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ECOLOGICAL STATUS OF THE SAZLIYKA RIVER AND ITS TRIBUTARIES (BLATNITSA AND SOKOLITSA) AS INDICATED BY THE COLOR POLYMORPHISM AND SEXUAL COMPOSITION OF THE POPULATIONS *PELOPHYLAX RIDIBUNDUS* (AMPHIBIA: RANIDAE)

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Abstract

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The present work studies the nature of the distribution of colour polymorphism and the sexual composition (among adult, sexually mature individuals involved in the breeding process) in populations of marsh frog (*Pelophylax ridibundus*) inhabiting different biotopes along one of the ecologically damaged rivers in Bulgaria (the river Sazliyka) and two of its left-side tributaries (the rivers Blatnitsa and Sokolitsa). The ecological conditions and the quality of the living environment were assessed based on these two population characteristics. It was found out that in habitats located in the upper reaches of the rivers Sazliyka and Sokolitsa, in the populations of *P. ridibundus* the two colour morphs of the coloration of the back (*striata*, *maculata*) are equally represented. At the same time, the ratio of individuals of both sexes is close to the theoretically expected 1:1 distribution in relatively clean waters. In habitats along the middle and lower reaches of the rivers Sazliyka and Sokolitsa and the one of the river Blatnitsa, the environmental conditions are highly impaired: in the populations of *P. ridibundus,* the dominant morph *striata* is expressed. Moreover, the sex ratio has changed resulting in a predominance of the females.

Key words: bioindication, *Pelophylax ridibundus*, morphs: *striata*, *maculata*, sexual composition, river Sazliyka

Introduction

One of the latest trends in modern ecology is the study on the formation and sustainability of ecosystems in areas subject to intense anthropogenic pressure. Worldwide, their size is constantly growing and today there are almost no landscapes left anthropogenically unchanged. Biota of such ecosystems respond to anthropogenic stressors with adaptive changes affecting both individual and population characteristics. Studying them provides us not only with an insight into the mechanisms of biodiversity modification, but also allows the development of indirect methods for assessment of the the environment damage.

Amphibians are a group of animals which is very susceptible to anthropogenic influence (Peskova, 2001; Nikashin, 2005; Vershinin, 2007; Zhelev, 2012a). The condition of their organism fully reflects the state of the environment, which makes them very convenient to act as bioindicators (Peskova, 2007; Zhelev, 2007, 2012b; Korzh et al., 2012; Zhelev et al., 2012, 2013a, 2013b, 2014a, 2014b, 2014c; Franco-de Sá and Val, 2014). Particular attention in this respect deserves the marsh frog *Pelophylax ridibundus* Pallas, 1771 (Speybroeck et al., 2010), a species strongly tied to the reservoir (rarely moves away and usually spends its life close to the breeding place) and at the same time tolerant to the anthropogenic stressors (Misyura and Sporadets, 2005; Zhelev et al., 2006, 2013b,

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2014c). It is known that under the conditions of anthropogenic pressure occur changes which affect the physiological state, the ratio of male to female individuals and the phenotype composition of populations (Vershinin, 2007; Zamaletdinov et al., 2008; Zaripova et al., 2009; Spirina, 2010; Zadorozhnjaja, 2013; Zhelev, 2011a, 2012a, 2014a, 2014c).

From the complex European green frogs (*Pelophylax esculentus* complex Linnaeus, 1758) the marsh frog *P. ridibundus* is a widely spread species in Bulgaria and it inhabits throughout the country; *P.* kl. *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; Stojanov et al., 2011).

The aim of this work was to study, along with the manifestations of fluctuating asymmetry (Zhelev et al., 2013b), the nature of colour polymorphism and sexual composition (among adult, sexually mature individuals involved in the reproduction) in the popuations of *P. ridibundus* inhabiting various biotopes along one of the ecologically damaged rivers in Bulgaria (the river Sazliyka) and two left-side tributaries (the rivers Blatnitsa and Sokolitsa). The ecological condition was assessed based on the characteristics of both populations and, parallelly and independently of the data of the physicochemical analysis, was made an assessment of the state of their living environment. In Bulgaria, a similar study is carried out for the first time.

Materials and Methods

The studies were conducted in the spring and summer of 2010 and 2011. In 2010, we studied seven biotopes situated on the left bank downstream the river Sazliyka from the village of Rakitnitsa to the mouth of the river to the south of the town of Simeonovgrad. And in 2011, we studies biotopes located on the left banks of two of the biggest tributaries: the rivers Blatnitsa and Sokolitsa (respectively biotopes 1 and 4) (Figure 1).

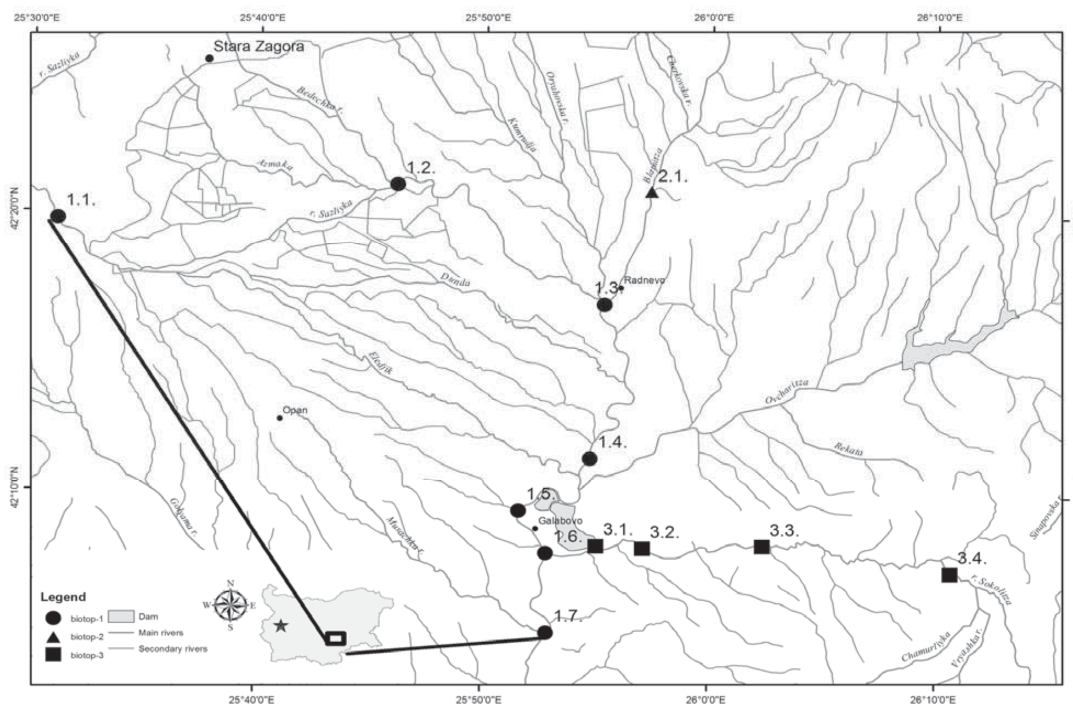

Fig. 1. Map of the investigated biotopes. Biotopes: 1.1 – the river Sazliyka downstream of the village of Rakitnitsa; 1.2 – the river Sazliyka downstream of the town of Stara Zagora (after the confluence with the river Bedechka); 1.3 – the river Sazliyka in Radnevo (downstream of the confluence with the river Blatnitsa) 1.4 – the river Sazliyka at the village Lyubenovo; 1.5 – north of the town of Galabovo (under the sluices surrounding the river to lead part of its flow towards "Brikel TPP"); 1.6 – the river Sazliyka in Galabovo (downstream of the confluence with the Sokolitsa River) 1.7 – the river Sazliyska to the mouth at the river Maritsa, 2.1 – the river Blatnitsa in Lyubenova Mahala; 3.1 – the river Sokolitsa in Obruchishte; 3.2 – dam "Rozov Kladenets"; 3.3 – the river Sokolitsa at the village Iskritsa; 3.4 – the river Sokolitsa near the village of Orlov Dol

Table 1
Recent data on the status of biotopes at the time of the study: Physicochemical analisis – surface water sample: mean annual parameters

Parameters	SI	Reg. 7/ 8.8.1986 category II	Reg. 7/ 8.8.1986 category III	year	1.1	1.2	1.3	1.4	1.6	1.7	2.1	3.1	3.2	3.3	3.4
pH	pH units	6.5-8.5	56.0-9.0	2010	8.34	7.8	7.8	7.97	7.97	7.9	8.07	7.64	8.04	8.21	7.96
				2011	8.06	7.75	7.7	7.81	7.88	7.9	7.86	7.91	8.21	8.01	7.82
Temperature	°C	Up to 3°from the seasonal average		2010	13.9	15.7	14.1	13.6	14.3	14.3	13.5	15.3	15.9	15.7	13.3
				2011	15.1	15.3	14.9	14.33	14.9	15.5	14.5	16.7	15.1	14.7	14.2
Insoluble substances	mg/dm^3	50	100	2010	5.8	16.8	31.8	31	64.0*	39	56.75*	25.67	81.0*	62.21*	3.6
				2011	6	25.8	41.8	56.5*	52.0*	64.0*	50.75*	21.1	83.28	56.3*	3.3
Conductance	µS/cm	1300	1600	2010	751.5	612	643	924	955	861	1295	2082**	1706**	1437*	820
				2011	891	612	596	912	945	1021	1286	2740**	1731**	1372*	834
Dissolved oxygen	mgO_2/dm^3	4	2	2010	8.1	7.1	5.2	4.95	6.1	6.4	6.98	7.57	6.1	6.6	9.6
				2011	7.88	5.3	5.5	4.88	5.9	5.8	6.13	7.73	6.6	7.2	9.4
Oxygen saturation	%	40	20	2010	81.5	52.2	52.3	48	59	61	66	72	76.5	61.8	86.3
				2011	74	52.8	56.5	48	57	59	64	73	75.3	60.9	88.3
$BCO_{5(biologic\ consumption\ of\ oxygen)}$	mgO_2/dm^3	15	25	2010	3.2	11	18.8*	8.06	10.5	12	7.54	6.3	6.9	2.45	3.1
				2011	1.8	9.7	9.3	10.35	7	8.2	4.69	6.21	6.4	2.34	3
$CCO_{(chemical\ consumption\ of\ oxygen)}$	mgO_2/dm^3	70	100	2010	4.9	30	66.2	44	54	55	29.6	41.7	34.6	57.5	3.9
				2011	6.7	29.1	42.2	43	37	40	26.2	37.4	33.7	47.2	4.1
Ammonium nitrogen $N\text{-}NH_4$	mg/dm^3	2	5	2010	0.07	2.18*	2.2*	1.04	0.6	0.301	2.04*	1.84	1.92	0.318	0.02
				2011	0.079	1.76	1.3	1.89	0.349	0.264	0.176	1.28	1.79	0.47	0.02
Nitrate nitrogen $N\text{-}NO_3$	mg/dm^3	10	20	2010	2.1	1.67	2.2	2.675	2.97	3	4.7	5.02	2.4	2.9	0.93
				2011	1.2	1.42	1.2	1.78	1.97	2.4	2.3	4.87	2.7	3.71	0.75
Nitrite nitrogen $N\text{-}NO_2$	mg/dm^3	0.04	0.06	2010	0.016	0.165**	0.2**	0.175**	0.148**	0.09**	0.235**	0.127**	0.079**	0.054*	0.011
				2011	0.012	0.138**	0.149**	0.158**	0.108**	0.087**	0.09**	0.1**	0.092**	0.042*	0.009
Orthophosphate P	mg/dm^3	1	2	2010	0.045	0.484	0.46	0.351	0.358	0.32	1.78*	–	–	1.09*	0.025
				2011	0.316	0.449	0.443	0.718	0.94	0.449	0.194	–	–	0.792	0.019
Total nitrogen	mg/dm^3	5	10	2010	2.4	6.0*	5.3*	6.3*	6.54*	5.4*	6.43*	–	–	–	–
				2011	1.8	5.43*	5.2*	6.3*	–	4.43	3.99	–	–	–	–
Total phosphorus - as P	mg/dm^3	2	3	2010	0.07	0.585	0.734	0.516	0.523	0.546	0.369	–	–	–	–
				2011	0.303	0.63	0.43	0.79	1.377	0.63	0.167	–	–	–	–
Sulphates	mg/dm^3	300	400	2010	–	–	55.7	181	281	178	306	578.0**	568.0**	484	–
				2011	–	–	57.8	247	46.3	–	450.0**	119.0**	573.0*	423.0*	–

* (above the maximum for category II), ** (above the maximum for category III), – (no measurement were made)

The river Sazliyka has a total length of 145.4 km – from the spring (Sarnena Sredna Gora) to the mouth (Maritsa River to the south of Simeonovgrad) and catchment area of 3300 km^2. Two of the large left-side tributaries flow in to the south of the towns of Radnevo (the river Blatnitsa, 54 km long) and Galabovo (the river Sokolitsa, 60.5 km long).

Based on the data of the annual report of the state of the environment (water) for the period 2001-2011, the Executive Environment Agency (http://eea.goverment.bg) and the data from the physical and chemical analysis of the water in the river Sazliyka (2001 to 2011) from the bulletins of the Basin Directorate of water management. In the region of the Eastern White Sea – Plovdiv, the river is considered one of the most polluted in Bulgaria exceeding the allowed norms for the country – Category I (clean), the expected Category II (slightly contaminated) and Category III (moderately contaminated) under Regulation № 7 of 8.08.1986 on indicators and standards for running waters in Bulgaria (SG, issue 96/12.12.1986). The main pollutants are nitrate nitrogen, phosphates, BOD_5 and suspended solids (Table 1). Along the river were located two "local hot spots" of national importance: the first following the town of Stara Zagora at the mouth of the river Bedechka and the second after Radnevo at the mouth of river Blatnitsa (Irikov and Atanassova, 2008). The river flows of Bedechka and Blatnitsa are consist almost entirely of household and fecal waste and industrial wastewater of respectively the towns of Stara Zagora (12 km of extremely badly affected area) and Nova Zagora (the whole stream following the town of Nova Zagora).

The subject of study is the marsh frog *P. ridibundus*. The animals were collected entirely in the evening, in the water and on the banks, using an electric torch. Sections with a length of 1 km and a width of 4 m of coastline were covered along the river after the respective inhabited areas (according to Sutherland, 2000). The studied biotopes of the river Sazliyka are conventionally numbered as follows: biotope 1.1 – to the south of the village of Rakitnitsa; 1.2 – the flow after the city of Stara Zagora and the mouth of the river Bedechka; 1.3 – after the town of Radnevo and the mouth of river Blatnitsa; 1.4 – following the village of Lyubenovo; 1.5 – north of the town of Galabovo (under the sluices surrounding the river to lead part of its flow towards "Brikel TPP"); 1.6 – south of the town of Galabovo following the confluence of river Sokolitsa; 1.7 – south of the village of Kalugerovo to its confluence into the river Maritsa. The researched population of the river Blatnitsa (2.1) inhabits the area south of the village of Lyubenova Mahala, and the studied biotopes of the river Sokolitsa are located north of the settlements along the river as follows: biotope 3.1 – the dam "Rozov Kladenets"; 3.2 – the village of Obruchishte; 3.3 – the village of Iskritsa; 3.4 – the village of Orlov Dol.

The distance between the biotopes is at least 12 km – 15 km and at most 40 km – 42 km in straight line, which excludes the exchange of animals between them. The analysis was performed on living animals after which they were returned back to nature. The determination of the age was based on the body size and the sex was determined on the basis of the degree of markedness of secondary sexual characteristics in males (lumps on the first finger and sac-resonator in the corners of the mouth). All test animals were adults (Snout-Vent Length > 60.0 mm, according to Bannikov et al., 1977) and sexually mature. The classification of animals according to the coloration of their backs was made based on the presence of longitudinal dorso-medial stripe – the morph *striata* and the absence of it (*non striata*) which is expressed by as potted phenotype: the morph *maculata* (Borkin and Tihonenko, 1979; Lada and Sokolov, 1999). Parallelly for each sample, we obtained the data (frequency of the asymmetric manifestation in an individual and the frequency of asymmetric manifestations of an indicator) connected to the integral indicator of the stability of development – fluctuating asymmetry FA (Zhelev et al., 2013b).

The *P. ridibundus* is listed in Appendix 4 to 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, capture permits for *P. ridibundus* are not issued if in use for scientific research.

The digital material was processed using the method of the pair comparisons based on χ^2 Pirson's, using the Statistical 7.0. Software (Statistica 2004).

Results and Discussion

The polymorphism in the groups of *P. ridibundus* related to the presence or absence of a central light stripe on the back only recently became used as a bioindication marker for assessing the condition of the environment. The analysis of the genetic nature of this indicator in the Ranidae (Rafinesque-Schmaltz, 1814) family shows that the morph *striata* is a monogenic mutant. The dominant allele of the diallelic autosomal gene *striata* determines the presence of stripe (complete dominance). This manner of inheritance is found out for *Rana arvalis* Nilsson, 1842 (Ishchenko and Shtupak, 1974; Shtupak, 1987) and *P. ridibundus* (Berger and Smielowski, 1982). The peculiarities of inheritance of this trait make it a convenient marker for monitoring changes in the genetic structure of populations.

In different species from the Ranidae family it is found out that for animals with the morph *striata* the total level of oxidation-reduction reactions and the amount of haemoglobin is higher, while the permeability of sodium in the skin is twice as low compared to those with the morph *maculata* (Vershinin, 2007). According to Sils (2008) the rate of the processes of physiological adaptation (a result of hereditary characteristics in haemopoiesis) in the animals with the morph *striata* is higher and is one of the reasons for the successful existence in unstable conditions and anthropogenically modified environments.

The related publications provide evidence that in relatively clean waters the ratio of sexually mature individuals of non-striped and striped frogs of the genus *Pelophylax* (Fitzinger, 1843), is close to equal to animals with the morph *maculata* or the latter are slightly predominant (Vershinin, 2004). In parallel, it is shown that with increasing levels of anthropogenic pressure on sexually mature individuals of *P. ridibundus*, the share of the morph *striata* increases (Fayzulin, 2004; Belova, 2009; Peskova and Zhelev, 2009; Vershinin, 2007, 2008; Zaripova et al., 2009; Zhelev, 2011a, 2012a, 2014a, 2014c).

Data on the distribution of both morphs in populations of *P. ridibundus* from various biotopes in the basin of the river Sazliyka and its two left-side tributaries (the rivers Blatnitsa and Sokolitsa) are shown in Table 2. Table 3 reflects the data obtained after the comparison using the criterion χ^2. Biotopes were divided according to the results of the physical-chemical analysis (Table 1) and evaluated based on the integral indicator of developmental stability – fluctuating asymmetry in populations of *P. ridibundus* in our previous work (Zhelev et al., 2013b) with a grade I – II (relatively clean) and those with the grade III – V (contaminated).

Table 2
Frequency of manifestation of the two morphs (*striata/maculata*) in the populations of *Pelophylax ridibundus* in the studied biotopes

Biotopes*		n	Category according to FA (Zhelev et al., 2013c)	striata		maculata		Sex composition			
				n (♂\♀)	%	n (♂\♀)	%	s ♂	m ♂	s ♀	m ♀
1	1.1	39	1(2)	13 8\5	33.33	26 15\11	66.67	20.51	38.46	12.82	28.21
	1.2	35	5	30 7\23	85.71	5 1\4	14.29	20	2.86	65.71	11.43
	1.3	26	5	24 6\18	92.31	2 0\2	7.69	23.08	0	69.23	7.69
	1.4	37	5	32 8\24	86.49	5 2\3	13.51	21.62	5.41	64.86	8.11
	1.5	34	4	24 9\15	70.59	10 7\3	29.41	26.47	20.59	44.12	8.82
	1.6	30	5	24 6\18	80	6 3\3	20	20	10	60	10
	1.7	38	4	25 11\14	65.79	13 7\6	34.21	28.95	18.42	36.84	15.79
2	2.1	30	5	27 5\22	90	3 0\3	10	16.67	0	73.33	10
3	3.1	32	3	19 8\11	59.37	13 7\6	40.63	25	21.87	34.38	18.75
	3.2	36	3	22 8\14	61.11	14 10\4	38.89	22.22	27.78	38.89	11.11
	3.3	36	2	16 9\7	44.44	20 12\8	55.56	25	33.33	19.45	22.22
	3.4	38	1	11 9\2	28.95	27 17\1	71.05	23.68	44.74	5.26	26.32

* (1 – the river Sazliyka), (2 – the river Blatnitsa), (3 – the river Sokolitsa)

Table 3
Comparisons between populations in relatively clean and in contaminated biotopes along the rivers Sazliyka, Blatnitsa and Sokolitsa

Biotopes	n	Morphs								χ^2
		striata		*maculata*		Sex composition (%)				
		n (♂\♀)	%	*n* (♂\♀)	%	*s* ♂	*m* ♂	*s* ♀	*m* ♀	
Relatively clean (1.1; 3.3; 3.4)	113	40 26\14	35.4	73 44\29	64.6	23.01	38.94	12.39	25.66	0.24ns
Contaminated (1.2; 1.3; 1.4 1.5; 1.6; 1.7; 2.1; 3.1; 3.2)	298	227 68\159	76.17	71 37\34	23.83	22.82	12.41	53.36	11.41	11.63*
χ^2					69.36*					

* (p<0.001), ns (p>0.05)

In each of the relatively clean biotopes in the upper reaches of the rivers Sazliyka (1.1) and Sokolitsa (3.3, 3.4) the percentage prevalence of the spotted non-striped morph *maculata* is less (3.3) or more expressed (1.1, 3.4) in the populations. Regardless of the ratio in habitat 3.4 where the female spotted non-striped morph compared to the spotted one is 5:1, the overall ratio is 35.4% *striata* to 64.6% *maculata* and does not account for statistically significant differences in the distribution of the two morphs between individuals of different sexes in the habitats with low levels of anthropogenic pressure on environment (χ^2 = 0.24; P > 0.05). As a whole, in the biotopes in the currents of both rivers under stable and close to the optimum environmental conditions, both morphs in populations of *P. ridibundus* are evenly represented. In the biotopes assessed with the grade III – V (contaminated), considered both separately and as a whole, individuals with the striped morph *striata* prevail statistically significantly over those with the morph *maculata*. In the individual biotopes the ratio *striata/maculata* varies – from a ratio of 59.37% / 40.63% to 92.31% / 7.69%. In the merged sample the difference is 76.17 to 23.83% (χ^2 = 11.63; P < 0.001). In conditions of increased anthropogenic pressure in populations of *P. ridibundus* in all three rivers among individuals of both sexes we observe a statistically significant increase of the share of those with the morph *striata* (χ^2 = 69.36; P < 0.001). Similar to this data were the data collected for the upper reaches of the river Don (Nikashin, 2007) and for the river Sviyaga in the Ulyanovskaya area of the Russian Federation (Spirina, 2010), where the share of individuals with the morph *striata* reaches 75.3% – 80.2% of the populations.

Considering the literature data and the obtained results about the relationship between the individuals of both morphs of *P. ridibundus*, we can conclude that the upper streams of the rivers Sazliyka and Sokolitsa are relatively clean and the environmental conditions are good for the biota inhabiting them. In the middle and lower reaches of the three rivers (Sazliyka, Blatnitsa and Sokolitsa), the level of anthropogenic pollution is significant and leads to serious disturbances in the genetic homeostasis of the populations of *P. ridibundus*. This results in both an increase in the degree of fluctuating asymmetry (Zhelev et al., 2013b) and in the modification of colour polymorphism (dominated by the morph *striata*).

The sexual composition – the ratio of male to female individuals, is one of the major population characteristics. Theoretically, it should be close to 1:1, so that this equal share of the sexes in the reproductive part of the population ensures a maximum chance for the individuals of the opposite sex to meet during the breeding period and thus reduces the degree of inbreeding (Kalmus and Smith, 1960). Any alteration of the sex ratio plays a role in the processes of population regulation and significantly defines the role of the population in the ecosystem and its response to changing conditions of the environment (Bolshakov and Kubantsev, 1984). The literature describes few data on changes in the ratio of male to female individuals in populations of *P. ridibundus* inhabiting anthropogenically polluted biotopes. For example, in the ecologically clean river Usa in the area Ulyanovskaya of the Russian Federation, a ratio of sexually mature animals of the type 1:1 was indicated, while in the polluted river Sviyaga it has changed towards a bigger share of the females – 1:4.4 (Spirina, 2010). In the populations of the species living in conditions of high anthropogenic pressure was noted a shift among the ratio among the sexually mature individuals in favour of the females - industrial pollution: the river Chapaevka, Samara region of the Russian Federation (Fayzulin, 2004), in ponds with pesticide contamination in the Western Predkavkazie region in Russia (Peskova and Zhukova, 2007), and in conditions of urbanization (Zamaletdinov et al., 2008). Similar changes in gender structure were found in populations of the green frog (*Bufo viridis* Laurenti, 1768) – in Germany (Sinsch et al., 2007), Poland (Mazgalska, 2009), Bulgaria (Zhelev, 2011b, 2012a) and Turkey (Bilal et al., 2011) – from ratios close or equal to 1:1 for habitation in an environment of relatively stable conditions to ratios with apparent predominance of females in conditions of anthropogenic pollution. According to Peskova (2002), a reduction of the number of females can only cause harm to the populations of the amphibians, as it reduces the reproductive potential and makes their genetic structure poorer. In connection to the deficit of males, the author notes that their loss under the influence of unfavourable factors is somewhat useful, too, because in this case the fertility of the population suffers significantly less than in the case of an increase of the females. At the same time, a selection of genotypes resistant to adverse factors is carried out, contributing to the micro evolutionary processes.

Data on the ratio of male to female individuals in the populations in each habitat of the flow of the three rivers studied by us are presented in Tables 4 and 5 contains the data on the sex ratio in the total comparison of populations from contaminated and relatively clean biotopes.

In biotopes 1.1 and 3.3, the ratio of male to female individuals is close to the theoretically expected 1:1. In biotope 3.4, the males are statistically significantly 2.2 times more in number than the females ($\chi^2 = 5.15$; $P < 0.05$). The average data on the three relatively clean biotopes indicates a statistically significant predominance of males in them – 1.6 times ($\chi^2 = 6.46$; $P < 0.01$).

In anthropogenically polluted habitats (Table 1) it is noteworthy that in each of the them assessed with a grade III and IV (namely 3.2, 3.3, 1.5, 1.7) by the method of FA, the ratio of male to female individuals is close to 1:1 ($P > 0.05$), while in each of the habitats receiving a grade IV. The females predominate statistically significantly (expressed the most in biotopes 1.2 and 2.1, where the ratio is of the type 1:3.4 and respectively 1:5. Therefore, given the total dominance of female individuals in the polluted biotopes of 1:1.8 ($\chi^2 = 25.96$; $P < 0.001$), an impaired reproductive structure of populations occurs only at high levels of anthropogenic pressure.

Based on the received data on the ratio of male to female individuals in populations of *P. ridibundus*, we can make the following generalization: in the clear upper reaches of the rivers Sazliyka and Sokolitsa and in areas with low anthropogenic pressure, the individuals of both sexes are in a ratio close to the theoretically expected one of 1:1, while in the populations occupying the most polluted parts of all three rivers the female individuals dominate.

Table 4
Distribution of the sexually mature individuals of *Pelophylax ridibundus* – in the studied biotopes along the rivers Sazliyka, Blatnitsa and Sokolitsa

Biotope		Number of individuals (♂\♀) absolute	relative	χ^2
1	1.1	23\16	1\0.7	1.26ns
	1.2	8\27	1\3.4	10.32***
	1.3	6\20	1\3.3	7.54**
	1.4	10\27	1\2.7	7.82**
	1.5	16\18	1\1.1	0.12ns
	1.6	9\21	1\2.3	4.80*
	1.7	18\20	1\1.1	0.12ns
2	2.1	5\25	1\5.0	13.34***
3	3.1	15\17	1\1.1	0.12ns
	3.2	18\18	1\1.0	0
	3.3	21\15	1\0.7	1.0ns
	3.4	26\12	1\0.5	5.15*

* (p<0.05), ** (p<0.01), *** (p<0.001), ns (p>0.05)

Table 5
Sex composition in populations in relatively clean and contaminated biotopes along the rivers Sazliyka, Blatnitsa and Sokolitsa

Biotopes	Number of individuals (♂\♀) absolute	relative	χ^2
Relatively clean (1.1; 3.3; 3.4)	70\43	1\0.6	6.46*
Contaminated (1.2; 1.3; 1.4 1.5; 1.6; 1.7; 2.1; 3.1; 3.2)	105\193	1\1.8	25.96**
χ^2		23.92**	

* (p<0.01), ** (p<0.001)

Conclusions

- In habitats located in the upper reaches of rivers Sazliyka and Sokolitsa in the populations of *P. ridibundus*, the two back coloration morphs (*striata*, *maculata*) are equally represented. At the same time, the ratio of individuals is close to theoretically expected distribution in relatively clean waters (1:1). Therefore, the environmental conditions here are close to optimal and the species populations are in a good condition.

- In habitats located in the middle and lower reaches of the rivers Sazliyka and Sokolitsa and the one of the river Blatnitsa, in the populations of *P. ridibundus* the dominant morph *striata* is expressed. At the same time, the sex ratio is changed towards a predominance of the female individuals. Therefore, in these areas of the three rivers the environmental conditions are worsened which leads to adaptive changes in the phenotype and sexual composition of the populations of the species.
- We confirm that the manifestations of colour polymorphism and changes in the sexual composition, along with the level of fluctuating asymmetry (Zhelev et al., 2013b) in the populations of *P. ridibundus* are good bioindicational methods for assessing the quality of the living environment. They effectively complement the data of physicochemical analysis (showing the status of the pond at the time of the sampling). At the same time and regardless of it, these methods objectively reflect the status of the populations in the considered environment because they represent a result of the long-term influence of the living conditions on the organism.

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