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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)

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Abstract: Levels of fluctuating asymmetry in two species of anuran amphibians, *Pelophylax ridibundus* and *Pseudepidalea viridis*, in sympatric and syntopic conditions at the dam lake "Studen Kladenets" located next to the Lead and Zinc Plant "Kardzhali", South Bulgaria, are studied. Materials were collected during the breeding season in three consecutive years.. Based on the indicators of fluctuating asymmetry in both species, grades characterizing the developmental stability of their populations are identified. These grades are indicative for the quality of the environment and are used for assessment of the biotope, carried out parallel and independently from the physicochemical analysis.

Key words: anthropogenic pollution, fluctuating asymmetry, bioindication, *Pelophylax ridibundus*, *Pseudepidalea viridis*

Introduction

In recent years, the biomonitoring became an integral part of the general environmental monitoring. Amphibians as inhabitants of two environments, aquatic and terrestrial, are successfully used as indicators of the damage of biotopes caused by anthropogenic pollution (Ustyuzhanina, Streltsov 2001, Nikashin 2005, Lada *et al*. 2012, Zhelev 2012, Zhelev *et al*. 2012). One of the simplest, easiest and cheapest methods of bioindication is the estimation of the fluctuating asymmetry (FA) as an integral indicator of developmental stability of anuran populations. It has been implemented as assessment of minor, untargeted deviations from the strict bilateral symmetry in the colour of the dorsal side of species of the genera *Pelophylax* Fitzinger, 1843 and *Pseudepidalea* Frost *et al*., 2006 or of the ventral side of species of the genus *Bombina* Oken, 1816 (Zakharov *et al*. 2000, Zakharov 2001). Most commonly, species of the complex of the European green frog *Pelophylax* kl. *esculentus* (L., 1758) are used as test subjects, and there are specially developed scales for assessing the deviations in developmental stability and the corresponding environmental conditions (Zakharov *et al*. 2000, Peskova, Zhukova 2007). In addition, recent studies also involve bioindicative assessment of FA in other anuran species living in sympatry and syntopy with the marsh frog (Zhelev, Peskova 2010, Peskova *et al.* 2011, Zhelev 2011a, 2011b).

The purpose of this work is to study the levels of fluctuating asymmetry in the populations of

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two species, *Pelophylax ridibundus* (Pallas, 1771) and *Pseudepidalea viridis* (Laurenti, 1768), as well as characteristics of their biotopes based on this integral indicator of their developmental stability and on parallel physical and chemical analysis of their environment.

Materials and Methods

The material for our study was collected during three consecutive breeding seasons, i.e. months March-April 2009, 2010 and 2011, in a biotope that is located in the area of "the tail" of the dam lake "Studen Kladenets" (29 km^2, 580 m altitude), in the vicinity of which the lead-zinc plant LZP "Kardzhali" is situated (Fig. 1).

Based on both data of physicochemical analysis of water for the period 2009-2011 from the newsletters of the Basin Directorate for Water Management in the East Aegean Region (http://www.bd-ibr.org) and data from the annual reports for the quality of waters in the Republic of Bulgaria produced by the Executive Environment Agency (http://eea.government.bg), it is obvious that the toxic agents (heavy metals) available in the dam lake exceed the limit values for pollutants and standards of water categorization in the Republic of Bulgaria, Order № 7/08.08.1986 (State Gazette, № 96, 12.12.1986) (Table 1).

The subjects of our study are the marsh frog *P. ridibundus* and the green toad *P. viridis*. Animals were collected at night using an electric torch and in the daytime during the time of reproduction in shallow coastal areas (Sutherland 2000). All amphibians captured, both males and females, were mature (L > 60.0 mm). After measuring body length and recording FA characteristics, the animals were released. We recorded and analyzed 10 morphological features of *P. ridibundus*: 1 – number of stripes on the dorsal side of the thigh, 2 – number of spots on the dorsal side of the thigh, 3 – number of stripes on the dorsal side of the shank, 4 – number of spots on the dorsal side of the shank, 5 – number of stripes on the foot, 6 – number of spots on the foot, 7 – number of stripes on the back, 8 – number of spots on the back, 9 – number of white spots on the plantar side of the third finger of the hind leg, 10 – number of white spots on the plantar side of the fourth finger of the hind leg (Zakharov *et al*. 2000). For *P. viridis*, we recorded and analyzed 12 morphological features: 1 – number of stripes on the dorsal side of the shank, 2 – number of spots on the dorsal side of the shank, 3 – number of stripes on the dorsal side of the thigh, 4 – number of spots on the dorsal side of the thigh, 5 – number of stripes on the dorsal side of the foot, 6 – number of spots on the dorsal side of the foot, 7 – number of stripes on the dorsal side of the back, 8 – number of spots on the dorsal side of the back, 9 – number of stripes on the dorsal side of the forearm, 10 – number of spots on the dorsal side of the forearm, 11 – number of stripes on the dorsal side of the wrist, 12 – number of spots on the dorsal side of the wrist (Peskova, Zhukova 2008).

We assessed FA in the two species by applying standard measures: FAMI (frequency of asymmetric manifestation of an individual – the ratio of the number of individuals exhibiting the asymmetric

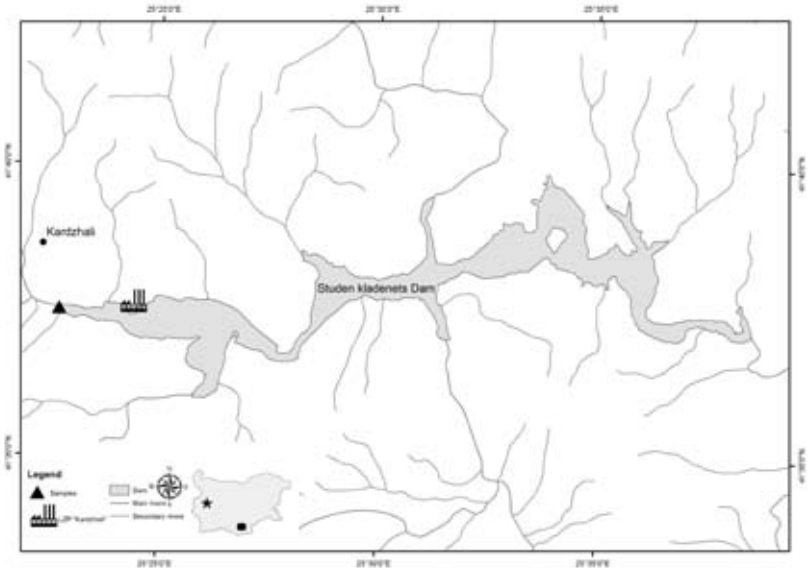

Fig. 1. Map of the studied biotope

Table 1. Recent data on the status of the dam lake "Studen Kladenets" at the time of the study (physicochemical analysis of surface water samples).

Parameters	Units SI	Surface water quality standards (Order №7/8.8.1986)	Average annual values		
		I	2009	2010	2011
pH	pH units	6.5-8.5	8.28	7.98	7.79
Temperature	°C	to 3° middle of the season	17.03	16.9	16.5
Insoluble substances	mg/dm^3	30.0	19.78	33.33	6.74
Electroconductivity	µS/cm	700.0	250.0	245.0	313.0
Dissolved oxygen	mgO_2/dm^3	6.0	10.30	9.77	9.64
Oxygenation	%	75.0	109.44	102.4	101.98
BOD_5	mgO_2/dm^3	5.0	2.71	1.4	2.68
COD	mgO_2/dm^3	25.0	18.25	11.62	18.59
Nitrate ammonium N – NH_4	mg/dm^3	0.1	0.12	0.07	0.08
Nitrate nitrogen N – NO_3	mg/dm^3	5.0	0.41	0.43	0.33
Nitrite nitrogen N – NO_2	mg/dm^3	0.002	0.001	0.002	0.03
Orthophosphates	mg/dm^3	0.2	0.15	0.05	0.02
Total nitrogen	mg/dm^3	1.0	0.43	1.73	0.93
Total phosphorus - as P	mg/dm^3	0.4	0.03	0.12	0.10
Sulphates (SO_4^{2-})	mg/dm^3	200.0	33.59	50.08	68.64
Cadmium (Cd)	mg/dm^3	SKOS– 0.005	0.012*	0.015*	0.014*
Copper (Cu)	mg/dm^3	SKOS– 0.05	0.011	0.007	0.015
Zinc (Zn)	mg/dm^3	SKOS– 1.0	0.619	3.366*	4.905*
Lead (Pb)	mg/dm^3	SKOS– 0.02	0.0225*	0.034*	0.027*
Nickel (Ni)	mg/dm^3	SKOS– 0.02	0.006	0.001	0.002
Chromium (Cr)	mg/dm^3	SKOS– 0.02	0.003	< 0.0005	< 0.0005
Note * – above SKOS: very poor condition					

Table 2. Rating scale for the aberrations in the status of *Pelophylax ridibundus* from the conventional standard.

Grade	The index value for stability of the development (FAMF or FAMI)	State of organism
	Southern part of the area (Peskova, Zhukova 2007)	
1	< 0.40	Conventional rate (clean water basin)
2	0.41 – 0.50	Minimal impact on organisms (slightly polluted water basin)
3	0.51 – 0.60	Satisfactory condition of organisms (average polluted water basin)
4	0.61 – 0.70	Unfavorable condition of organisms (heavily polluted water basin)
5	≥ 0.71	Critical condition of organisms (very heavily polluted water basin)

features towards the total number of tested individuals), and FAMF (frequency of the asymmetric manifestation of a feature – the ratio of the number of asymmetrical features towards the total number of examined features). The individuals of both sexes were tested together, as the literature rarely indicates sex differences in the manifestation of FA. The grade rates for the state of populations (and the relevant state of the biotope) were obtained on the basis of the features of FA. We used the specified scale for *P. ridibundus* in the southern part of the area (Peskova, Zhukova 2007) (Table 2). A total of 101 individuals of *P. ridibundus* and 92 *P. viridis* were used for the morphological analysis.

The data were processed according to Lakin (1990) using the software package STATISTICA for Windows 7.0. The data about the studied characters features are evenly distributed (Shapiro-Wilk - test; $p > 0.05$) and this allows comparing of the mean values with a parametric Student's t-test at a level of significance $\alpha = 5\%$ ($p < 0.05$).

Results and Discussion

For both species studied, there were no statistically significant differences between the values of FAMF and FAMI ($p > 0.05$). For that reason, the discussion hereinafter is based solely on the FAMI features. It is obvious that the values of the feature for FA in the populations of both species (Table 3) are assessed with a grade rate 3 (moderate level of disorder in developmental stability) by the scale for *P. ridibundus* in 2009 and a grade rate of 4 (unfavorable condition of the body) during the breeding seasons of 2010 and 2011. Therefore the degree of contamination of the water body and hence the deterioration of the living conditions have increased over the years (for each of the two species the values of FAMI increased statistically significantly in 2010 and 2011: $p < 0.001$). The values of the integral indicator for developmental stability confirm the data of physicochemical analysis of the water body. This indicates that there is significant and persistent anthropogenic pollution in the area (Table 1).

Although the values of FAMI for both species were assessed with the same grade in each of the three years of study, they are higher in *P. viridis*. In our previous study (Zhelev 2011a) on manifestations of FA in the same two species, living in conditions of syntopy, in ponds with varying degrees of anthropogenic pollution in southern Bulgaria, we found a similar situation as the aforementioned one. In the biotope that is located next to the waste collectors of the chemical plant "Neochim" (pollution with phenols and heavy metals), we found higher rates of FAMI (0.63 ± 0.04) in the green toad populations and hence the grade 4, compared to the values of this indicator in the population of the marsh frog (0.54 ± 0.02) and grade 3 for the disorders in the developmental stability. In this study, the grades are the same in both species by the scale of Peskova, Zhukova (2007). The comparison (Table 3) shows that in conditions of long-term contamination of the environment in the early stages of their development the green toad reacts with more significant disorders of the genetic homeostasis in comparison with *P. ridibundus*. The main morphological features in color in *P. viridis* according to Chikin (2001) are formed in the first year of development and there are no further changes with age.

Table 3. Indicators of FA in *Pelophylax ridibundus* and *Pseudepidalea viridis* from the studied biotope (Range; Mean ± standard errors of means, Number of individuals).

Year	*Pelophylax ridibundus*			*Pseudepidalea viridis*		
	FAMI	**FAMF**	**Grade**	**FAMI**	**FAMF**	**Grade**
2009	0.30–0.70 0.53±0.02 34	0.41–0.71 0.53±0.03 10	3	0.50–0.70 0.57±0.01 32	0.31–0.66 0.57±0.03 12	3
2010	0.40–0.80 0.63±0.02 35	0.37–0.80 0.63±0.02 10	4	0.50–0.90 0.69±0.02 31	0.42–0.74 0.68±0.03 12	4
2011	0.50–0.80 0.63±0.01 32	0.47–0.81 0.63±0.02 10	4	0.50–0.90 0.67±0.02 29	0.45–0.72 0.67±0.03 12	4
Comparisons FAMI	2009/2010 t = 3.33*** 2010/2011 t = 0.0 2009/2011 t = 5.0***			2009/2010 t = 6.0*** 2010/2011 t = 0.67 – none 2009/2011 t = 5.0***		
	Pelophylax ridibundus / Pseudepidalea viridis					
	2009 t = 2.0* 2010 t = 2.0* 2011 t = 2.0*					

Note: * - $p < 0.05$ (significant); ** - $p < 0.01$ (more significant); *** - $p < 0.001$ (most significant); none - $p > 0.05$ (non-significant).

Conclusions

1. Based on the disorders found in the features for developmental stability in both species, we ascertain a persistent deterioration of the ecological situation in the dam lake "Studen Kladenets" with a tendency toward progressive deterioration of the living conditions as a result of the anthropogenic pollution.

2. Both *P. ridibundus* and *P. viridis* are good bioindication markers in conditions of syntopic habitats for assessing the ecological status of water by the method of fluctuating asymmetry. They clearly show features of developmental stability or disorders and, therefore, they objectively reflect the longtime changes in ecological conditions of the environment. Our work confirms that adult amphibians of various species, inhabiting during the mating period the same waters, can appropriately be used for bioindication purposes.

3. The indicators of fluctuating asymmetry in *Pseudepidalea viridis* are higher than those in *Pelophylax ridibundus* when they live together in conditions of continuous anthropogenic pollution.

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