Research Article

ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., Suppl. 8, 2017: 169-176

Health Status of *Pelophylax ridibundus* (Pallas, 1771) (Amphibia: Ranidae) in a Rice Paddy Ecosystem in Southern Bulgaria: Body Condition Factor and Fluctuating Asymmetry

***Zhivko M. Zhelev*[1,*]*, Stefan V. Tsonev*[2]* & Desislava N. Arnaudova*[3]

[1]Department of Human Anatomy and Physiology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski", 24 Tsar Assen Street, 4000 Plovdiv, Bulgaria
[2]AgroBioInstitute, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
[3]Department of Zoology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski", 24 Tsar Assen Street, 4000 Plovdiv, Bulgaria

Abstract: Effects of pollutants on the morphological parameters of *Pelophylax ridibundus* populations were examined at two sites in southern Bulgaria: the Rice Fields Tsalapitsa (RF) and the river Vacha (Reference site, RS). Morphological analysis showed significantly different values of the index for fluctuating asymmetry (FAMI) and body condition factor (CF) in frogs from RF compared to those from RS. These findings provide information on the long-term background pollution of RF. The lower values of body CF and the high levels of FA of *P. ridibundus* populations from RF are considered as a consequence of the negative effects of the presence of xenobiotics in paddy cages – probably pesticides and fertilizers. The present result prove the potential for practical application of integral indicator for developmental stability, i.e. the fluctuating asymmetry in the *P. ridibundus* populations, in bioindication analyses for assessment of the ecological status of agroecosystems.

Key words: *Pelophylax ridibundus*, developmental stability, morphological parameters, pesticides, bioindication

Introduction

Tsalapitsa rice fields is one of the biggest agroecosystems in Bulgaria. It is a complex consisting of watered areas surrounded by low dikes, canals and wet meadows located in the vicinity. The main habitats have been formed as a result of human activities and their existence largely depends on the use of the land, primarily for rice production. Paddy cages have different water level (water-filled in the end of April and emptied in the end of September) and it creates conditions suitable for birds and other animals, including amphibians. The rice fields Tsalapitsa was proclaimed as a protected area aiming the conservation of birds and their habitats based on the Directive 2009/147/EU (State Gazette, № 21. 2007). Although the legislative status of the protected area prohibits the use of indiscriminate means of pest control, the poor control and the lack of effective protection measures allow the use of fertilizers and pesticides in the paddy cells. Agrochemical contaminants can affect amphibians, as they directly reduce biodiversity and destroy the low levels of food chains and further affects consumers of the higher trophic levels (Blaustein *et al.* 2011). In addition, there are negative effects at the individual level such as deviation in growth, metabolism, metamorphosis, development, behaviour and the frequency of producing morphological abnormalities (Hayes *et al.* 2006, Relyea 2009, Krishnamurthy & Smith 2011, Aliko *et al*. 2012, Jelodar & Fazli 2012, Thammachoti *et al*. 2012, Hegde & Krishnamurthy 2014).

*Corresponding author. E-mail: zhivko-m@mail.bg

It is not a routine practice to monitor bottom sediments (where concentration of pesticides is greater); the monitoring procedures accepted by the national authorities in Bulgaria cover mostly surface waters based on the Water Framework Directive (Directive 2000/60/EU) and this makes it impossible to obtain an accurate picture of the ecological status of the environment and living conditions in the habitats (paddy cages). Bioindication analyzes can be applied as an alternative to expensive and labour-intensive analyzes about pesticide content in paddy field cages.

The amphibians are excellent bioindicators of environmental change due to their susceptibility to chemicals during the freshwater stage of their life cycles (Venturino *et al*. 2003) and their highly permeable skin (Duellman & Trueb 1994). Haematological parameters and the integral indicator for developmental stability in *P. ridibundus* populations, i.e. the fluctuating asymmetry, are particularly suitable as biomarkers (Romanova & Romanova 2003, Romanova & Egorikhina 2006, Peskova & Zhukova 2007, Zhelev *et al*. 2013a, 2013b, 2014a, 2014b, 2015a, 2016). The present work examines the possibilities for application of bioindication based on the health status of amphibians in agroecosystems.

The aim of the present study is to assess the health status of *P. ridibundus* populations from the rice fields Tsalapitsa – a region with intensive use of fertilizers and pesticides, as well as to compare it with that of populations living in a less disrupted biotope used as a reference site (river Vacha). We analyze the values of the integral indicator for developmental stability: Fluctuating asymmetry (FA) and body condition factor (CF). On the basis of the results obtained, a bioindication assessment of the status of the environment in the region of the rice fields Tsalapitsa is to be done, which is independent from the assessment based on physicochemical analyses. The present work is part of a complex study of the basic morphological and haematological parameters of *P. ridibundus* populations that inhabit the rice fields Tsalapitsa. Here we present morphological results of our study. The results of the haematological examinations are to be published elsewhere.

Materials and Methods

Study sites and data from physicochemical analysis of the water ecosystems: The studies were conducted during the spring of 2013 in two biotopes in southern Bulgaria: the river Vacha (Reference site, RS), in the south of the town of Krichim, altitude 200 m, and the rice fields Tsalapitsa (RF) in the north-west of the town of Plovdiv, altitude 192 m (Fig. 1).

The river Vacha is in the western Rhodope Mountains. According to the data from physicochemical monitoring performed by the Basin Directorate for Water Management in the Eastern Aegean Region, Plovdiv (http://www.bg-ibr.org), water in the river meets the first category (clean) according to surface water categorization in Republic of Bulgaria, Regulation № 7/08.08.1986 (State Gazette 96, 12.12.1986). Sampling for the purposes of the present study was carried out on 27.05.2013 in the region south of the town of Krichim (42°34′ N, 24°47′ E).

Roughly in the middle of the rice fields Tsalapitsa, north-southwards, a third-class road is connecting the village Tsalapitsa with the town Saedinenie and Trakia Motorway. Samples were collected on 05.16.2013 (42°23′ N, 24°58′ E), in paddy cages located on both sides of the road. The water used to fill paddy cages comes from a system of secondary channels connected with the complex irrigation canal (CIC) Lesichevo-Stryama. Data of the physicochemical monitoring of water in CIC Lesichevo-Stryama obtained in the process of development of the Management Plan for the Protected Area Rice Field Tsalapitsa), that coincide with the time of our study, showed no deviation from the permissible limits: all 14 tested parameters are in the norms for the first and second categories according to surface water categorization in Republic of Bulgaria (including Ammonium Nitrate $N\text{-}NH_4$ – 0.22 mg/dm^3, Nitrogen Nitrate $N\text{-}NO_3$ – 1.42 mg/dm^3, Nitrogen Nitrite $N\text{-}NO_2$ – 0.007 mg/dm^3 and Total Nitrogen – 1.9 mg/dm^3) are the norm ($N\text{-}NH_4$: 0.1–2.0 mg/dm^3, $N\text{-}NO_3$: 5–10 mg/dm^3, $N\text{-}NO_2$: 0.002–0.04 mg/dm^3, Total Nitrogen: 1–5 mg/dm^3) (Management Plan for the Protected Area for Birds BG0002086 Rice Field Tsalapitsa 2013).

Frog capturing and methods of analyses: This study was carried out with the marsh frog *P. ridibundus*, determined on the base of its morphological characteristics; namely, *P. ridibundus* differentiated from *P.* kl. *esculentus* in metatarsal tubercle size (Biserkov *et al*. 2007). The animals were caught from the water along the banks in the evening time with an electric torch. The catch was selective in terms of quantity and sex, aiming for a roughly equal sample in each of the two biotopes (25 males and females from each biotope). 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. The analyses were done with live animals, after that they were returned back to their natural habitat. Their age

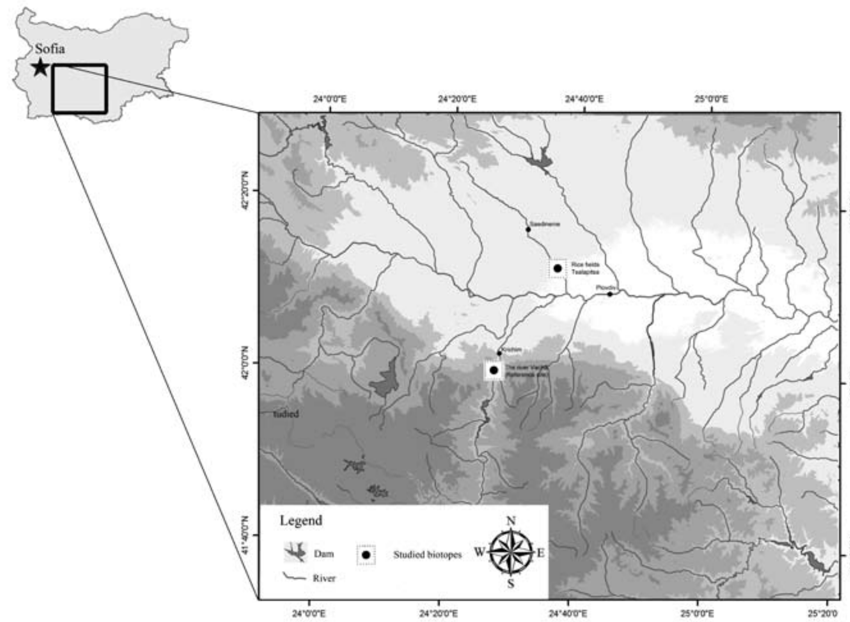

Fig. 1. Geographical location of the water ecosystems. The map has been prepared by the help of the Geographic information system (GIS). **Legend**: **Sites**: 1 – the river Vacha (Reference site, RS); 2 – the rice fields Tsalapitsa (RF)

was determined on the basis of their body size. All examined animals were adults (SVL>60.0 mm) and sexually mature (see Bannikov *et al*. 1977).

Morphological measurements: According to the ethical standards for handling of animals Beaupre *et al*. (2004), the live frogs had been anaesthetized with ether (Stetter 2001). We used a calliper to measure the Snout-Vent Length (SVL) with an accuracy of 0.1 mm and we weighed the animal – Body Weight (BW). For the animals from each biotope we calculated the "Factor for body nutritional status" of individuals, or Condition Factor (CF) which is used in ichthyology, by the formula: $(BW/SVL^3) \times 10^2$ proposed by Pauly (1983), also applicable for tailless amphibians and previously used in this frog species (Spirina 2009, Jelodar & Fazli 2012, Zhelev *et al*. 2015b).

Fluctuating asymmetry: As a method for assessing the developmental stability, we used fluctuating asymmetry in 10 morphological traits, as suggested by Chubinishvili (1997) and Zakharov *et al*. (2000). The level of asymmetric manifestation for each of the ten traits was recorded for each individual; it may vary from 0 (no asymmetry) to 1 (all the traits are asymmetric). It is possible for some of the traits not to express asymmetry but only in very rare cases it is possible for all 10 traits to be bilateral (see Zhelev *et al*. 2014b, 2015c). Using the FA in meristic traits in amphibians, as a method for assessing the developmental stability, is justified, since they show a greater variability in comparison with metric signs (Zakharov 1987, Palmer 1994, Eisemberg & Bertoluci 2016). Moreover, the meristic traits are mostly used as diagnostic markers because they are more sensitive to environmental changes rather than the metric ones (Zakharov *et al*. 2000). The fluctuating asymmetry was defined by the index frequency of asymmetric manifestation of an individual (FAMI). FAMI = $(\Sigma\ d_{L-R}/N)$ and calculated as the ratio of the sum of the number of individuals expressing asymmetric traits towards the total number of individuals (Zakharov *et al*. 2000). The grade rating for the status of the populations (respectively for the corresponding biotope) achieved on the basis of the FAMI values defined by a specfic scale for *P. ridibundus* in the southern part of its geographical range (see Peskova & Zhukova 2007, Zhelev *et al*. 2014b, 2015c).

Statistical analysis: Mathematical processing of the data was carried out using standard statistical procedures, using statistical package R – 3.1.2. Normality in the distribution of the parameters was verified with the Shapiro-Wilk test, and a standard distribution of: $p>0.05$ was found. Morphological examination data were analyzed by a two-way analysis of variance (Yoshida 1995), which included factorial analyses of group (sites: Reference site RS and Rice fields RF), sex and group-sex interaction, using the values from individual frogs. A statistical significance of the between-groups differences were evaluated using Tukey HSD post hoc test. Results with $p<0.05$ [α=5%] were considered significant. Data

Table 1. Two-way ANOVA: tests of between-subjects effects for morphological parameters in Pelophylax ridibundus populations from two investigated sites in southern Bulgaria

Parameters	Fixed effects	Df Sum	Sum Sq	Mean Sq	F value	Pr(>F)
SVL	Sites	1	1.21	1.21	2.629	0.108 ns
	Sex	1	55.35	55.35	120.253	<2e^{-16} ***
	Sites: Sex	1	0.27	0.27	0.587	0.445 ns
	Residuals	96	44.19	0.46	–	–
BW	Sites	1	4227	4227	43.769	2.1e^{-09} ***
	Sex	1	19560	19560	202.550	< 2e^{-16} ***
	Sites: Sex	1	621	621	6.425	0.0129 *
	Residuals	96	9271	97	–	–
CF	Sites	1	125.10	125.10	82.255	1.48e^{-14} ***
	Sex	1	0.06	0.06	0.039	0.843 ns
	Sites: Sex	1	0.17	0.17	0.109	0.742 ns
	Residuals	96	146.01	1.52	–	–
FAMI	Sites	1	2.7889	2.7889	554.086	<2e^{-16} ***
	Sex	1	0.0009	0.0009	0.179	0.673 ns
	Sites: Sex	1	0.0001	0.0001	0.020	0.888 ns
	Residuals	96	0.4832	0.0050	–	–

Significance codes: *** (<0.001), ** (<0.01), * (<0.05), ns (>0.05).

Table 2 Descriptive statistics and results from the between-groups differences analysis of the morphological parameters and indicator of development stability of *Pelophylax ridibundus* individuals from the investigated sites in southern Bulgaria

Parameters	Sites				Tukey HSD
	Reference site		Rice field		
	Female (1) (n=25)	Male (2) (n=25)	Female (3) (n=25)	Male (4) (n=25)	
	Means ± standard errors of means Minimum–Maximum				
SVL (cm)	8.24±0.19 (7.10–10.20)	6.65±0.08 (6.10–7.20)	7.92±0.15 (7.10–9.60)	6.54±0.07 (6.10–7.20)	**1/3ns**, p=0.335 **1>4**, p=0.000 **2<3**, p=0.000 **2/4ns**, p=0.930
Tukey HSD	**1>2**, p=0.000		**3>4**, p=0.000		
BW (g)	69.71±2.69 (53.17–93.67)	36.75±0.95 (30.61–45.12)	51.72±2.64 (39.25–78.06)	28.73±0.55 (24.26–35.55)	**1>3**, p=0.000 **1>4**, p=0.000 **2<3**, p=0.000 **2>4**, p=0.024
Tukey HSD	**1>2**, p=0.000		**3>4**, p=0.000		
CF	12.59±0.39 (8.83–14.86)	12.46±0.19 (10.99–14.75)	10.27±0.14 (8.39–11.59)	10.31±0.18 (8.69–11.95)	**1>3**, p=0.000 **1>4**, p=0.000 **2>3**, p=0.000 **2>4**, p=0.000
Tukey HSD	**1/2ns,** p=0.982		**3/4ns**, p=0.999		
FAMI	0.37±0.01 (0.30–0.50)	0.37±0.01 (0.30–0. 50)	0.71±0.01 (0.60–0.80)	0.70±0.01 (0.60–0.80)	**1<3**, p=0.000 **1<4**, p=0.000 **2<3**, p=0.000 **2<4**, p=0.000
Tukey HSD	**1/2ns**, p=0.997		**3/4ns**, p=0.978		

Legend: SVL – Snout-Vent length, BW – Body weight, CF – Condition factor; FAMI – Frequency of asymmetric manifestation of an individual; n – Number of individuals. The sings < and > compare mean values of the parameters. Significance codes: ns (>0.05).

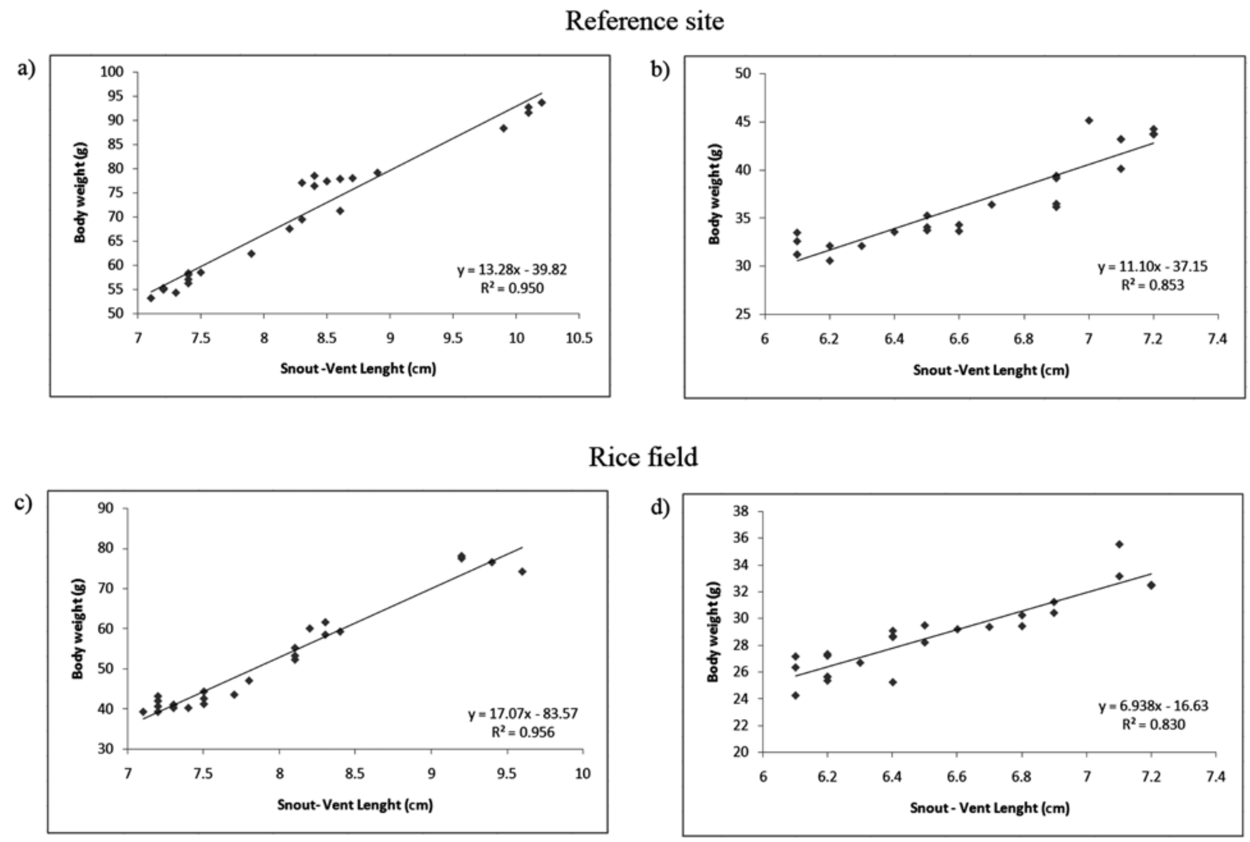

Fig. 2. Regression analysis of relationships between body weight and snout-vent length of *Pelophylax ridibundus* from the sites investigated in southern Bulgaria. **Ledend:** Females – a, c; Males – b, d

are given as Mean±SEM, a Min–Max. The weight-length relationships of *P. ridibundus* individuals from each site were performed by linear regression analysis.

Results

Results from two-way ANOVA analysis are presented in Table 1.

There were statistically significant differences in the mean values of the parameters BW, CF and FAMI for individuals from the two sites (without taking into account the sex). The mean values of the parameters SVL and BW, recorded for the individuals of both sexes showed statistically significant difference (without taking into account the sites). Analysis of the interaction Sites x Sex reported a statistically significant difference in just one parameter – BW. Further analysis of the interaction Sites x Sex showed that it is due to the bigger effect of sex in the Reference site, where the sex explains ~73.5% of the variation observed, while in the Rice fields the sex explains barely 60% (not shown). In order to better understand the potential effect of the agricultural pollution on the studied parameters, we analyzed all possible between-group differences using Tukey-HSD test. The results from the analysis are presented in Table 2.

SVL and BW: The values of the two parameters of females from Reference site and Rice fields Tsalapitsa were statistically significantly higher than those of males. This is normal in view of the existence of sexual dimorphism in *P. ridibundus*. For the parameter SVL the comparisons between same-sex individuals from RS and RF showed no statistically significant differences, while BW had reliably higher values in males and females from RS. CF: There were no sex differences in the parameter values between males and females from RS, and between those from RF. The individuals of both sexes inhabiting RS had statistically significantly higher levels of the body CF as compared with females, and in comparison with males inhabiting RF. The weight-length relationship of *P. ridibundus* individuals from the two sites compared are shown in Figure 2. The values of the coefficient of determination (R^2) were higher in females from the two sites compared to its values in males. Meanwhile, the differences between same-sex animals from RS and RF were minor. The scaling coefficients in females from RF (17.07) were higher than that in the individuals of the same sex inhabiting RS (13.28). A reverse situation was found with males: in these from RF, the scaling coefficients had lower values (6.93) than the animals from RS (11.10).

FAMI: The value of this parameter did not show differences of sex in both: the comparisons between males and females from RS and between those from RF. The individuals of both sexes inhabiting RF had statistically significantly higher values of FAMI as compared with females, and in comparison with males inhabiting RS.

Discussion

Previous data suggest that the values of BW and body CF in *P. ridibundus* from populations inhabiting conditions of anthropogenic pollution have significantly lower values than those of individuals from populations inhabiting less disrupted territories (Spirina 2009, Jelodar & Fazli 2012, Zhelev *et al.* 2015b). In our study the analysis of morphological parameter values, reflecting the general physical state of the individuals of *P. ridibundus* inhabiting the two sites, shows that the individuals of both sexes inhabiting the rice fields Tsalapitsa had serious growth problems. The results from linear regression analysis are indication of similarity in growth processes, but also of a different model in the course of physical development of the animals from RS and RF: in similar linear dimensions in individuals of both sexes, weight of the animals inhabiting RF was lower than that of these from RS. The reliably lower values of CF in individuals of both sexes in RF are also a confirmation of a serious delay in their physical development (more pronounced in males). The reasons for this could be sought in the deteriorated nutrient base in RF, as well as in disturbances in metabolism caused by xenobiotics. The analysis results for the values of body conditions factor are in support of our thesis, the tailless amphibian *Fejervarya limnocharis* (Gravenhorst, 1829) living in agrochemical contaminated rice fields in Western Ghats in India and Thailand. In this study the values of body CF of amphibians inhabiting rice fields are reliably lower than those of amphibians inhabiting reference sites (see Thammachoti *et al.* 2012, Hegde & Krishnamurthy 2014).

The analysis on the values of fluctuating asymmetry showed much higher values of the index FAMI in the population from RF. On the basis of the values, recorded for the integral indicator for developmental stability, RF was assessed with a rating grade 4/5 according to the scale of Peskova & Zhukova (2007) whereas RS was rated with grade 1. These values clearly show that conditions in the rice fields have worsened much (the environment is polluted), which resulted in higher levels of FA in *P. ridibundus* populations. The results obtained in this study are compatible with those obtained by a previous examinations in the rice fields Tsalapitsa (Zhelev 2011); moreover, in comparison with the previous study, these results trace out darker trends. In a study carried out in 2009 with the populations of two amphibian species, *P. ridibundus* and *Bombina bombina* L., 1761, the following levels of asymmetry were recorded, respectively *P. ridibundus*: FAMI ♂ 0.68±0.02, ♀ 0.58±0.02, and *B. bombina*: FAMI = ♂ 0.62±0.05, ♀ 0.61±0.06, respectively. On the basis of the values for FAMI, the environmental conditions of the rice fields Tsalapitsa were assessed in 2009 with a rating grade ¾ according to the scale of Peskova & Zhukova (2007). The comparisons show that the conditions in the rice fields Tsalapitsa are not getting better; on the contrary, they become worse. Similar high levels of asymmetry, with FAMI 0.70–0.80, were reported for the populations of *P. ridibundus* from the rivers Topolnitsa (polluted with heavy metals) and Sazliyka (with domestic sewage pollution) (Zhelev *et al.* 2013b, 2015c).

The physicochemical data presented in Management Plan for the Protected Area for Birds BG0002086 Rice Field Tsalapitsa (2013) show that the water used for filling paddy cages does not contain toxicants of anthropogenic origin above the permissible standards in the country. In our view, the changes in the condition of the studied morpho-physiological parameters of *P. ridibundus* individuals in the rice fields Tsalapitsa reflects the negative effects of the long-term influence of xenobiotics, probably pesticides, which have been applied in paddy cages. This could be confirmed by carrying out analyzes on the pesticide content of the bottom sediments of the paddy cages but, even without such examinations, the bioindication test gives grounds for a serious concern about the extent of human impacts on the biota in this sensitive agroecosystem and refers to the need for measures to control human intervention.

Conclusion

There are significant changes in amphibian morphological parameters: lower values of body CF and high levels of FA. In our view, these changes in the populations of *P. ridibundus* that inhabit the rice fields Tsalapitsa, probably have been caused by the pesticides and fertilizers introduced in the paddy cages in the rice production processes. The present work confirms the reliable role of fluctuating asymmetry as a biomarker in *P. ridibundus* populations and it illustrates the opportunities for practical application of the method in biomonitoring on agroecosystems.

References

Aliko V., Adiola B., Eldores S. & Alketa G. 2012. Effects of pollution on amphibian blood parameters (Ranidae: *Rana balcanica* & *Rana lessonae*) from the Albania's Costal zone. International Conference on Marine & Coastal Ecosystems, 25–28 April, Tirana, Albania, 7 p.

Bannikov A. G., Darevskii I. S., Ishtenko V. G., Rustamov A. K. & Shterbak N. N. 1977. A Guide to the Amphibians and Reptiles of the USSR. Moscow (Prosveshtenie Publishing House). 414 p. (In Russian).

Beaupre S. B., Jacobson E. R., Lillywhite H. B. & Zamudio K. 2004. Guidelines for use of live amphibians and reptiles in field and laboratory research. 2nd revised edition, Lawrence, Kansas, USA: Herpetological Animal Care and Use Committee (HACC), American Society of Ichthyologists and Herpetologists. (www.asih.org/files/hacc-final.pdf).

Biserkov V., Naumov B., Tchankov N., Stoyanov A., Petrov B., Dobrev D. & Stoev P. 2007. A field guide to amphibians and reptiles of Bulgaria. Sofia (Green Balkans). 196 p. (In Bulgarian).

Blaustein A. R., Barbara A. H., Relyea R. A., Johnson P. T. L., Buck J. C., Gervasi S. S. & Kats L. B. 2011. The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses**.** *Annals of the New York Academy of Sciences*, **1223**: 108–119.

Chubinishvili A. T. 1997. The status of Natural Populations of the *Rana esculenta*-complex in Response to Anthropogenic Influences. A Morphogenetic Approach. In: Kuz′min S. L. & Dodd C. K. (Eds): Advances in Amphibian Research in the Former Soviet Union. Sofia-Moscow (Pensoft), 117–124. (In Russian).

Duellman W. E. & Trueb L. 1994. Biology of Amphibians. Baltimore (John Hopkins University Press). 669 p.

Eisemberg C. C. & Bertoluci J. 2016. Fluctuating asymmetry in populations of the South American frog *Physalaemus cuvieri* (Leptodactylidae) in areas with different degrees of disturbance. *Journal of Natural History* **1**: 1–9.

Hayes T. B., Case P., Chui S., Chung D., Haeffele C., Haston K., Lee M., Mai V. P., Marjuoa Y., Parker J. & Tsui M. 2006. Pesticide mixtures, endocrine disruption and amphibian declines: Are we underestimating the impact? *Environmental Health Perspectives* **114**: 40–50.

Hegde G. & Krishnamurthy S. V. 2014. Analysis of health status of the frog *Fejervarya limnocharis* (Anura: Ranidae) living in rice paddy fields of Western Ghats, using body condition factor and AChE content. *Ecotoxicology and Environmental Contamination* **9**(1): 69–76.

Jelodar H. T. & Fazli H. 2012. Monthly changes in condition, hepatosomatic index and bioavailability in frogs (*Rana ridibunda*). *Research Journal of Biology* **2**: 9–14.

Krishnamurthy S. V. & Smith G. R. 2011. Combined effects of malathion and nitrate on early growth, abnormalities, and mortality of wood frog (*Rana sylvatica*) tadpoles. *Ecotoxicology* **20**: 1361–1367.

Management Plan for the Protected Area for Birds BG0002086 Rice Field Tsalapitsa(2013), 243 p.

Palmer A. R. 1994. Fluctuating asymmetry analyses: A primer. In: Markow T. A. (ed), Developmental Instability: Its Origins and Evolutionary Implications. Dordrecht (Kluwer Academic Publishers), pp. 335–364.

Pauly D. 1983. Some simple methods for the assessment of tropical fish stocks. Rome (FAO Fisheries Technical Paper). 52 p.

Peskova T. Y. & Zhukova T. I. 2007. The usage of amphibians for bio-indication of water pollution. *Science Kuban* **2**: 22–25. (In Russian).

Regulation № 7of 08.08. 1986 for indicators and standards for determining the quality of flowing surface water in the Republic of Bulgaria. Promulgated, State Gazette, № 96. 12.12.1986 (Annulled 05.03.2013).

Relyea R. A. 2009. A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* **159**: 363–376.

Romanova E. B. & Romanova O. Y. 2003. Peculiarities of leukocytic formula of peripheral blood of green frogs under conditions of anthropogenetic load. *Journal of Environmental Physiology* **39**(4): 480–484.

Romanova E. B. & Egorikhina M. N. 2006. Changes in haematological parameters of *Rana* frogs in a transformed urban environment. *Russian Journal of Ecology* **37**(3): 188–192.

Spirina E. V. 2009. Morphophysiological adaptations in *Rana ridibunda* Pall. under the influence of pollution. *Bulletin of the Altai State Agrarian University* **12**(62): 64–68. (In Russian).

Stetter M. D. 2001. Fish and amphibian anesthesia. *Veterinary Clinics of North America: Exotic Animal Practice*, **4**: 69–82.

Thammachoti P., Khonsue W., Kitana J. & Varanusupakul P. 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.

Venturino A., Rosenbaum E., Caballero de Castro A., Anguiano O. L., Gauna L., Fonovich de Schroeder T. & Pechen de D'Angelo A. M. 2003. Biomarkers of effect in toads and frogs. *Biomarkers* **8**: 167–86.

Yoshida M 1995. Design of Experiments for Animal Husbandry. Tokyo (Youkendo Ltd). 162 p. (In Japanese).

Zakharov V. M. 1987. Asymmetry of Animals. Moscow (Science). 216 p. (In Russian).

Zakharov V. M., Baranov A. S., Borisov V. I., Valetsky A. V., Kryazheva N. G., Chistyakova E. K. & Chubinishvili A. T. 2000. Health of environment: Methods of assessment. Moscow (Center for Russian Environmental Policy). 234 p. (In Russian).

Zhelev Z. M. 2011. Bio-indication assessment of the two habitats in Southern Bulgaria on the basis of fluctuating asymmetry and phenotypic structure of populations of the marsh frog *Rana ridibunda* Pallas, 1771 (Amphibia: Anura: Ranidae) and fire-bellied frog *Bombina bombina* Linnaeus, 1761 (Amphibia: Anura: Discoglossidae) in syntopic habitat. *Science Prospects* **7**(22): 7–18. (In Russian, English summary).

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

Zhelev Z. M., Arif M., Popgeorgiev G. S., Rauf M. & Mehterov N. H. 2014a. Haematology of *Pelophylax ridibundus* (Amphibia: *Ranidae*) of *striata* and *maculata* morphs in populations living in conditions of anthropogenic pollution in Southern Bulgaria. *Open Journal of Animal Sciences* **4**: 206–216.

Zhelev Z. M., Popgeorgiev G. S. & Georgieva Z. K. 2014b. Fluctuating Asymmetry in the Populations of *Pelophylax ridibundus* and *Pseudepidalea viridis* (Amphibia: Anura) in the Region of the Lead and Zinc Plant "Kardzhali" (South Bulgaria). *Acta Zoologica Bulgarica* **66**(1): 83–87.

Zhelev Z. M., Popgeorgiev G. S. & Mehterov N. H. 2015a. Haematological Parameters of *Pelophylax ridibundus* (Amphibia: Ranidae) from the region of the lead and zinc plant "Kardzhali" (South Bulgaria) and their use in the Environmental Quality Assessment. *Acta Zoologica Bulgarica* **67**(2): 271–282.

Zhelev Z. M., Popgeorgiev G. S. & Mehterov N. H. 2015b. Changes in the hepatosomatic index and condition factor in the populations of *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria. Part II. *Bulgarian Journal of Agricultural Science* **21**: 534–539.

Zhelev Z. M., Popgeorgiev G. S., Arnaudov A. D., Georgieva K. N. & Mehterov N. H. 2015c. Fluctuating asymmetry in *Pelophylax ridibundus* (Amphibia: Ranidae) as a response to anthropogenic pollution in South Bulgaria. *Archives of Biological Sciences* **67**(3): 1009–1023.

Zhelev Z. M., Mehterov N. H. & Popgeorgiev G. S. 2016. Seasonal changes of basic erythrocyte-metric parameters in *Pelophylax ridibundus* (Amphibia: Ranidae) from anthropogenically polluted biotopes in Southern Bulgaria and their role as bioindicators. *Ecotoxicology and Environmantal Safety* **124**: 406–417.