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Original Contribution

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

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ABSTRACT
This work presents data on the occurrence of colour polymorphism, sex ratio and age structure (among adult individuals actually involved in the breeding process) in populations of two tailless amphibian species – *Pelophylax ridibundus* and *Pseudepidalea viridis* inhabiting anthropogenically polluted biotopes in Southern Bulgaria. The habitats are adjacent to the reservoirs "Rozov Kladenets" (pollution of household waste origin: nitrite nitrogen, suspended solids and of industrial origin: coal dust, sulfates) and "Topolnitsa" (heavy metal pollution). It was established for both biotopes with *P. ridibundus* populations that the individuals with the *striata* morph predominate in both sexes. In *P. viridis* populations, in the two sexes, individuals with morphs A and C predominate. In the biotope contaminated with heavy metals (the "Topolnitsa" reservoir) the female individuals predominate in both tailless amphibian populations. In both biotopes the main part of the breeding marsh frogs are of the middle age group (2+). While in the "Rozov Kladenets" reservoir the males predominate in this age group, in the "Topolnitsa" reservoir predominate the females. In animals of the senior age groups in both biotopes predominate the females (mainly of the 3+age group). In *P. viridis* populations in both biotopes among the breeding animals (in both sexes) predominate individuals from the 2+ and 3+ age groups. In both amphibians, animals of the age groups 4 + and 5 + are very rare (females). It was found that under bad living conditions in ponds used for reproduction, the populations of both tailless amphibians grow better in the area of the "Rozov Kladenets" reservoir.

Key words: anthropogenic pollution, biomonitoring, marsh frog, green toad, *striata*, *maculata* morphs, sexual, age differentiation.

INTRODUCTION

Currently, anthropogenic pollution is a global phenomenon and world-wide, there are almost no areas unaffected by this problem.

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Amphibians, being consumers on several levels and inhabiting the border between two environments - land and water, are an important link in the food chain of an ecosystem. They are sensitive to changes in the environment and react with various modifications affecting both the functional systems of the organism and the population characteristics. This explains the increased interest in this class in recent years in the context of its use for the purposes of bioindication and biomonitoring (1-5).

The marsh frog (*Pelophylax ridibundus* Palas, 1771) and the green toad (*Pseudepidalea viridis* Laurenti, 1768) are tailless amphibians which are common in Southern Bulgaria (6-7). During the breeding season they often use the same reservoirs for laying their eggs. Usually, the marsh frog remains in the same reservoir in which its development has taken place after its metamorphosis (going periodically on dry land in the area of the lake). The green toad leaves it and continues its life on dry land. Referring to the reproduction characteristics of both tailless amphibians, we used the opportunity to perform a bioindicational evaluation of the ecological status of two reservoirs in Southern Bulgaria contaminated with different types of toxicants. The evaluation is parallel to the physicochemical analysis data and is based on the developmental stability indicators (fluctuating asymmetry) in the populations (8).

The objective of this work is to present the results from a parallel evaluation of colour polymorphism, sex ratio and age structure of a representative, random sample from the populations of two amphibian species (*P. ridibundus* and *P. viridis*) having their reproduction together in the same reservoirs (8) and inhabiting the areas around them.

The work is an integral part of our extensive research carried out in populations of both tailless amphibian species in Southern Bulgaria in the period 2009-2012. It aims to find out whether they can be introduced as test objects in the system of bioindication and biomonitoring. It is a natural continuation of a series of previous publications which describe the results (8-19).

MATERIAL AND METHODS

The area of investigation

The material for the work was collected in the spring of 2010 in the area located along the eastern banks of two reservoirs with different types of anthropogenic pollution in Southern Bulgaria: the biotopes are symbolically referred to in this work as Biotope 1 and Biotope 2. This was performed during the simultaneous reproduction of the two studied tailless amphibian species.

Biotope 1 – the “Rozov Kladenets” reservoir (42°8′34″ N, 25°54′11″ E, 3.6 km^2, 200 m above sea level) is located near the town of Galabovo. Its water is taken from Sazliyka River (expected Categories II and III: pollution from household waste). The reservoir is used for the needs of TPP “Brikel” and TPP “AES Galabovo” located nearby and burning lignite coal; the turbine cooling industrial waste waters flow into it. Ambient air in the area is contaminated with industrial coal dust and sulfur oxides, often going over the limit values (20). Biotope 2 – The “Topolnitsa” reservoir (42°27′18″ N, 23°59′54″ E, 9.6 km^2, 470 m above sea level) is situated on the river with the same name. The copper quarry complex “Aurubis” and CPC “Asarel Medet” are located in the river valley.

Data from physicochemical analysis done in the water ecosystems

Based on data from the annual report on the environmental status (waters) for the period 2000-2010 of the Executive Environmental Agency (EEA) (21) and the data of the physicochemical analysis of the waters in the dams “Rozov Kladenets” and “Topolnitsa” for 2010 in the newsletters of Basin Directorate of water management in the East Aegean Sea – Plovdiv (22), it is clear that toxicants exceeding the permissible standards for the country Category I (clean) are permanently present in the two basins. Their values also exceed the expected Category II (slightly contaminated) and Category III (normally contaminated) water categories under Regulation № 7/08.08.1986 on indicators and standards for running waters in Bulgaria (23).The main pollutants in the “Rozov Kladenets” reservoir are nitrite nitrogen, sulfates and suspended solids, and in the “Topolnitsa” reservoir – heavy metals (copper, iron, manganese, lead, arsenic) **(Table 1).**

Subject of study and methods of analysis

The subjects of the study are *P. ridibundus* and *P. viridis*, two of the most flexible and tolerant to anthropogenic pressure tailless amphibian species (15-18, 24-27). The taxonomy used in this work is according to (7) *P. ridibundus* is closely connected to its habitat and spends its entire life near or within a small radius of the basin used for reproduction; the *P. viridis* is inclined to migrations. In both biotopes studied by us (around the reservoirs) there are large open areas without any major obstacles for the amphibians. Around the “Topolnitsa” reservoir lies an area of several tens of km^2 covered with low grass and reaching the nearby village of Poybrene.

Table 1. *Current data on the reservoirs "Rozov Kladenets" (Biotope 1) and "Topolnitsa" (Biotope 2) at the time of the study (physicochemical analysis – surface water sample).*

Parameters	Units SI	Ordinance №7/8.8.1986г Category			Biotop1 (2010)	Biotop2 (2010)
		I	III	II.	4.1	5.1
pH	pH units	6.5-8.5	6.0-9.0	6.0-8.5	8.04	7.82
Temperature	°C	Up to 3˚from the seasonal average			15.9	12.5
Insoluble substances	mg/dm^3	30	100	50	81.0*	1.51
Conductance	μS/cm	700	1600	1300	1706**	468
Dissolved oxygen	mgO_2/dm^3	6	2	4	6.1	5.92
Oxygen saturation	%	75	20	40	76.5	63
BOD_5 (biological oxygen demand)	mgO_2/dm^3	5	25	15	6.9	–
COD (chemical oxygen demand)	mgO_2/dm^3	25	100	70	34.6	–
Ammonium nitrogen ($N\text{-}NH_4$)	mg/dm^3	0.1	5	2	1.92	–
Nitrate nitrogen ($N\text{-}NO_3$)	mg/dm^3	5	20	10	2.4	–
Nitrite nitrogen ($N\text{-}NO_2$)	mg/dm^3	0.002	0.06	0.04	0.079**	0.005
Orthophosphate	mg/dm^3	0.2	2	1	–	0.004
Total nitrogen	mg/dm^3	1	5	10	–	–
Total phosphorus - as P	mg/dm^3	0.4	3	2	–	–
Sulphates (SO_4^{2-})	mg/dm^3	200	400	300	568**	111
Iron - total (Fe)	mg/dm^3	SKOS-0.1			< 0.001	0.21″
Manganese (Mn)	mg/dm^3	SKOS-0.05				0.22″
Copper (Cu)	mg/dm^3	SKOS-0.022				0.06″
Arsenic (As)	mg/dm^3	SKOS-0.25				0.003
Lead and its compounds (Pb)	mg/dm^3	SKOS-0.0072				0.005
Nickel and its compounds (Ni)	mg/dm^3	SKOS-0.02				0.05″

* Above the admissible concentration limit for category II, ** above the admissible concentration limit for category III, ″ above SKOS - assessment index: very poor condition, – no measurements done.

Close to the "Rozov Kladenets" reservoir, as noted above, is located TPP "Brikel" where due to the continuous process in the evening there are many large lit spaces attracting insects (basic food of amphibians). It is likely that adult *P. viridis* individuals inhabit quite a large perimeter (including the nearby settlements, the town of Galabovo and the village of Obruchishte near Biotope 1 and the village of Poybrene near Biotope 2) but concentrate around the two reservoirs namely during the reproduction period. Since the objectives of this study did not include producing a quantitative report on the breeding animals in the studied reservoirs but presenting the relative status of colour polymorphism and sex ratio in populations of breeding amphibians of the two species, the summing of the animals was performed once (near the "Rozov Kladenets" reservoir on 16.05.2010 and on 21.05.2010 in the area of the "Topolnitsa" reservoir, respectively) as a kind of a modified "report on the test sites" (according to 28-29). At the end of April, on the eastern banks of both reservoirs were formed numerous small and shallow temporary ponds with an area of several square meters and a depth of 10-25 cm. For the "Rozov Kladenets" reservoir these ponds were in the area of the confluence of the

TPP "Brikel" effluent, and for the "Topolnitsa" reservoir – in the area of the confluence of the river "Topolnitsa". In the period of active reproduction, which began in late April for the marsh frog, and in about a week later for the green toad, in these temporary reservoirs numerous individuals of both species reproduced themselves. The special nature of these small flooded areas and the shallow water in them allowed a single total collection of the amphibians, including the breeding ones within the area of the shallow areas (1-1.5 m away from the coast) on the eastern shores of the two lakes. The total area of collection for both studied biotopes was about 1-1.5 km^2. The catch of *P. ridibundus* was performed in the evening (19-22 h.) using a bag and an electric lamp, and of *P. viridis* – in the light part of the day as well (12-16 h), the air temperature in both cases being above 15°C. The division into age groups was not performed using the osseous chronological method (method for determining the age by analysis of bone sections) because of local (30) and European legal Directives (31-33) for animal protection which include both studied amphibian species. The method based on the body dimensions measurement, which also gives good results (34-36), was used to separate the animals into age groups. All captured breeding animals were adults: Snout-Vent Length > 60.0 mm (according to 37). The animals were divided into a female and a male group based on their secondary sex characteristics – lumps on the first finger and sac-resonator in the corners of the mouth in males. The colour polymorphism in populations of both tailless amphibian species was determined based on variations in the coloration of the dorsal side of the body. For the marsh frog, the distinction of animals was performed based on the presence of a longitudinal dorsal-medial stripe – the *striata* morph and its absence (*non striata*); spotted phenotype: the *maculata* morph (38-39). For the green toad in Southern Bulgaria are established 4 colour morphs: with an even background: 1) light or 2) dark; green spots against this background: a) individual, small or b) merging. Accordingly, the following 4 morphs can be distinguished: A – light back background, individual spots; B – light back background, merging spots; C – dark back background, individual spots; D – dark back background, merging spots (4). These designations are used further in our work. After the analyses, the animals were returned to nature.

For the purposes of the study were processed in total 273 *P. ridibundus* individuals – in Biotope 1: 166 (71 ♂, 95 ♀); in Biotope 2: 107 (25 ♂, 82 ♀); and 218 *P. viridis* individuals – in Biotope 1: 133 (48 ♂, 85 ♀); in Biotope 2: 85 (37 ♂, 48 ♀).

Statistical procedures

All numerical data on the distribution of individuals with the relevant colour morphs and the gender comparisons in the populations of both tailless amphibian species were processed statistically by means of χ^2 – the Pearson criterion. The method of pair comparisons was used (at significance level $\alpha = 0.05$), which is included in the software package "STATISTIKA 7.0" (40). The descriptive statistics for each size and age groups of both tailless amphibians includes a minimum and maximum value (Range), mean value and error (Mean ± SEM).

RESULTS AND DISCUSSION

The results of the comparison between individuals of the two tailless amphibian species based on the different colour morphs in Biotopes 1 and 2 are shown in **Table 2.**

Overall in *P. ridibundus* populations, individuals with the *striata* morph dominate in both biotopes. Despite the higher percent age of animals with this morph in Biotope 2 (80.37%), the ratio of males to females here shows no statistically significant difference – individuals with the *striata* morph were three times higher than individual of the corresponding sex with the morph *maculata*. The females prevail over the males by the same quantity in both morphs. The statistically significant difference in the ratio of males to females with the *striata* and *maculata* morphs in Biotope 1 is due to definite predominance of female individuals with the *striata* morph (nearly three times more than females with the *maculata* morph and about twice as many as the total count of the males with both morphs). Overall, a statistically significant reduction is observed in males from both morph types in the two biotopes, as well as in the females with the *maculta* morph in Biotope 2.

Table 2. *Frequency of occurrence of colour morphs in populations of Pelophylax ridibundus and Pseudepidalea viridis in the studied biotopes in Southern Bulgaria.*

Biotopes	n	Morphs: *striata* n (♂/♀)	*striata* Morph share %	*maculata* n (♂/♀)	*maculata* Morph share %	Sex ratio of the morphs %: *s* ♂	*m* ♂	*s* ♀	*m* ♀	χ^2
1	166	112 41/71	67.47	54 30/24	32.53	24.70	18.07	42.77	14.46	5.33*
2	107	86 19/67	80.37	21 6/15	19.63	17.75	5.61	62.62	14.02	0.39 ns
χ^2	11.63**									

Pseudepidalea viridis

Biotopes	n (♂/♀)	Morph	n (♂/♀)	Morphs share % ♂	Morphs share % ♀	χ^2
1	133	*A*	21/49	43.75	57.65	2.54 ns
		B	6/7	12.50	8.23	
		C	16/23	33.33	27.06	
		D	5/6	10.42	7.06	
2	85	*A*	12/26	32.43	54.17	4.48 ns
		B	10/7	27.03	14.58	
		C	9/8	24.32	16.67	
		D	6/7	16.22	14.58	
χ^2	15.96 ns					

* $p<0.05$, ** $p<0.01$, ns - $p>0.05$.

Literature data show that in living conditions of increased anthropogenic pressure *P. ridibundus* individuals with the *striata* morph survive better than those with the *maculata* morph. This is established for urbanized environments (41-42) and for anthropogenically contaminated biotopes (9, 43-46). The results obtained in this study support this view. In both studied biotopes in both sexes dominate animals with the *striata* morph. But it is clear that in the presence of toxicants of different types, mortality of males is higher within this morph. The resistance of animals of both sexes is higher within the *striata* morph in Biotope 2. An interesting fact is that the literature provides evidence of cases where the ratio of individuals with the striped to those of the non-striped morph is almost equal and even of cases of reduction of the individuals with the *striata* morph in anthropogenically transformed habitats (47-48). In most of these cases, however, the local populations live in specific environmental conditions or in isolates in which microevolutionary processes take place. Such is the case of the population of the pond-settler of TPP “Brikel” in which the "bottleneck effect" is observed (49).

For the other studied species – *P. viridis*, which breeds in both studied biotopes, the results do not detect statistically significant differences in the number of individuals of the four morphs in both sexes in each Biotope and also when comparing the two biotopes (Table 2). The distribution of individuals with light background morphs (A+B) and ones with dark background morphs (C+D) is roughly even among males (56.25/43.75 for Biotope 1 and 59.46/40.54 respectively for Biotope 2). In females from both biotopes, proportions of light to dark background is approximately equal (65.88/34.12 for Biotope 1 and 68.75/31.25 for Biotope 2), the light background being a little more but the difference was not statistically significant. However, it is noteworthy that the presence of individuals with the morph A and C among both sexes in both biotopes is higher than the rest of the morphs (more explicit in the Biotope 1). In our previous works (10-11), we showed that *P. viridis* individuals from these two morph types are statistically significantly prevalent in areas with anthropogenic pollution. We expressed the opinion that animals of both sexes with morphs A and C probably have some selective advantages compared to individuals with the other two morphs of this species, allowing their easy adaptation and survival in anthropogenically transformed environment (12). The established proportions concerning the distribution of the colour morphs among green toad individuals from the biotopes studied in this work once again highlight the greater stability of animals with the morphs A and C under conditions of anthropogenic pollution. These proportions also refer to the toxicant type and the degree of contamination of the pond used for breeding. On the other hand, should be taken into account that the green toad spends most of its life as a terrestrial species, i.e. the effect of the ambient air pollutants should be considered. The results connected to the other indicator studied by us – sex ratio of both tailless amphibians populations in the breeding period are shown in **table 3.**

Table 3. *Number of sexually mature individuals (males – numerator, females – denominator) in populations of Pelophylax ridibundus and Pseudepidalea viridis during the breeding season of 2010 in the studied biotopes in Southern Bulgaria. The number of males is set to one.*

Pelophylax ridibundus			
Biotopes	Number of male and female individuals: Absolute value	Number of male and female individuals: Relative value	χ^2
1	71/95	1/1.3	3.46 ns
2	25/82	1/3.3	30.36***
χ^2	10.75***		
Pseudepidalea viridis			
1	48/85	1/1.8	10.30***
2	37/48	1/1.3	1.42 ns
χ^2	1.21 ns		

*** $p<0.001$, ns - $p>0.05$.

In Biotope 1 the ratio of male to female *P. ridibundus* individuals is not different to the theoretically expected 1:1 ratio, whereas regarding the other species – *P. viridis*, it is statistically significantly indicated that the females are three times as much as the males. The opposite situation is reported in Biotope 2: the individuals of both sexes of *P. viridis* are equally spread, where as in *P. ridibundus*, the females are statistically significantly twice as much as the males.

The literature (50-51) shows that a 1:1 ratio (which contributes to higher reproductive potential, maximum meeting chances between individuals of the opposite sex during the breeding period and reduces the degree of inbreeding) is characteristic of populations living in stable environmental conditions. The deviation from this ratio is often found in anthropogenically transformed areas whereby the males are eliminated in most cases (this is justified from a biological point of view regarding the preservation of the reproductive performance and the genetic pool of the population). Similar data are presented for populations of *P. ridibundus* (44, 52-53) and also for *P. viridis* populations (13, 54-57) from anthropogenically polluted biotopes.

The data relating to the size and age composition of breeding individuals in populations of both tailless amphibians in from Biotopes 1 and 2 are of interest and are shown in **Tables 4 and 5**.

Table 4. *Characteristics of the size and age structure of breeding individuals in the populations of the two tailless amphibian species in Biotope 1 (the "Rozov Kladenets" reservoir) in Southern Bulgaria.*

Species	Indicator		Age Group											
			2+			3+			4+			5+		
Pelophylax ridibundus	L (♂±♀)	Range	61.5-70.0			70.5-85.0			86.0-91.5			92.0-100.0		
		Mean±SEM	63.72±0.03			78.43±0.04			88.51±0.05			96.43±0.05		
	Share in the population %		♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀
			36.14	13.86	50.0	6.63	22.89	29.52	–	12.65	12.65	–	7.83	7.83
Pseudepidalea viridis	L (♂±♀)	Range	62.5-71.5			72.0-84.6			85.0-92.4			–		
		Mean±SEM	65.71±0.04			80.5±0.03			87.43±0.05			–		
	Share in the population %		♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀
			12.03	18.05	30.08	19.55	27.82	47.37	4.51	18.04	22.55	–	–	–

Table 5. *Characteristics of the size and age structure of breeding individuals in the populations of the two tailless amphibian species in Biotope 2 (the "Topolnitsa" reservoir) in Southern Bulgaria.*

Species	Indicator		Age group								
			2+			3+			4+		
Pelophylax ridibundus	L (♂±♀)	Range	61.0-70.0			71.0-85.0			86.0-89.0		
		Mean±SEM	62.83±0.03			77.41±0.03			87.41±0.05		
	Share in the population, %		♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀
			16.82	36.45	53.27	6.54	31.78	38.32	–	8.41	8.41
Pseudepidalea viridis	L (♂±♀)	Range	61.5-70.0			71.0-83.0			86.5-91.0		
		Mean±SEM	62.46±0.03			75.77±0.03			88.63±0.03		
	Share in the population, %		♂	♀	♂±♀	♂	♀	♂±♀	♂	♀	♂±♀
			22.35	11.76	34.11	21.18	31.77	52.95	–	12.94	12.94

In both reservoirs the majority (more than 50%) of the breeding marsh frogs are of average age (2+). But while in Biotope 1 in this age group predominate males (36.14%), in Biotope 2 females prevail (36.45%). In animals of the older age groups the considerable percentage of individuals in age group 3+ makes an impression. Nevertheless, in both biotopes it is at the expense of expressed presence of females. In Biotope 1 the marsh frog individuals reach 4+ and 5+ years of age, in Biotope 2+–4+ years but in both biotopes these are only females constituting an insignificant share of the population.

In *P. viridis* populations in both biotopes is observed a similar situation: among breeding animals the majority is aged 2+ and 3+ and the distribution of males and females in each age group is approximately proportional. Single 4+-year-old animals were seen (in Biotope 2 – only female individuals).

Data from the literature suggest that the age structure of the population of tailless amphibians changes significantly with the increase of the degree of contamination of water basins: the maximum life expectancy is reduced and the share in the 2+–3+-year-old animals (58-60). The established sex ratio in populations of *P. ridibundus* and *P. viridis* and the characteristics of the age composition of the populations living in conditions of sympatric and syntopic in both anthropogenically polluted biotopes in Southern Bulgaria once again gives us grounds to assert that the nature of the toxicants has an essential role in the environmental tolerance. In Biotope 1 the population of *P. ridibundus* inhabiting reservoir polluted mainly by nitrates and sulfates (Table 1), practically preserves its relatively stable condition, while the population inhabiting Biotope 2, which is contaminated by heavy metals, is in ‘intense environmental situation’ – females markedly dominate in it (males of all age groups get eliminated).

On the other hand, against the background of the relatively steady state of the *P. viridis* populations in both biotopes, the reason for the predominance of females in the population breeding in the Biotope 1 must be sought in anthropogenic pollutants present in the ambient air in this region.

Based on the population characteristics test results, obtained in this study for the two tailless amphibians living in conditions of sympatric and syntopic, and on the basis of previously obtained (8) indicators for the sustainability of development (fluctuating asymmetry) in *P. ridibundus* and *P. viridis*, we can conclude that the living conditions in both basins are worsened - but the conditions in the “Rozov Kladenets” reservoir are better than those in the “Topolnitsa” reservoir.

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