpha=5\%$] were considered significant.

Results

To facilitate the commentary in the work, each sample from the population inhabiting the corresponding biotope was marked with its number. All the animals tested were adult: SVL (cm) [population 1 (♂6.76±0.10, ♀7.76±0.14), population 2 (♂7.53±0.18, ♀9.47±0.28), population 3 (♂6.71±0.13, ♀8.16±0.23,] and BW (g) [population 1 (♂56.91±1.89, ♀61.81±1.83), population 2 (♂61.54±1.62, ♀65.78±1.88), population 3 (♂31.43±1.53, ♀44.22±2.42)].

Some of the basic statistical indicators and descriptive statistics of the morphophysiological parameters in *P. ridibundus* populations and the statistically reliable differences found among the populations for each of the studied parameters are presented in Table 2 and Figures 2, 3, 4 and 5.

For each of the morphophysiological parameters studied we made comparison to certify the statistically significant differences in *P. ridibundus* populations (post-hoc LSD test) from the three biotopes as follows: a) between individuals of both sexes in each of the three populations; b) between individuals of the respective sexes (♂/♂ and ♀/♀) from the populations in the polluted biotopes (2 and 3) and the control group (1); c) between individuals of the respective sexes from the populations in the biotopes with different type of toxins – 2 (Domestic sewage polution) / 3 (Heavy metal pollution). Our findings are presented below:

Heart

a) In the population from the relatively clean biotope, the value of cardiosomatic index in females was statistically significantly higher than that in males (♀0.24±0.01/♂0.18±0.01, $p<0.001$). The sign showed no gender differences in the two polluted biotopes.

b) In the polluted biotopes, the values of CSI in male [0.31±0.01 (2), 0.33±0.01 (3)] and female [0.30±0.01 (2), 0.32±0.02 (3)] individuals are statistically significantly higher than those in individuals from the respective sex in the control group ($p<0.001$).

c) There are no statistically significant differences in the values of CSI between individuals from the respective sexes in the populations from the two anthropogenically polluted biotopes (Figure 2).

Table 2
One-way ANOVA – Analysis of Variance (data: morphophysiological parameters) among populations *Pelophylax ridibundus* in the investigated biotopes in southern Bulgaria

Parameters	SS Effect	Df Effect	MS Effec	SS Error	Df Error	MS Error	F	P
CSI	0.66	5	0.133	1.45	196	0.0074	17.98291	0.00~1
SSI	0.03	5	0.006	0.04	196	0.0002	26.09367	0.00~1
RSI	1.34	5	0.268	2.18	196	0.0111	24.07328	0.00~1
GSI	1045.03	5	209.007	1153.51	196	5.8853	35.51362	0.00~1

Spleen

a) The only statistically significant gender difference is in population 2, where the value of SSI in female individuals (0.05±0.003) is higher than that in males (0.04±0.003).

b) With equal value of SSI (0.03±0.002) for individuals of both sexes in the control group, the values of the sign are statistically significantly lower in comparisons with both males and female individuals from populations 2 and 3 (0.06±0.003).

c) The value of SSI in male individuals from population 2 is statistically significantly lower than that in males from population 3; there are no differences between females in the two biotopes (Figure 3).

Kidney

a) In the population from the relatively clean biotope, the value of RSI is statistically reliably higher in female individuals (♀0.24±0.01/♂0.19±0.01, $p<0.05$), while in that

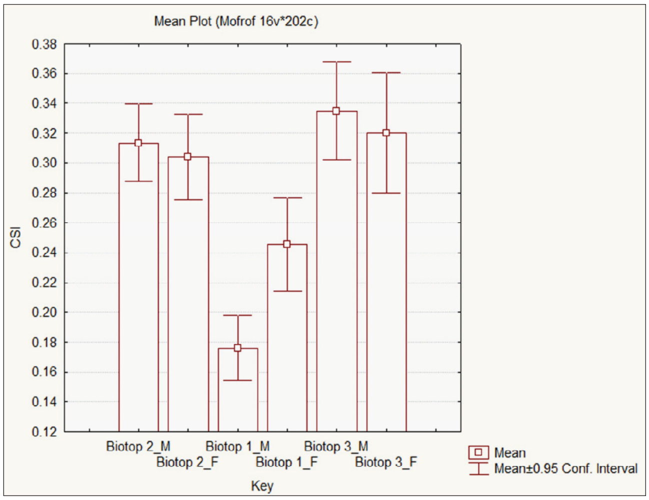

Fig. 2. Cardiosomatic index (M – males, F – females) of *Pelophilax ridibundus* individuals from populations in the investigated biotopes in southern Bulgaria

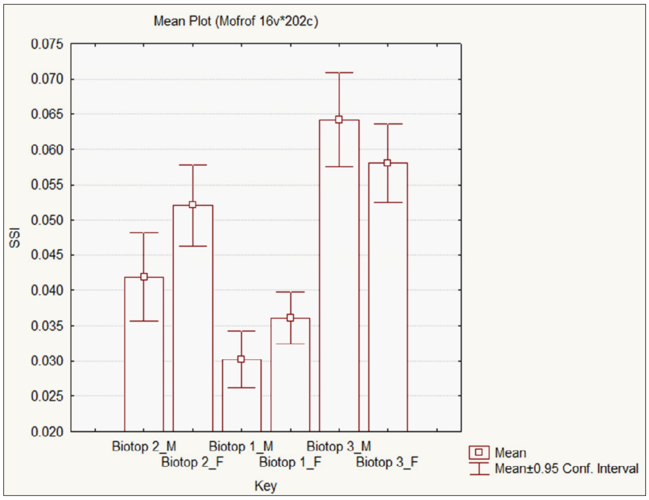

Fig. 3. Spleenosomatic index of individuals (M – males, F – females) of *Pelophilax ridibundus* populations in the investigated biotopes in southern Bulgaria

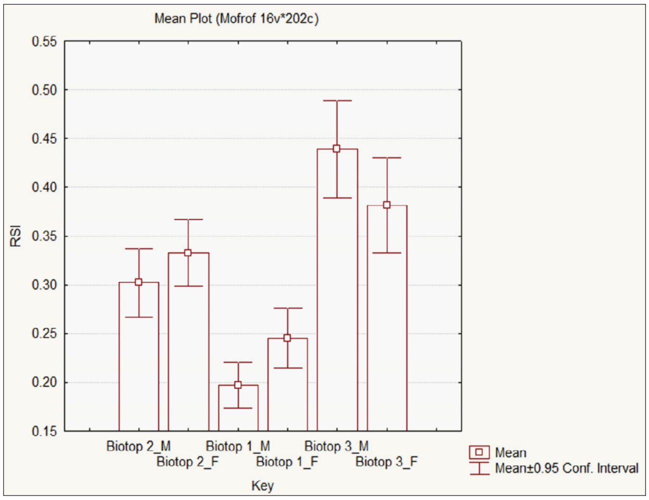

Fig. 4. Renosomatic index of individuals (M – males, F – females) of *Pelophilax ridibundus* populations in the investigated biotopes in southern Bulgaria

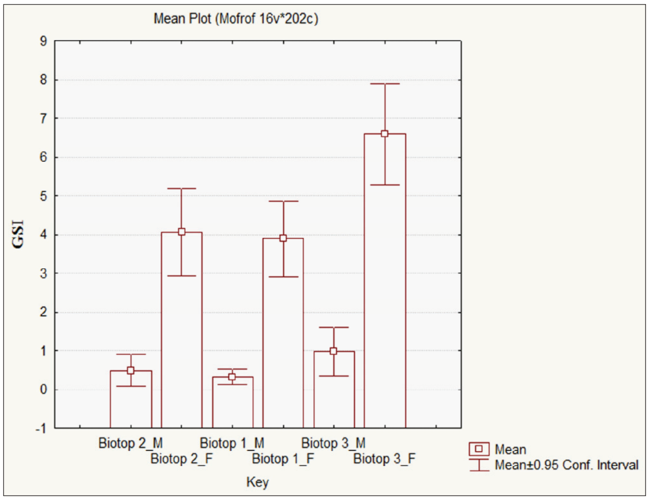

Fig. 5. Gonadosomatic index (ovarian somatic index and testis somatic index) for individuals (M – males, F – females) of *Pelophilax ridibundus* populations in the investigated biotopes in southern Bulgaria

from the biotope with heavy metal pollution – in males (♂0.44±0.02/♀0.38±0.02, $p<0.01$). There are no gender differences in biotope 2.

b) In conditions of pollution (population 2 and 3), the values of RSI statistically reliably increase in both male and female individuals, compared to individuals of the respective genders in the control group.

c) In comparing the two anthropogenically polluted biotopes, the value of RSI in male individuals from population 3 is statistically reliably higher than that in males from population 2. There are no reliable differences in the values of RSI between female individuals from both populations (Figure 4).

Gonads

a) In each of the three populations, the values of GSI are statistically reliably higher in female individuals ($p<0.001$).

b) Testis somatic index (TSI) There is no statistically significant difference in any of the comparisons between the values of the index in the populations from biotopes 2 (0.49±0.19) and 3 (0.98±0.31) and that in population 1 (0.32±0.09); ovarian somatic index (OSI) in the control group has a statistically reliably lower value (3.89±0.48) than in population 3 (6.59±0.64), $p<0.001$ and does not differ to that in population 2 (4.05±0.5).

c) While the values of TSI between populations 2 and 3 do not show a statistically significant difference, those of OSI are statistically reliably higher in individuals in the population from the biotope with heavy metal pollution (Figure 5).

Discussion

In individuals of both sexes of *P. ridibundus* we found statistically reliable increase in the relative weight of, heart, spleen, kidneys (for these organs it was more distinct in male individuals), and the relative weight of gonads in females, in conditions of anthropogenic pollution, which is due to the presence of toxins in the water. Regardless the different nature of pollution (domestic sewage pollution and heavy metal pollution) the reaction in the amendments of, heart, spleen and kidney in individuals of both sexes is in one direction: the relative weight increases as compared with those living in the relatively clean environment. There is a specific reaction only in female individuals – the relative weight of gonads increases reliably in the environment polluted with heavy metals.

There are very few studies about the changes in the values of CSI, SSI and RSI in amphibians. This is even more valid for research on the changes of these indices in Anura in anthropogenically transformed areas. The bulk of research in this direction have been conducted with species of the Ranidae (Rafinesque-Schmaltz, 1814) family in the eastern and central parts of the area (Russian Federation, Ukraine and other countries of the former USSR), where the majority of species are abundant and they are not under legal restrictions. It should be noted that the findings concerning changes in the values of the three indices, in relation to the characteristics of the environment are quite contradictory. Thus, in the eastern part of the species habitat (Kazakhstan) in two populations of *P. ridibundus*, first of them inhabiting the vicinity of a copper processing plant and the other 250 km remote, no differences were found in the values of RSI, while those of CSI were statistically reliably higher in the population that live in conditions of pollution (Toktamysova, 2005). According to the data of Zhukova and Kubancev (1982) for the same species in southern Russia, there were no reliable differences in the values of CSI for populations from rice paddies polluted with pesticides and the control group. In a later study conducted in the same territory (Zhukova et al., 1986), they reported a reliable increase of the relative weight of the heart in conditions of pesticide contamination (2.9-3.9‰), compared with the control from a fish farming lake (2.1-3.3‰) in the surroundings of the town of Krasnodar. In previous research of ours, carried out in the territory of southern Bulgaria (Peskova and Zhelev, 2009; Zhelev, 2012a), we did not find any statistically significant differences in the values of CSI, RSI and SSI in populations of *P. ridibundus* living in areas with developed energy industry (TPP “Maritza East-1”, near the town of Galabovo) and chemical industry (“Neochim SA”, near the town of Dimitrovgrad), compared with the control from a relatively clean area (the town of Harmanli). However, there are data (the region of the Volga-Akhtuba Valley in southern Russia) for a statistically significant increase of RSI in a lake polluted with wastewater from a chemical complex: 4.5-4.6‰ against 3.6-3.9‰ in the control (Kosareva and Vasiukov, 1976). In Ukraine, in three species of Anura (*P. ridibundus*, *Bombina bombina* Linnaeus, 1761 and *Pelobates fuskus* Laurenti, 1768) that inhabit a zone polluted with waste industrial water, the relative weight of kidneys, heart and spleen reliably grow in comparison with populations from relatively clean areas (Misura and Sporadets, 2005; Misyura et. al., 2007). Peskova (2002) reports higher values of CSI and RSI in *B. bombina* from polluted with pesticides rice paddies in southern Russia, in comparison with the control, and Thammachoti et al. (2012) report similar data about the values of RSI in the rise frog *Fejervarya limnocharis* Gravenhorst, 1829 from a rice system with pesticide contamination in Nan Province, Thailand. In the context of the examples discussed the results of this study that allude to a bigger heart in amphibians, in population 2 and 3, living in conditions of pollution in southern

Bulgaria, may be a result of their greater mobility in this environment. According to Schwartz et al. (1968), the values of RSI are in inverse proportion to the size of the body, which may partly be associated with the data of increased values of the index in male individuals in the two population from the polluted biotopes (especially in population 3, where SVL has the lowest values), but not in females (in population 2 and 3 the female individuals are larger than those in the control group). Another more probable reason for the increased values of RSI in individuals from the anthropogenically polluted biotopes, as mentioned above, can be found in their accelerated metabolism in conditions of pollution, which increases the role of kidneys as organs that take wastes out of the metabolic exchange. This could also be regarded as adaptive response. It is difficult to assess whether the increased sizes of the heart is also a result of increased metabolism and give some big advantages of the amphibians in the polluted biotopes, as the bigger requirements to the functions of this organ could also be implemented in other ways: at the expense of enhancement of myocardial contractility or on account of pulse rate acceleration, without being associated with the increased size of the organ (Vershinin, 2007). The main function of the spleen in amphibians is associated with formation, storage and destruction of blood cells. In the spleen of young individuals there is formation of various types of leukocytes (together with the liver) and erythrocytes, and in mature individuals – predominantly lymphocytes, as the main erythrocyte mass is destroyed simultaneously. The higher values of SSI found in the two anthropogenically polluted biotopes find their explanation in relation to the increased functional requirements to the organ in conditions of stimulating the formation of blood cells in individuals from these two populations: statistically reliable erythrocytosis and leukocytosis compared with the control group (Zhelev et al., 2013b). To support our thesis is the data for *P. ridibundus* populations, living in conditions of anthropogenic pollution in the Ukrainian Pridneprovie, where with higher values of SSI and RSI a high percentage of individuals have two spleens and three kidneys (Misyura et al., 2007).

Regarding the changes in the values of GSI in the populations of Anura that inhabit anthropogenically polluted biotopes, the data are also contradictory: from no differences with the values of the index in individuals from relatively clean biotopes (Zhelev, 2012a), via reduction of TSI and OSI, which is associated with oppression of reproduction ability (Marchenkovskaya, 2005; 2006; Misura et al., 2007), to their increase, which clearly speaks for an enhancing fertility in conditions of pollution and it is an adaptive process (Zaripova and Fayzulin, 2012). A question of great interest is the data associated with enlargement of gonads in conditions of pollution (when there are no changes in the values of TSI), cases similar to that found by us in population 3 from South Bulgaria. Such data are given for *P. ridibundus* (Zhukova and Kubancev, 1982), *B bombina* (Peskova, 2001), *F. limnocharis* (Thammachoti et al., 2012) in conditions of pesticide contamination. It was found that certain herbicides (atrazine) may induce the growth of gonads through endocrine system disorders (McLachan et al., 2008) and they lead to estrogenic effects (Hayes et al., 2006, 2010; Kloas and Lutz, 2006). This makes the question controversial – to what extent the increase in size of gonads (in terms of greater fertility) is an adaptation or pathology. The data of Thammachoti et al. (2012) comes to support this opinion, and they report bigger gonads of *F. limnocharis* during the dry season, a time during which the species does not reproduce.

Conclusions

In *P. ridibundus* populations that inhabit anthropogenically polluted biotopes in southern Bulgaria (whatever the nature of toxins is), in individuals of both sexes, the relative weight of vital organs increases: heart, spleen and kidney. In various types of pollution (domestic sewage pollution and heavy metal pollution), the relative weight of gonads grows reliably in the biotope with heavy metal pollution).

The changes in the values of the cardiosomatic index (CSI), the spleenosomatic index (SSI), the renosomatic index (RSI) and the gonadosomatic index (GSI) in *P. ridibundus* populations objectively reflect the physiological state of the organismin environmental conditions of anthropogenic contaminaton of different degrees. They have their practical application as biomarkers in the biomonitoring system, especially in countries lacking legislative restrictionson catching frogs.

Achnowledgements

We are indebted to Mr. M. V. Angelov (Basin Directorate - Plovdiv, Bulgaria) for his technical support.

References

Bannikov, A. G., I. S. Darevskii, V. G Ishtenko, A. K. Rustamov and N. N. Shterbak, 1977. A Guide to the Amphibians and Reptiles of the USSR. *Prosveshtenie*, Moscow, 414 pp. (Ru).

Biserkov, V., B. Naumov, N. Tchankov, A. Stoyanov, B. Petrov, D. Dobrev and P. Stoev, 2007. A Field Guide to Amphibians and Reptiles of Bulgaria. *Green Balkans*, Sofia. 196 pp. (Bg).

Blaustein, A. R., J. M. Romansic, J. M. Kiesecker and A. C. Hatch, 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions*, **9**: 123-140.

Garey, C. and C. J. Bryant, 1995. Possible interrelations among environmental toxicants, amphibian development and decline of amphibian populations. *Environmental Health Perspective*, **103**: 13-17.

Hayes, T. B., P. A. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker and M. Tsui, 2006 Pesticide mixtures, Endocrine disruption and Amphibian declines: Are we underestimating the impact? *Environmental Health Perspectives*: **114** (Supplement 1): 40-50.

Hayes, T. B., V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adame, E. Chan, D. Buchholz, T. Stueve and S. Gallipeau, 2010. Atrazine induces complete feminization and chemical castration im male African clawed frogs (*Xenopus leavis*). *Proceedings of the National Academy of Sciences United States of America*, **107** (10): 4612-4617.

Korzh, A. P., V. Y. Zadorozhnyaya and V. V. Moseyko, 2012. Methodological aspects of usage of batrachans representatives in bioindicative researches. *Journal of Zaporizhzhya National University*, **3**: 46-56 (Uk).

Kosareva N. A. and I. L. Vasiukov, 1976. Influence of the anthropogenic factors over amphibians Volga-Ahtubin region. In: B. S. Kubancev (Editor) Anthropogenic influences of nature complexes and ecosystems, *Volgograd State Pedagogical Institute publishing*, Volgograd, pp. 84 - 93 (Ru).

Kloas, W. and I. Lutz, 2006. Amphibians as model to study endocrine disrupters. *Journal of Chromatography A*, **1130** (1): 16-27.

Loumbourdis, N. S. and A. K. Vogiatzis, 2002. Impact of cadmium on liver pigmentary system of the frog *Rana ridibunda*. *Ecotoxicology and Environmental Safety*, **53**: 52-58.

Marchenkovskaya, A. A., 2005. Effects of urbanization on species diversity and population status of amphibians. Predneprovya. In E. M. Pisanets (Editor) Proceeding of the 1th Conference of the Ukrainian Herpetological Society, *Zoomuseum NMNH NAS of Ukraine*, Kyiv, pp. 105-106 (Ru).

Marchenkovskaya, A. A., 2006. Characteristics of biochemical parameters of two hydrophilic species of amphibian from biotopes in Dnieper-Donbas. *Bulletin of the University of Dnipropetrovsk, Biology, Ecology*: **14** (2): 114-118 (Ru).

McLachan, J. A., E. Simpson and M. Martin, 2008. Endocrine disrupters and female reproductive health, Best practice and research. *Clinical endocrinology and metabolism*, **20** (1): 63-75.

Misyura, A. N. and A. A. Marchenkovskaya, 2001. Condition of amphibian populations in anthropogenic influence. In: N. B. Ananjeva (Editor) Problems of herpetology, Proceedings of the 1th Meeting of the Nikolsky Herpetological Society, *Scvience*, Pushchino, Moscow, pp. 197-200 (Ru).

Misyura, A. N. and D. A. Sporadets, 2005. Influence of chemical waste on eco-physiological characteristics in tailless amphibians from different habitats. *Bulletin of the University of Dnipropetrovsk, Biology, Ecology*, **3** (2): 128-134 (Ru).

Misyura, A. N., A. A. Marchenkovskaya, and S. V. Chernyshenko, 2008. Characteristic of indices of microelements accumulation in Amphibians from biotopes being under influence of different industrial wastes. In: E. M. Pisanets (Editor) Proceedings of the Ukrainian Herpetological Societ, *Zoomuseum NMNH NAS of Ukraine*, Kyiv, pp. 47-54 (Ru).

Misyura, A. N., A. A., Marchenkovskaya, S. V. Chernyshenko and I. N. Zalipuha, 2007. Using the tailless amphibians in biomonitoring system for assessing the impact on zoocenoses, caused by companies for uranium ore waste processing. Bulletin *of the University of Dnipropetrovsk, Biology, Ecology*, **2** (19): 102-106 (Ru).

Papadimittriou, E. A. and N. S. Loumbourdis, 2003. Copper kinetics and hepatic metallothionein leves in the frog *Rana ridibunda*, after exposure to $CuCl_2$. *BioMetals*, **16**: 271-277.

Peskova, T. Yu., 2001. Influence of anthropogenically polluted environment of amphibians. *Volgograd State Pedagogical Institute publishing*, Volgograd, 156 pp. (Ru).

Peskova, T. Yu., 2002. Structure of populations of amphibians as a bioindicator for anthropogenic pollution. *Science*, Moscow, 132 pp. (Ru).

Peskova, T. Yu. and Zh. M. Zhelev, 2009. Comparative assessment of population variability of marsh frog *Rana ridibunda* from reservoirs in Bulgaria. *Problems of Contemporary Science and Practice*, **3**: 16-23 (Ru).

Schwartz, S. S., V. S. Smirnov and L. N. Dobrinskiy, 1968. Method of morphological indicators in the ecology of terrestrial vertebrates. *Proceedings of the Institute of Plant and Animal*, Sverdlovsk, 387 pp. (Ru).

Shapiro, S., M. Wilk and H. Chen, 1968. A Comparative study of various tests of normality. *Journal of American Statistical Association*, **63**: 1343-1372.

Speybroeck, J., W. Beukema and Piere-Andre Crochet, 2010. A tentative species list of the European herpetofauna (Amphibia and Reptilia) – an update. *Zootaxa*, **2492**: 1-27.

Statistica for Windows 2004 version 7.0. Statsoft Inc. Tulsa, USA.

Stetter, M. D., 2001 Fish and amphibian anesthesia. Veterinary Clinic North America. *Exotix Animmal Practice*, **4**: 69-82.

Steven, J., E. R. Jacobson, H. B. Lillywhite and K. Zamundo, 2004. Guidelines for use of Amphibians and Reptiles in field and laboratory research. Second Edition, Revised by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists. (http://www.asih.org/files/hacc-final.pdf)by).

Stojanov, A. J., N. Tzankov and B. Naumov, 2011. Die Amphibien und Reptilien Bulgariens. *Chimaira*, Frankfurt am Main, 588 pp.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodriges, D. L. Fischman and R. W. Waller, 2004. Status and trends of Amphibian declines and extincions worldwide. *Scisense*, **306**: 1783-1786.

Thammachoti, P., W. Khonsue, J. Kitana and P. Varanusupakul, 2012. Morphometric and gravimetric parameters of the rise frog *Fejervarya limnocharis* living in areas with different agricultural activity. *Journal of Environmental Protection*, **3**: 1403-1408.

Toktamysova, Z. S., 2005. Morphophysiological indicators of the marsh frog populations exposed to anthropogenic impact. In: A. E. Pakhomov (Editor) Biodiversity and Role of Zoocenosis in Natural and Anthropogenic Ecosystems, *Dnipropetrovsk National University publishing*, Dnipropetrovsk, pp. 380-381. (Ru).

Vershinin, V. L., 2007. Biota of urban areas. *UroRAN Academy press*, Yekaterinburg, 85 pp. (Ru).

Vershinin, V. L., 2008. Morpha striata in the members of the genus *Rana* (Amphibia, Anura), the reasons of adaptability to environmental changes. *Journal General Biology*, **69**: 65-71.

Vogiatzis, A. K. and N. S. Loumbourdis, 1998. Cadmium accumulation in liver and kidneys and hepatic metallothionein and glutathione levels in *Rana ridibunda*, after exposure to $CdCl_2$. *Archives of Environmental Contamination and Toxicology*, **34**: 64-68.

Zaripova, F. F. and A. I. Fayzulin, 2012. Characteristic of morphophysiological parameters of population of the marsh frog *Rana ridibunda* (Anura, Amphibia) in urban areas in the republic of Bashkotorstan. *Proceedings of the Samara Scientific Center of Russian Academy of Sciences*, **14** (5): 145-149 (Ru).

Zhelev Zh. M., 2007. Investigation on the blood differential formula in *Rana ridibunda* (Amphibia: Anura) from the area of the Maritsa-Istok-1 Steam Power Plant. *Acta Zoologica Bulgarica*, **59**: 181-190.

Zhelev Zh. M., 2012a. Ecology of tailless amphibians living under the influence of anthropogenic factors in Bulgaria. Usage of some population characteristics of three common amphibian species for the biomonitoring purposes. *Lambert Academic publishing*, Saarbrucken, 272 pp. (Ru).

Zhelev, Zh. M., 2012b. Erythrocyte pathologies in the peripheral blood of *Rana ridibunda Pallas*, 1771 (Amphibia: Anura: Ranidae) in populations inhabiting two river ecosystems with different types of Anthropogenic pollution in Southern Bulgaria. *Global Scientific Potential*, **4** (13): 13-19 (Ru).

Zhelev, Zh. M., G. S. Popgeorgiev and Zh. K. Georgieva, 2013a. Ecological status of the river Sazliyka and its tributaries (Soutern Bulgaria) as indicated by developmental stability of *Pelophylax ridibundus* (Amphibia: Ranidae). *Acta Zoologica Bulgarica*, **65** (3): 371-380.

Zhelev, Zh. M., G. S., Popgeorgiev and M. V. Angelov, 2013б. Investigating the changes in the morphological content of the blood of *Pelophylax ridibundus* (Amphibia: Ranidae) as a result of anthropogenic pollution and its use as an environmental bioindicator. *Acta Zoologica Bulgarica*, **65** (2): 187-196.

Zhelev, Zh., At., Arnaudov and P. Boyadzhiev, 2014. Colour polymorphism, sex ratio and age structure in the populations of *Pelophylax ridibundus* and *Pseudepidalea viridis* (Amphibia: Anura) from anthropogenically polluted biotopes in Southern Bulgaria and their usage as Bioindicators. *Trakia Journal of Sciences*, **1**: 1-12.

Zhelev, Zh. M., A. D. Arnaudov, G. S. Popgeorgiev and H. A. Dimitrov, 2012. Assessment of ecological status of two rivers with different types of anthropogenic pollution in Southern Bulgaria based on the level of fluctuating asymmetry in the populations of marsh frog *Rana ridibunda* (Amphybia: Ranidae). *Acta Zoologica Bulgarica*, **4** (Supplement): 225-231.

Zhukova, T. I. and B. S. Kubancev, 1982. Influence of pesticide water pollution on some morpho physiological characteristics of the marsh frog. In: B. S. Kubancev (Editor) Anthropogenic impacts on ecosystems and their components, *Volgograd State Pedagogical Institute publishing*, Volgograd, pp. 104-120. (Ru).

Zhukova T. I., B. S., Kubantsev and T. L. Burlachenko, 1986. Some reactions of the marsh frog populations to pesticide water pollution In: B. S. Kubancev (Editor) Human impacts on animal populations, *Volgograd State Pedagogical Institute publishing*, Volgograd, pp. 61-81 (Ru).

Received February, 1, 2014; accepted for printing September, 2, 2014.