ECOLOGIA BALKANICA

2021, Vol. 13, Issue 2 December 2021 pp. 257-287

Synopsis

Fluctuating Asymmetry in Pelophylax ridibundus (Anura: Ranidae) and Bufotes viridis (Anura: Bufonidae) Meristic Morphological Traits as Indicators of Ecological Stress and a Method for Assessing Environmental Quality of Their Habitats – 9 years Monitoring in Bulgaria: Systematic review

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Abstract. This paper summarizes and presents the results from our years-long *in situ* analyses of the populations of the Marsh frog *P. ridibundus* and the Green toad *B. viridis* inhabiting freshwater ecosystems in South Bulgaria with different degrees of anthropogenic transformation. It covers the period 2009-2017 with tests of the application of fluctuating asymmetry in meristic morphological traits as a marker for deviation assessment of the developmental stability of individuals of both species as well as for parallel and independent from physicochemical analyses assessment of the quality of their living environment. The analyses held on the territory of South Bulgaria are divided into three phases: first (2009-2010), second (2011-2015) and third (2016-2017). The results of our investigations conducted over a period of 9 years, show that changes in asymmetry levels in meristic morphological traits in adult and sexually mature representatives of *P. ridibundus* and *B. viridis* are a very good bioindication method.

Key words: Marsh frog, Green toad, fluctuating asymmetry, developmental stability, anthropogenic pollution, environmental health.

Introduction

Anthropogenic pollution and its role for global decline of amphibians. Anuran amphibians and their potential as indicators of ecological stress in biomonitoring in situ analyses

In the 21[st] century, the industrial revolution that started in the end of the 18[th] century became the reason for a drastic change in the relations between modern man and surrounding nature. Today, the concept of Anthropocene is defined as a new geological period (Crutzen, 2006). Currently, the role of the anthropogenic factor is key to the change of geological and climatic processes on planet Earth and involves the functioning of all ecosystems (Dirzo *et al.*, 2014; El Idrissi *et al.*, 2019). Agricultural revolution is inextricably related to industrial revolution (Green *et al.*, 2004). Accelerated agriculture in the past

Union of Scientists in Bulgaria – Plovdiv
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and current century is characterized not only by the use of new arable land at the expense of cutting down the last large forests on the planet but also by the increased use of various fertilizers and chemical protection preparations (Mann *et al*., 2009; Relyea, 2009). It is not by accident that the first places in the list of most significant pollutants of anthropogenic origin are taken by such related to industry and agriculture: polycyclic aromatic hydrocarbons, polychlorinated biphenyls, anticholinesterase pesticides, carbamate, pyrethroid, organophosphate and organochlorine insecticides, endocrine disruptors (estrogen mimics such as DDT, polychlorinated hydrocarbons, surfactant alkyl phenolic compounds), heavy metals, sulfur dioxide, products resulting from the oxidation of sulfur, petroleum products and wastewater from industrial enterprises (Raimondo *et al*., 2007; Selgrade, 2007; Spraling *et al*., 2010).

The negative effects of anthropization in the 20th and 21st century strongly affect different types of continental freshwater basins and the natural wetland connected with them (Czech & Parsons, 2002; Dupler *et al*., 2019). In the context of increasing anthropization, pollution of continental freshwater systems is a serious social problem because freshwater is not only a habitat of its natural biome but is also used for human agricultural activities such as irrigation, fish farming, and tourism. This requires not only knowledge of the mechanisms of functioning of these sensitive ecosystems but also development of efficient measures for their protection, restoration and efficient management (Vollmer *et al*., 2018).

Amphibians are the first vertebrates colonizing land; they survived the periods of the five top planetary extinctions (Schoch, 2014). Anthropocene is the period of the sixth mass extinction on a planetary scale (Wake & Vredenburg, 2008) and the amphibians are the most vulnerable vertebrate group with over 1800 (32%) species directly threatened with extinction (Baillie *et al*., 2004). Global changes in the planet's climate, together with local changes in temperature, precipitation and sunshine (ultraviolet radiation), combined with strong anthropogenic pressure are cited as the main reason for the global decline in amphibian populations (Stuart *et al*., 2004; Whittaker, 2013). Tailless amphibians (Anura) are an important link in the trophic chains connecting aquatic and terrestrial ecosystems (Crump, 2010; Dupler *et al*., 2019) because they can be predators feeding on insects and arthropods (Ferrie *et al*., 2014) as well as prey for many waterfowl and some mammals (Scheffer *et al*., 2006) at the same time. The larval stages of most tailless amphibians thrive entirely in water, feeding mainly on algae, and adults of many species are strongly attached to the water body in which reproduction took place, even after they move to land (Vitt & Caldwell, 2014; Navas *et al*., 2016). This complex life cycle, combined with highly permeable skin, exposes them to the direct action of a variety of toxic agents entering their habitats with anthropogenic wastewater. The chances of survival of the species under altered environmental conditions are determined by the hereditary biological characteristics of individuals (genetic limits of variation of traits) and the associated individual adaptive potential (Burraco & Gomez-Mestre, 2016), and hence the conservation opportunities of the specificity in the structure of the populations and the plasticity of their polymorphism (Vershinin, 2005, 2007). When toxic agents are present in static concentrations (albeit in sub lethal doses) for a long time in the habitat (chronic action or pulse emissions) or increase their concentrations, this leads to depletion of the organism's resistance and its death (Earl & Whiteman, 2009; Montiglio & Royauté, 2014; Saaristo *et al*., 2018). The death of amphibians in the presence of toxic substances is due to the immediate disruption of the organism’s homeostasis most often as a result of strong oxidative stress at the cellular and tissue level (Verma & Srivastava, 2003; Liendro *et al*., 2015; Prokic´ *et al*., 2017), or is a consequence of changes in the behavior of animals under the influence of toxicants (Boone *et al*., 2007; Romero & Wingfield, 2016; Zhang *et al*., 2018). Environmental stress caused by toxicants leads to immunosuppression, which weakens the organism's defenses of amphibians, and hence reduces their resistance to disease and parasites (Corn, 2000). Many species of tailless amphibians cannot live in high-toxicant environments and die, but there are species that survive and adapt to life under environmental stress (Sparling *et al*., 2010; Whittaker, 2013). These species are extremely suitable as test objects in bioindication analyses (Venturino *et al*., 2004; Gonçalves *et al*., 2019). Biomonitoring studies are a valuable risk assessment tool as they rely on biomonitoring species to understand temporal and spatial changes in the quality of the environment and its impact on wildlife (Huggett *et al*., 2018; Conan *et al*., 2021). Biomonitoring (indicator) species are organisms that provide quantitative information on habitat quality through various somatic, physiological or biochemical measures (Brodeur *et al*., 2020a). A number of important characteristics in some species of tailless amphibians (despite the global decline of amphibians in general), such as relatively large abundance and wide range (population level), clear taxonomy and low dispersal capacity increase their potential as bioindicators (Hellawell, 1986; Marques *et al*., 2009). This potential is greater in species that respond to environmental stress through changes in various morphological and physiological traits (Gondim *et al*., 2020; Brodeur *et al*., 2020b).

Analyses performed immediately after the capture of animals, often in natural conditions (*in situ*) are a powerful tool for objective and realistic assessment of the state of populations of different anuran species (health status of individuals, number, density, phenetic, sexual and demographic structure of populations) living in specific ecological conditions of the environment. The information obtained from *in situ* analyses is correct, complements the physicochemical analyses, and often reflects the state of the living environment much more objectively (Otero *et al*., 2018; Zhang *et al*., 2018). The reason for this is that physicochemical analyses take a "snapshot" of the state of the water body (reflecting the presence or absence of toxicants at the time of sampling), while changes in the biological parameters of test objects reflect the long-term effects of toxic agents. (Prokic´ *et al*., 2017; Saaristo *et al*., 2018; Pollo *et al*., 2019). The study of the effects of the chronic action of toxicants on the various functional systems of test animals allows not only to obtain an objective picture of the state of their living environment, but also to reveal the mechanisms and levels of adaptation of these animals to environmental stressors (including those of anthropogenic origin). The advantages of using bioindicator organisms and biomarkers for integrated assessment of biosystems with different levels of organization and complexity is that they respond not only to individual pollutants, but also to a whole complex of influencing substances (Mahmood *et al*., 2016; Jayawardena *et al*., 2017). Chemical analysis of water and soils is a direct evidence of the nature and extent of pollution, but sensory organisms can reveal the state of the dynamic scenario and the long-term effects. Biomarkers complement and improve the reliability of chemical monitoring data by offering an integrated assessment of the impact of pollutants on the health of organisms (Hansen, 2003; Van der Oost *et al*., 2003; Cazenave *et al*., 2009). Many amphibian species usually have the required population density for *in situ* analyses with a sufficient number of individuals, do not perform large spatial migrations (Flickinger & Nichols, 1990) and are also highly philopathic, which with a high degree of confidence means that adults analyzed in specific habitats have most probably undergone their larval development in them (Sinsch, 1990). Endangered species of tailless amphibians are difficult to study due to their low abundance, legal measures for their protection and the difficulties associated with issuing permits for their capture. However, widespread species with abundant populations can serve as biological indicators of ecosystem health (Linder *et al*., 2003; Venturino *et al*., 2004; Marcogliese *et al*., 2009). However, the development of non-lethal sampling techniques is of paramount importance, especially when working with endemic and / or endangered species of tailless amphibians (Narayan, 2013; Barriga-Vallejo *et al*., 2015).

Use of fluctuating asymmetry (FA) as a biomarker for the assessment of the effects of various anthropogenic stressors on the stability of the development of tailless amphibians and the quality of their habitat

One way to assess the impact of various toxic agents on individuals (populations) of tailless amphibians is through their developmental stability (DS) – that is, the ability to neutralize stress during individual development: tadpoles, larvae periods, adults (Van Dongen & Lens, 2000; Zakharov *et al*., 2020). According to the classical notion, developmental stability (morphogenetic homeostasis) is considered as the ability of the genome of each organism to form a stable phenotype, with relatively constant parameters of the environment in which it develops (Zakharov & Graham, 1992; Thornill & Møller, 1997; Klingenberg, 2003). Even in the most balanced and structured genome, random stochastic phenomena can lead to disturbances in the processes at the molecular and cellular level, and hence to the impossibility of reproducing the predetermined phenotype (Palmer & Strobeck, 1986, Zakharov *et al*., 2001). According to modern notions, each phenotypic trait and the degree of its manifestation depends on the influence of two opposing forces: developmental stability and developmental noise (ontogenetic noise) (Palmer, 1994, 1996; Zakharov *et al*., 2001). Noise is a characteristic of a living system and is a reflection of its instability in development - a strong system is characterized by minimal noise (Zakharov *et al*., 2020). When developmental noise is stronger than developmental stability in the developing organism, small accidental errors can occur in the pre-set parameters of cellular, biochemical and physiological processes (Mc Adams & Arkin, 1999). The result of developmental noise is the formation of a phenotype with characteristics fluctuating above the level of natural variations (Palmer & Strobeck, 1986; Polak, 2003). Ontogenetic disturbances in developmental stability can be caused by various genetic (Frankham, 2005; Garrido & Perez-Mellado, 2014) or environmental stressors (Anciães & Marini, 2000; Wright & Zamudio, 2002; Schmeller *et al*., 2011). Since developmental stability and noise cannot be observed independently, but together lead to a certain level of asymmetry, their components of variation cannot be easily separated (Zakharov, 1992; Lens *et al*., 2002).

As far as "ideal forms" practically do not exist in nature, the measurement and presentation in an integral form of the deviations from the strict bilateral symmetry in different morphological features of the organism is proposed as a measure of development instability (Van Valen, 1962). Fine deviations from symmetry are most often described by frequency distributions on the right and left side and can be represented in integral form (Van Valen, 1978; Palmer, 1994). After describing the three asymmetry types (Van Valen, 1962): directional asymmetry (DA), antisymmetry (AS) (in both asymmetry types the characteristics on one of the sides are more frequent i.e. the average is always different from zero), and fluctuating asymmetry (FA) that measures the fine accidental fluctuations from perfect bilateral symmetry, with average always equal to zero), the scientific community decided that FA is most suitable for describing disturbances in the stability of individuals from "wild populations" of various animal organisms (Leary & Allendorf, 1989; Parsons, 1992; Zakharov, 1992; Palmer, 1994). The main advantage of FA is that it is one of the few morphological characteristics for which the norm is known (i.e. perfect symmetry is known) and it is not under strict gene regulation (Zakharov, 1987; Palmer, 1996; Parsons, 2005; Graham *et al*., 2010). At the same time, large morphological differences between the sides are observed in the other two asymmetry types and they are usually strictly generically determined (Palmer, 2005; Zakharov *et al*., 2020). The other important advantage is that FA measurements do not require expensive equipment and are performed without killing the animals. It is the humanity of the method that is its strength, because it allows it to work even with endangered species. Susceptibility to a particular stressor and tendency to fluctuate from symmetry may differ among individuals and populations of the same species in different localities (Sanseverino & Nessimian, 2008), and different species may be related to the intensity of stress in different ways, both negative and positive (Piha *et al*., 2007; Oda *et al*., 2016; Gondim *et al*., 2020). One of the major problems with in situ analyses assessing FA levels in wild populations is related to finding reference populations (those that are not affected by FA). In many cases, this can be problematic, as most habitats are in complex ecosystems and are subject to many major stressors (Sanseverino & Nessimian, 2008). According to Sanseverino & Nessimian (2008), the choice of control areas away from the studied stress-exposed habitat is often impractical due to the difficulty of combining environmental characteristics (same habitat types, comparable physicochemical characteristics, etc.). This requires a very careful approach in field analyses studying the manifestations of FA in order to minimize similar errors, and a possible tool for solving the problem is the use of parallel physical-chemical analyses. In a number of environmental studies, elevated levels of FA have been reported in animal populations (including tailless amphibians) subjected to various types and levels of environmental stress, including anthropogenic (Leung & Farbes, 1996; Leamy & Klingenberg, 2005; Loehr *et al*., 2012; Trokovic *et al*., 2012; Jumawan *et al*., 2016; Saber *et al*., 2016; Shadrina & Vol'pert, 2016; Gondim *et al*., 2020). Despite these positive results, however, in the last years of the past century and at present there is evidence that FA is not the "universal stress indicator" sought by scientists and perceptions of the role of FA and its application in bioindication analyses need to be reconsidered and revised (Bjorksten *et al*., 2000; Van Dongen & Lens, 2000; Rasmuson, 2002; Costa, & Nomura, 2016; Niemeier *et al*., 2019). Most skeptical opinions are grouped around controversial issues regarding the choice of the appropriate test type, the type of characteristics used to analyze FA (meristic traits or metric signs), their number (single or a group of characteristics), error control in measuring asymmetry (Swain, 1987; Whitlock, 1998; Bjorksten *et al*., 2000; Van Dongen, 2000). An additional problem is the lack of consensus on the methods of statistical processing of the obtained data (Whitlock, 1996; Van Dongen & Lens, 2000) and last but not least the presence of heterogeneity in the relationship between FA, developmental stability, stress, quality of life and fitness (Clarke, 1998; Bjorksten *et* *al*., 2000; Van Dongen & Lens, 2000). This, however, does not mean a refusal to conduct research related to the study of FA levels in natural animal populations living under stress or to address environmental issues based on the values of this indicator (Lens *et al*., 2002; Beasley *et al*., 2013; Breno *et al*., 2013). Despite the existing pessimism, recent studies convincingly show that FA is an informative indicator of environmental stress in animal populations, including tailless amphibians (Cánovas *et al*., 2015; Lajus *et al*., 2015; Matías-Ferrer & Escalante, 2015; Natividad *et al*., 2015; Eisemberg & Bertoluci, 2016; Peligro & Jumawan, 2016; Coda *et al*., 2017; Guo *et al*., 2017; Gondim *et al*., 2020; Rodríguez-Gonzáleza *et al*., 2020; Conan *et al*., 2021) and has its place in bioindication examinations along with physicochemical analyses and other bio-tests. According to Lens *et al*. (2002), researchers' efforts should focus on better clarifying why, in some cases, but not in others, FA reflects stress and fitness, and in particular, what are the factors that cause this discrepancy.

The present paper summarizes and presents the results of our long-term *in situ* analyses conducted in populations of marsh frog *Pelophylax ridibundus* (Pallas, 1771) and green toad *Bufotes viridis* (Laurenti, 1768), synonymous with *Pseudepidalea viridis* (Frost *et al*., 2006, 2013), inhabiting freshwater ecosystems in South Bulgaria with varying degrees of anthropogenic transformation. It covers the period from 2009 to 2017 and includes studies related to the application of fluctuating asymmetry in meristic morphological traits as a marker for the assessment of deviations in the stability of development of individuals of both species, as well as for parallel and independent of physicochemical analyses assessment of the quality of their living environment. The two species of tailless amphibians were not randomly selected as test subjects. They are ecologically plastic, resistant to anthropogenic stress and widespread throughout the country (Beschkov & Nanev, 2002; Stojanov *et al*., 2011). In the course of studies, different and specific working hypotheses related to the objectives of the respective studies were tested, but the common denominator was the assumption of expected differences in the values of fluctuating asymmetry in the populations of tailless amphibians living under anthropogenic stress in comparison with values for individuals from reference populations. The other main hypothesis was based on the assumption that the obtained FA values for the populations of *P. ridibundus* and *B. viridis* can be the basis of an objective, parallel and independent from physicochemical analyses assessment of the ecological status of each of the studied habitats in the short and short term.

The analyses conducted on the territory of South Bulgaria can be divided into three phases: first (2009-2010), second (2011-2015) and third (2016-2017). In each phase, goals were set in accordance with our current capabilities for conducting analyses in the populations of the two test subjects of tailless amphibians. Below we present in detail the goals set and the tasks performed for their realization, during the different phases of the research:

<u>2009-2010</u>

The main goal set in this period was to establish for the first time on the territory of Bulgaria the levels of fluctuating asymmetry (FA) in populations of *P. ridibundus* and *B. viridis*, inhabiting anthropogenically polluted habitats (different degree of pollution) and to use them as a marker to assess developmental stability disorders in these species of tailless amphibians. Another main goal of the research was, based on the obtained values of FA in the populations of *P. ridibundus* and *B. viridis* to assess the quality of the environment in their habitats, an analysis also applied for the first time in Bulgaria.

<u>2011-2015</u>

The main goals of the research during this time period were aimed at expanding the use of FA as a method for assessing developmental stability disorders in the populations of *P. ridibundus* and *B. viridis* and their use as a marker for assessing the degree of anthropogenic disturbances in their living environment. In the course of the analyses the following tasks were solved:

1) Use of FA levels as a biomarker for environmental quality assessment, in parallel and independently of the physicochemical analyses of the water, in large freshwater ecosystems (rivers and dams) on the territory of South Bulgaria.

2) Determining whether FA values differ in populations of *P. ridibundus* inhabiting relatively clean waterbodies of running (rivers) and still (dams) type.

3) Determining whether FA values differ in populations of *P. ridibundus* inhabiting water bodies with identical type of pollution of flowing and standing type.

4) Determining whether FA values differ in populations of *P. ridibundus* inhabiting water bodies with different type of toxicants.

5) Determining whether FA levels in populations of *P. ridibundus*from biotopes with different degrees and different types of pollution change over time.

6) Study of FA values in populations of *P. ridibundus* and *B. viridis* in conditions of syntopic inhabitation of freshwater ecosystems on the territory of South Bulgaria and on the basis of the obtained results to perform assessment of the ecological condition of the respective water bodies.

<u>2016-2017</u>

During this time the research was focused on the practical application of FA as a method for monitoring the ecological status of a large freshwater ecosystem on the territory of South Bulgaria - Sazliyka River. The study was conducted in the spring of 2017 (5 years after the construction of a large treatment plant after the city of Stara Zagora) and not only compared FA values obtained from river analyses performed in 2015, but FA was also directly opposed to physicochemical analyses in order to assess of its objectivity in determining the ecological quality of the environment in different locations along the river. The obtained results allowed not only to perform a parallel and independent of physicochemical analyses assessment of the living environment along the river, but also on the basis of FA values to assess the effects of the treatment plant on water quality in the Sazliyka River.

Another main task in this time period was the study of FA levels in populations of *B. viridis*, living in conditions of increased urbanization and their use as a method for assessing the living environment in their habitats – two syenite hills located in the central part of Plovdiv (places lacking permanent natural water sources, which makes it impossible to apply physicochemical analyses).

Materials and Methods

The analyzed material was collected during the spring-autumn seasons of 2009-2017 from rivers, dams, rice fields and other types of smaller water bodies (natural and artificial reservoirs) located on the territory of South Bulgaria.

Test subjects (indicator species of tailless amphibians) in in situ analyses performed on the territory of South Bulgaria

The main tested species of tailless amphibian used in the full research period (2009-2017) is the marsh frog *P. ridibundus*. This species of aquatic anurans has a wide range in Eurasia: from Denmark and Finland to the north, through Central Europe, to the southern regions of Russia, Turkmenistan, Iraq and Iran to the south, with isolated habitats in Saudi Arabia and Bahrain (Amphibia Web, 2021). In Bulgaria the species is distributed throughout the country: from the coast to 1400 m above sea level and up to 2000 m in Belasitsa (Beschkov & Nanev, 2002; Stojanov *et al*., 2011). The species is protected: BDA (IV), 92/43 (V), BERN (III), IUCN (LC) (Biserkov *et al*., 2007). According to Article 42, Article 41 and Appendix II of Article 41 of BDA, permits for capturing *P. ridibundus* are not issued for the purpose of scientific research. The other type of tailless amphibian that is a test subject in our *in situ* bioindicator analyses perform in the period 2009-2017 is green toad *B. viridis*. Only the nominal species *B. viridis* (Dufresnes *et al*., 2019) inhabits the territory of Bulgaria although the diploid complex of green toads (*B. viridis* complex) includes at least 12 evolutionary lines and at least 14 morphologically similar species distributed throughout Europe, much of Asia and North Africa (Stöck *et al*., 2008, Özdemir *et al*., 2014, Vences *et al*., 2019).

In Bulgaria *B. viridis* inhabits a wide range of habitats (including highly urbanized areas) and is distributed throughout the country (Stojanov *et al*., 2011; Mollov, 2019). The species is protected: BDA (III), 92/43 (IV), BERN (II), IUCN (LC) (Biserkov *et al*., 2007). According to Article 49, Paragraph 1 and Article 48, Paragraphs 2, 5 from BPA permits for capturing *B. viridis* for scientific purposes are issued. Analyses using *B. viridis* in the period 2009-2015 are performed in compliance with permit by Ministry of Environment and Water of Bulgaria No 637/26.05.2015, and the ones in the period 2016-2017 in compliance with permit by MOEW No 701/06.04.2017.

Note: BDA (2002): Biological Diversity Act of Bulgaria. Appendix III - species protected in the whole of the country; Appendix IV - species under the mode of protection and regulated use of nature (State Gazette, No 77/9.08.2002).

DCE'92/43 from 21.05.1992: Annex IV - animal and plant species of community interest in need of strict protection; Annex V - animal and plant species of community interest who is taking in the wild and exploitation may be subject to management measures (EO 1992).

BERN (1979) (Convention on the Conservation of European Wildlife and Natural Habitats, Bern, 19.09.1979): Appendix II - strictly protected fauna species (status in force since 1 March 2002); Appendix III - protected fauna species (status in force since 1 March 2002).

IUCN (International Union for Conservation of Nature and Natural Resources): LC - Least Concern (a taxon is Least Concern when it has been evaluated against the criteria and does not qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened. Widespread and abundant taxa are included in this category.

Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research

2009-2010

Field analyses in 2009-2010, which were the first bioindication analyses of this type in Bulgaria were held in the locations of different types of water bodies in the southern part of the country, the lands of Galabovo (Stara Zagora district), Dimitrovgrad and Harmanli (Haskovo district), Plovdiv and Stamboliyski (Plovdiv district). A summary map with exact coordinates of the locations is presented in Zhelev *et al*. (2012a).

2011-2015

In situ analyses in the period 2011-2015 were held in large freshwater ecosystems: the rivers Sazliyka (after the village of Rakitnitsa), Topolnitsa (after the village of Chavdar) and Vacha (south of the town of Krichim) and the dams Rozov Kladenets (near the village of Obruchishte), Topolnitsa (near the village of Poibrene), Vacha (near the village of Mihalkovo), Studen Kladenets (in the "tail") and Tsalapitsa rice fields on the territory of South Bulgaria. Detailed maps with exact coordinates of the locations where the analyses were performed are presented in Zhelev *et al.* (2011b, 2012b. 2013, 2014, 2015, 2016, 2017).

<u>2016-2017</u>

The field analyses in the period 2016-2017 were conducted in the region of Plovdiv in the central highly urbanized city area – two of the natural syenite hills in the city. The natural formations "NL "Halm Bunardzhik" Hill" (22.0 hectares, with peak altitude at 265 m a.s.l.) and "NL "Mladezki Halm" Hill" (36.2 hectares with highest point at 285.5 m a.s.l.) are located in the center of Plovdiv and are originally syenite hills formed during the Paleogene. Both hills have been declared protected areas by the Ministry of Environment and Water (MOEW) in order to protect the natural landscape and unique geomorphological formations. Despite the prohibition of public and business activities, these hills are used for recreation and tourism and, especially on holidays, they are visited by a significant number of people. In addition, the hills were built-up to a high degree before they were declared protected areas (Mollov, 2019). The hills lack permanent natural water sources. A map with the exact coordinates of the places where the frogs were caught is presented in Zhelev *et al.* (2021).

In the spring of 2017, a large-scale survey was conducted on the Sazliyka River, 5 years after the construction of a modern water treatment plant downstream the city of Stara Zagora (2011), whose wastewater is one of the main sources polluting the river. Detailed maps with the exact coordinates of the locations along the Sazliyka River (before the villages of Kazanka and Rakitnitsa, after the village of Dinya and after the towns of Radnevo and Galabovo, as well as at the mouth of the Maritsa River south of Simeonovgrad) are presented in Zhelev *et al.* (2019).

The monitoring and control over the condition of the surface waters in the Republic of Bulgaria is performed by the National System for Environmental Monitoring (NSMOS). All *in situ* analyzes performed in the period 2009-2017 are compliant and performed on the basis of basic data on the ecological status of each of the water bodies in which frogs were caught, obtained on the basis of physicochemical analyzes of water performed by order of the Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, Ministry of the Environment and Waters (2021) in the full period of research (Newsletters on the state of water in each of the studied water bodies – rivers and dams, for the period 2009-2017). Physicochemical analyses measure the values of the priority substances for each of the water bodies, determined according to the monitoring Basin Directorate of Water Management in the East Aegean Sea – Plovdiv, in accordance with the Water Framework Directive WED 2000/60/EO (EC, 2000) and Ordinance No H-4/14.09.2012 (Ordinance, 2012) and No 256/1.11.2010 (Ordinance, 2010) for characterization of surface waters in Bulgaria. Until 2013 on the grounds of Ordinance No 7/08.07.1986 on the indicators and norms in determining the quality of running surface water and Annex No 3 to Order No RD – 272/03.05.2001 of the Minister of Environment and Water, pursuant to cl. 118 (2) of the Water Act of 1999, the waters in the Republic of Bulgaria were divided into four categories: first category (drinking water), second category (slightly polluted water), third category (moderately polluted water) and fourth category (heavily polluted water). Ordinance No 7/8.08.1986 was canceled, starting on 05.03.2013. Since 2013, the provisions of Ordinance No H-4/14.09.2012 on the characteristics of surface waters in Bulgaria (State Gazette No 22/5.03.2013) have introduced new terminology (ecological status and ecological potential) in the categorization of surface waters in Bulgaria, in accordance with annex V of the Water Framework Directive (EC, 2000).

The rivers Sazliyka (after the town of Stara Zagora) and Topolnitsa are one of the most polluted in Bulgaria. Along the Sazliyka River, two "local hotspots" have been marked nationally: the first after the town of Stara Zagora (148 887 citizens, on 15.06.2017) at the confluence of the Bedechka River and the second after the town of Radnevo (12 850 citizens on 15.03.2016) at the confluence of the Blatnitsa River. The main share of the water quantities in the rivers Bedechka and Blatnitsa falls on the waste domestic-fecal and industrial waters of the cities of Stara Zagora (12 km extremely severely affected section) and Nova Zagora – the whole course after the town of Nova Zagora (22 238 citizens on 15.12.2017). The pollution of the Rozov Kladenets Dam connected with the river is of the same type. The main polluters of the Topolnitsa River and the associated dam of the same name are the Aurubis copper plant (formerly Pirdop), the Asarel-Medet MOK and the Chelopech-Mining tailings pond. Heavy metals are the main toxicants in the Topolnitsa River and the Topolnitsa Dam. Pollution with high doses of heavy metals is also reported for the Studen Kladenets Dam. The water in Vacha Dam is clean, without data for anthropogenic pollution. The locations in the upper reaches of the Sazliyka River (near the villages of Rakintitsa and Kazanka) are also relatively clean. Water bodies without data for exceedances of the studied physicochemical parameters of water (according to the above-mentioned statutory acts in Bulgaria) are accepted as conditional (conventional) controls in the analyses. Detailed data (physicochemical analysis of water) on the status of each of the studied water bodies are presented in Zhelev *et al.* (2011b; 2012a; b; 2013; 2014; 2015; 2016; 2017; 2019).

Experimental analyses

Adult individuals of both sexes were used for all analyzes performed in the period 2009-2019 (SVL > 60.0 mm, according to Bannikov *et al.*, 1977). The distinction by sex was made on the basis of the degree of development of secondary sexual characteristics in male individuals (nuptial pads on 1 finger and vocal sacs). The catch was made in the evening by the light of Petromax lamps placed on the water body shore, or using a flashlight. In the second case, sections with length of about 1-2 km and width of 4 m along the river or along the shoreline in the water area of the water bodies were surveyed, according to Sutherland (2000). All manipulations were performed in compliance with ethical standards for working with live animals (Steven *et al.*, 2004). After the analyses, the frogs were returned in the wild.

Identification and measurement of fluctuating asymmetry (FA) in P. ridibundus and B. viridis

As a method for assessing the developmental stability in *P. ridibundus,* we used fluctuating asymmetry in 10 morphological traits, as suggested by Chubinishvili (1997) and Zakharov *et al.* (2000a) (Fig. 1). As a method for assessing the developmental stability in *B. viridis*, we used fluctuating asymmetry in 11 meristic morphological traits, as suggested by Peskova (2006) and Peskova *et al*. (2011) (Fig. 2).

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) (see Fig 3). It is possible for some of the traits not to express asymmetry, but only in very rare cases it is possible for all 10 (11) traits to be bilateral (Zakharov *et al*., 2000a; b). The advantage of the method is that it does not require killing animals and according to the methodologies proposed by Zakharov *et al.* (2000a; b), a minimum of 20 adults is sufficient for the tests. The fluctuating asymmetry was defined by the index frequency of asymmetric manifestation of an

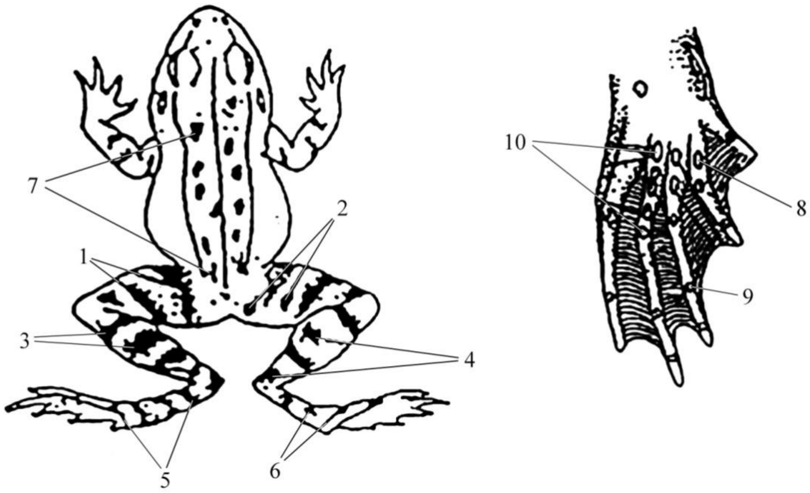

Fig. 1. Diagram of morphological features for assessing the developmental stability in *Pelophylax ridibundus*. This diagram is published in Zhelev (2012b; 2016) and Zhelev *et al*. (2015). *Legend*: 1 – number of stripes on the dorsal side of the thigh (femur), 2 – number of spots on the dorsal side of the thigh (femur), 3 – number of stripes on the dorsal side of the left shank (crus), 4 – number of spots on the dorsal side of the right shank (crus), 5 – number of stripes on the left foot (pes), 6 – number of spots on the right foot (pes), 7 – number of stripes and spots on the back (dorsum), 8 – number of white spots on the ventral side of the second finger of the hind leg, 9 – number of white spots on the ventral side of the third finger of the hind leg, 10 – number of white spots on the ventral side of the fourth finger of the hind leg.

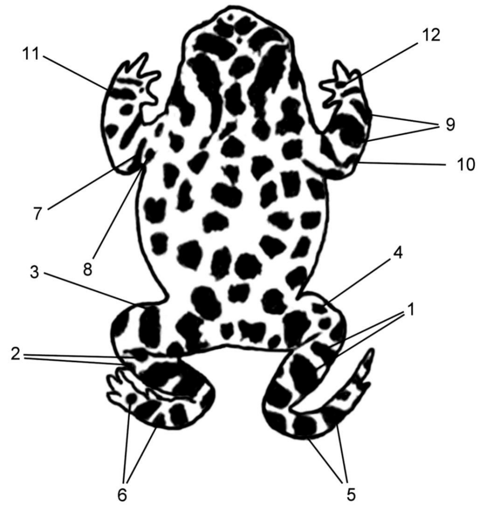

Fig. 2. Diagram of morphological features for assessing the developmental stability in *Bufotes viridis*. This diagram is published in Zhelev (2012b; 2016). *Legend*: 1 - number of spots on the dorsal side of the right shank (crus); 2 - number of spots on the dorsal side of the right shank (crus); 3 – number of stripes on the dorsal side of the thigh (femur); 4 – number of spots on the dorsal side of the thigh (femur); 5 - number of stripes on the left foot (pes); 6 – number of spots on the right foot (pes); 7 - number of stripes on the dorsal side of the right back; 8 -number of spots on the dorsal side of the right back; 9 - number of stripes on the dorsal side of the left forearm; 10 - number of spots on the dorsal side of the left forearm; 11 - number of stripes on the dorsal side of the right wrist; 12 - number of spots on the dorsal side of the right wrist.

Fig. 3. Photos of the most variable meristic morphological traits in *P. ridibundus* individuals found in our investigation in 2017. This photo is published in Zhelev *et al*. (2019). ***Legend*:** a) trait 1 – number of stripes on the dorsal side of the thigh (femur); b) trait 2 – number of spots on the dorsal side of the thigh; c) trait 3 – number of stripes on the dorsal side of the shank (crus); trait 4 – number of spots on the dorsal side of the shank; d) 5 – number of stripes on the foot (pes); trait 6 – number of spots on the foot; e) trait 7 – number of stripes and spots on the back (dorsum); f) trait 8 – number of white spots on the ventral side of the second finger of the hind leg; trait 9 – number of white spots on the ventral side of the third finger of the hind leg; trait 10 – number of white spots on the ventral side of the fourth finger of the hind leg.

individual (FAMI): FAMI = (∑X_i) / n, where X_i measures the asymmetry of an individual and is the number of asymmetric characters (traits) in each specimen divided by the number of used characters and *n* is the number of individuals in the sample (Palmer, 1994; Zakharov *et al*., 2001). Based on the observed mean FAMI values the status of the populations (respectively for the corresponding biotope) was rated, using a special scale suggested for the marsh frogs (Zakharov *et al*., 2000b; Peskova & Zhukova, 2007), but also successfully applied to *Bufotes sp.* (Chikin, 2001; Peskova *et al*., 2011; Guo *et al*., 2017). The scale classifies the quality of the habitat as follows FAMI < 0.4 [grade 1: conventional rate (clean water basin)], 0.41 ≤ FAMI ≤ 0.5 [grade 2: minimal impact on organisms (slightly polluted water basin)], 0.51 ≤ FAMI ≤ 0.6 [grade 3: a satisfactory condition of organisms (average polluted water basin)], 0.61 ≤ FAMI ≤ 0.7 [grade 4: an unfavourable condition of organisms (heavily polluted water basin)] and FAMI ≥ 0.71 [grade 5: a critical condition of organisms (very heavily polluted water basin)], respectively.

Number of test animals used for the analyses in the period 2009-2017

<u>2009-2010</u>

For the whole period in water bodies of different type (rivers, dams, small temporary reservoirs, rice fields), in the locations specified in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research, a total of 352 individuals of *P. ridibundus* and 115 individuals of *B. viridis* were collected and treated. Details of the exact number of individuals and the dates of the catches in each of the water bodies at the locations indicated in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research, are presented in Zhelev & Peskova (2010) and Zhelev *et al*. (2011a; b; 2012a; b).

<u>2011-2015</u>

On the Sazliyka River, 7 biotopes (microhabitats) located on its left bank from village ot Rakitnitsa, to the mouth of Maritsa River south of the town of Simeonovgrad, and on the left banks of two of its large tributaries: the rivers Blatnitsa and Sokolitsa were studied. A total of 411 individuals of *P. ridibundus* were used for the analyzes. Details with the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al*. (2013; 2015; 2016).

A total of 1020 individuals of *P.ridibundus* were used for the studies of the river ecosystems of the rivers Sazliyka, Topolnitsa and Vacha and the dams Rozov Kladenets, Topolnitsa, Vacha and Studen Kladenets. Details with the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al*. (2012b; b; 2015; 2016).

Studies of FA levels and assessment of developmental stability in the populations of *P. ridibundus* and *B. viridis* inhabiting in sympatric and syntopic conditions the region of the Studen Kladenets Dam in the period 2009-2011 were performed with a total of 101 individuals of *P. ridibundus* and 92 individuals of *B viridis*. Details of the exact number of individuals and catch dates in each of the years of analysis are presented in Zhelev *et al*. (2014; 2016). Similar analyzes in the populations of the two species inhabiting habitats in the regions of the Rozov Kladenets and Topolnitsa Dams in spring 2010 were held with 69 individuals of *P. ridibundus* and 65 individuals of *B. viridis* (Zhelev, 2011b).

Fifty frogs (25 individuals of each sex) were used for the research held in the Tsalapitsa rice fields in spring 2013. The Vacha River, south of the town of Krichim, was used as a reference site for this study. 50 frogs were also captured there – equal number of both sexes (for details see Zhelev *et al*., 2017).

2016-2017

For the research conducted in spring 2017 on the river ecosystem of the Sazliyka River, 5 years after the construction of a treatment plant after the town of Stara Zagora (2011), a total of 300 individuals of *P. ridibundus* were used. Frogs (25 male and 25 female) were caught in 6 biotopes (microhabits) listed in subsection Geographical locations and summarized data from the physicochemical monitoring of the studied aquatic ecosystems at the time of research. Details of the exact number of individuals and catch dates in each of the habitats are presented in Zhelev *et al*. (2019).

The studies of *B. viridis* conducted in spring 2017 in the city of Plovdiv were conducted with a total of 120 individuals - 60 frogs (30♂, 30♀) caught from each of the two syenite hills located in the central part of the city (for details see Zhelev *et al*., 2021).

Statistical analyses

The scientific community is currently debating which ways to represent the levels of asymmetry in meristic traits are most appropriate: whether to present the data in integral form and then compare them numerically, or to use mathematical models (Lens *et al*., 2002). In the period from 2009 to 2015, in order to derive the statistical reliability of the data, we compared the FAMI values obtained for the different habitats with parametric analysis methods (at normal data distribution and homogeneous of samples): Students t-test, or one-way analysis of variance (ANOVA). In order to examine the odds for observing asymmetry in the studied traits in the individuals from each habitat (site), in the 2016-2017 period, a generalized linear model (GLM) using logit link function was fitted to the FAMI data (Molenberghs, 2003). Statistical processing of the data was performed, using the Statistica 7.0 Software (Statistica, 2004), or R language (version 3.1.2, R Development Core Team, 2015).

Results and Discussion

2009-2010

The results obtained regarding the values of the integral indicator for FA in the populations of the two species of tailless amphibians, from studies conducted in the period 2009-2010, are summarized in Table 1.

The Table 1 shows that FA values in *P. ridibundus* that inhabit 14 different habitats from 8 water bodies: small natural and artificial reservoirs, rice fields, dam lakes and three rivers – Sazliyka River (3 sites), Maritsa River (2 sites) and Topolnitsa River (2 sites) are determined in this period. In parallel, FA values in *B.viridis* in 7 different habitats (7 water bodies) in which the reproduction and larval development of the species originated) were studied (see Table 1). The results presented in Table 1 show that lowest FA levels in *P. ridibundus* were found in three water bodies (A, B and F) and on this basis these three water sources receive the minimum degree 1 according to Zakharov *et al*. (2000a) and Peskova & Zhukova (2007). The populations of *P. ridibundus* in these habitats were found to be in a stable condition, under optimal developmental environmental conditions. The anthropogenic pressure on them is far from the levels at which processes leading to disturbances in developmental stability begin. In the other species, *B. viridis*, with reproduction and early development taking place in water bodies A, B and F, FA levels are a little higher for the populations from habitats A and B and correspond to degree 2 of developmental disorder according to Zakharov *et al*. (2000a) and Peskova & Zhukova (2007). In habitat F, FA levels are identical to the ones of *P. ridibundus*. In our opinion, these facts suggest that in the early stages of its development *B. viridis* is a slightly more sensitive to disturbances in environmental factors than *P. ridibundus*. In water bodies D and G, FA levels in *P. ridibundus* also correspond to degree 2 of disorders in developmental stability. This means that these populations also develop under relatively good environmental conditions, but they have a reaction (albeit weak) to environmental stressors. In habitats J (degree 4), C, K, L, M and N (degree 5), higher FA levels were detected. This means that these populations are under conditions of severe stress caused by anthropogenic activity: introduction of pesticides into habitat J, phosphates (PO^{3-}_4) and petroleum products in habitat E, wastewater from TPP "Maritsa - East 1" in habitat C, sewage domestic pollutants and reactive forms of nitrogen (NO^-_3-N, NO^-_2-N) in habitats K and L and heavy metals in habitats M and N. The conclusion to be drawn is that at high levels of contamination, regardless of the type of toxicants in the populations of both species of anuran amphibians, high asymmetry levels are observed (Students t test > 3.0, $p < 0.001$). Details and detailed comments on the research in populations of *P. ridibundus* and *B. viridis* in the 2009-2010 period can be found in Zhelev & Peskova (2010) and Zhelev *et al*. (2011a; b; 2012a; b; c).

Most of the studies related to the analysis of the levels of FA in meristic traits of anurans are conducted on the territories of Russia, Ukraine, Georgia, Belarus, Moldova, Kazakhstan and Uzbekistan (Chubinishvili, 1998; Zakharov *et al*., 2000a; 2000b; Chikin, 2001; Loginov & Gelashvili, 2005; Ustyuzhanina & Streltsov, 2001a; b; Nikashin, 2005; Fomin, 2006; Maksimov 2007a; b; Spirina, 2009; Lada *et al*., 2012; Peskova *et al*., 2011). There are single studies conducted in China (Guo *et al*., 2017) and Turkey (Dönmaz & Şişman, 2021). In most of these studies, low FA levels were reported for populations of anurans (mainly *P. ridibundus*), living under low environmental stress conditions – in protected areas or in habitats far from settlements, agricultural lands or industrial enterprises (Zakharov,1987; Hitsova *et al*., 2004; Peskova & Vasyutina, 2005). High asymmetry levels (degree 4 and 5) are usually reported for anurans populations under severe anthropogenic stress. It is noteworthy that FA values are not influenced by the type of the toxicants, but only by their levels. At high levels of polluting agents such as chemical contaminators (Chubinishvili, 1998; Spirina, 2009), heavy metals (Loginov & Gelashvili, 2005; Nikashin, 2005; Fomin, 2006; Guo *et al*., 2017; Dönmaz & Şişman, 2021), nutrients and phosphates (Erdeneev & Zvolinski, 2002; Peskova & Zhukova, 2007; Vasilev & Vasileva, 2009) or pesticides (Ustyuzhanina & Streltsov, 2001a, 2001b), are reported to cause high levels of FA in populations of frogs.

2011-2015

The results from the research on FA levels in populations of the two species of tailless amphibians in various types of water bodies: running (rivers) and still (reservoirs) and with different types of anthropogenic pollution (domestic sewage pollution and heavy metal pollution) conducted in 2011-2015 period are presented in Table 2.

The Table 2 shows that in this period FA levels in the populations of *P. ridibundus* inhabiting three large river ecosystems: the rivers Sazliyka (12 different sites), Topolnitsa (two sites) and Vacha (one site) and four reservoirs (the dam lakes Vacha, Rozov Kladenets, Topolnitsa and Studen Kladenets) were determined. In parallel, FA levels were determined in the populations of *P. ridibundus* and *B. viridis*, inhabiting under syntopic conditions the region of Studen Kladenets Dam Lake for three years period.

This research shows that the highest levels of FA were found in *P. ridibundus* populations under conditions of permanent anthropogenic pollution, in rivers, at high concentrations of toxicants, regardless of their nature. The grade rating by Peskova & Zhukova's scale (2007) is 5 – the maximum for the six sites (habitats) from the two rivers (B, C, D, F, M and N), with the highest FAMI values. Similar high levels of FA (FAMI = 0.65-0.87) were found in the populations of *P. ridibundus* in the northern and central parts of the area in conditions of different types of anthropogenic pollution: the Voronezh River – metallurgic industry in the region of the town Novolipetsk (Nikashin, 2005), the Hadazhka River – a region of a pig farm in the West Caucasus (Peskova & Zhukova, 2007), the Sviyaga River, near the town of Ulyanovsk, pollution from chemical industry (Spirina, 2009) and the Karasu River in

Turkey polluted with heavy metals (Dönmaz & Şişman, 2021). In water bodies of still type (dam lakes), irrespective of the nature of toxicants, values of FA (FAMI index) in the populations of *P. ridibundus* are lower than the ones in populations inhabiting water bodies of running type (rivers) (ANOVA, $F_9 = 180.077$, $p < 0.001$). The research conducted in the 2011-2015 periods reaffirms the opportunities for assessing the developmental stability of *P. ridibundus* populations that live in conditions of anthropogenic pollution through the method of FA. It supplements the data of FAMI values in *P. ridibundus* populations from rivers and dam lakes polluted with heavy metals and domestic sewage pollution. The results provide better opportunities to use FA in *P. ridibundus* populations for bioindication and biomonitoring, and for parallel and independent analyses on the physicochemical analyses assessment of the environmental condition (for details see Zhelev *et al.*, 2013, 2014, 2015, 2017).

The levels of fluctuating asymmetry in *B. viridis* were higher than those in *P. ridibundus* when they live together in sympatric and syntopic conditions at the dam lake Studen Kladenets for the whole three years period of the research (Students t test > 2.0, $p < 0.05$) (for details see Zhelev *et al.*, 2014, 2016).

2016-2017

The results of the study on the Sazliyka River, conducted in 2017, the 5 years after the construction of a modern, wastewater treatment facility near the town of Stara Zagora are presented in Table 3.

Table 1. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al.*, 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2009-2010 periods. *Legend:* *Sites: A – the Galabovo town (water body with groundwater sources); B – the Galabovo town (Sazliyka River); C – the Galabovo town (sedimentation lake of TPP "Maritsa - East 1"); D – the Stamboliyski town (tailings of a pulp and paper factory); E – the Dimitrovgrad town (Maritsa River in the industrial collectors of "Neohim"); F – the Plovdiv town (Maritsa River in the sugar factory); G – the Plovdiv town (the Chaya River flows into Maritsa River); H – the Galabovo town (Rozov Kladenets Dam lake); I – the Poybrene (Topolnitsa Dam lake); J – the Plovdiv town (Tsalapitsa rice fields); K – the Stara Zagora town (Sazliyka River); L – the Radnevo town (Sazliyka River); M – the Pirdop town (Topolnitsa River); N – the Chavdar village (Topolnitsa River); – no data.

	Pelophylax ridibundus 2009							Publications
	A	B	C	D	E	F	G	
Sites* (habitats)	0.22±0.03 1	0.37±0.06 1	0.73±0.03 5	0.53±0.09 2	0.57±0.06 3	0.37±0.02 1	0.53±0.06 2	Zhelev& Peskova (2010); Zhelev (2012b)
	Bufotes viridis 2009							
	0.48±0.01 2	0.47±0.03 2	0.67±0.05 5	–	0.63±0.04 4	0.39±0.03 1	–	Zhelev (2011a, 2012b)
	Pelophylax ridibundus 2010							
	H	I	J	K	L	M	N	
	0.57±0.023 3	0.54±0.01 3	0.63±0.11 4	0.82±0.02 5	0.80±0.02 5	0.73±0.02 5	0.75±0.01 5	Zhelev (2011b, 2012b, 2012c)
	Bufotes viridis 2010							
	0.56±0.03 3	0.63±0.04 4	–	–	–	–	–	Zhelev (2012a, 2012b)

Table 2. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al*., 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2011-2015 periods. *Legend:* *Sites: the Sazliyka River south of village Rakitnitsa (A), downstream of the Stara Zagora town and the confluence with Bedechka River (B), downstream of the Radnevo town and the confluence with Blatnitsa River (C), downstream of the Lyubenovo village (D), north of the Galabovo town, under the sluices which block the river to discharge part of it to "TPP Brikel" (E), south of the Galabovo town, downstream of the confluence with Sokolitsa River (F), south of the Kalugerovo village until it runs into Maritsa River (G), the Blatnitsa River south of the Lyubenova Mahala village (H), the Sokolitsa River in the Rozov Kladenets Dam (I), east of the Obruchishte village (J), east of the Iskritsa village (K), east of the Orlov Dol village (L); the Topolnitsa River below the bridge on the road Panagyurishte – Pirdop (M), below the village of Chavdar (N), the Vacha Dam lake (O), the Rozov Kladenets Dam lake (P), the Topolnitsa Dam lake (Q), the Vacha River (S) and the Tsalapitsa rice fields (T).

Sites* (habitats)	*Pelophylax ridibundus* 2011-2013							Publications
Rivers: Sazliyka, Blatnitsa, Sokolitsa, Topolnitsa and Vacha; Dams: Vacha, Rozov Kladenets and Topolnitsa; Tsalapitsa rice fields	A	B	C	D	E	F	G	Zhelev *et al*. (2013, 2015, 2017); Zhelev, (2016)
	0.47±0.01 1	0.82±0.02 5	0.80±0.02 5	0.72±0.03 5	0.64±0.02 4	0.72±0.02 5	0.63±0.03 4	
	Pelophylax ridibundus 2011-2013							
	H	I	J	K	L	M	N	
	0.85±0.02 5	0.55±0.01 3	0.57±0.02 3	0.49±0.01 2	0.33±0.01 1	0.73±0.01 5	0.76±0.01 5	
	Pelophylax ridibundus 2011-2013							
	O	P	Q	S	T			
	0.38±0.01 1	0.59±0.01 3	0.54±0.01 3	0.37±0.01 1	0.71±0.01 5			

	Pelophylax ridibundus		*Bufotes viridis*		
Studen Kladenets Dam lake	2009	0.53±0.03 3	2009	0.57±0.03 3	Zhelev *et al*. (2014); Zhelev (2016)
	2010	0.63±0.02 4	2010	0.68±0.03 4	
	2011	0.63±0.02 5	2011	0.67±0.03 4	

Table 3. The values of FAMI index (Means±SEM) and values of grade scale (Zakharov *et al*., 2000a; Peskova & Zhukova, 2007) of two anuran species from investigated habitats (sites) in South Bulgaria in 2016-2017 periods. ***Legend:*** *Studied sites on the Sazliyka River: A – before the village of Kazanka, B – before the village of Rakitnitsa, C – below the village of Dinya, D – below the town of Radnevo, E – below the town of Galabovo and F – at the mouth of Maritsa River south of the town of Simeonovgrad; Studied sites in the Plovdiv town: G – Halm Bunardzhik Hill and H – Mladezki Halm Hill.

	Pelophylax ridibundus 2017						Publications
Sites* (habitats)	A	B	C	D	E	F	
	0.39±0.01 1	0.49±0.01 2	0.76±0.02 5	0.82±0.02 5	0.84±0.02 5	0.64±0.01 4	Zhelev *et al.* (2019)
	Bufotes viridis 2017						
	G	H					Zhelev *et al*. (2021)
	0.27±0.01 1	0.25±0.01 1					

The obtained FA levels in the populations of *P. ridibundus* inhabiting in 6 sites are compared with the data from the 2011 survey (see Table 2 and Zhelev *et al.*, 2015). No significant differences between years were found except for the site 5, in which there were two, times higher odds to observe asymmetries in the year of 2017 in comparison to 2011 (GLM, $Z = 4.201$, $p < 0.001$) (for details see Zhelev *et al.*, 2019).

What is the possible explanation for the established lack of positive effects on the condition of the Sazliyka River water in the section after the town of Stara Zagora, 5 years after the construction of wastewater treatment plant Stara Zagora? In our opinion, the reasons can be found in the technical characteristics of WTP Stara Zagora set in its design. Wastewater treatment plant Stara Zagora is with capacity for 256 300 citizens and has facilities for treatment (reduction of parameters)as follows: total phosphorus (TP) – until 1 mg/l, total nitrogen (TN) – until 10 mg/l, biological oxygen demand five days (BOD_5) – until 25 mg/l, chemical oxygen demand (COD) – until 125 mg/l. The quantity of treated water entering the Sazliyka River is 33 500 000 m^3/year, (Register of the issued permits for use of a water body for discharging wastewater 2010-2019, Permit №33140101/11.05.2011. This treated waste water constitutes 25% of the resource of the water body: Q_{WB} - 136 235 520 m^3/year (Final report of the National Institute of Meteorology and Hydrology - Bulgarian Academy of Sciences on implementation of the agreement with the Ministry of Environment and Water for 2015). The technically set purification degrees according to the above indicators meet the requirements of Commission Directive 98/15/EC (EC, 1998) for settlements with over 100 000 citizens but they fail to meet the standards for high water quality of inland surface water. The quantity of treated water from WTP Stara Zagora is evenly distributed throughout the year. The river flow of the Sazliyka River has been greatly altered due to the transfer of water from another river catchment (Koprinka Dam). This water enters the river before Stara Zagora through a derivation tunnel. Before entering the Sazliyka River, the water passes through two cascading hydroelectric power plants (north of Stara Zagora, before the treatment plant). The transferred water is used for irrigation. River outflow is seasonal and depends on the irrigation season. From mid-April to mid-October; the water levels in the river are approximately twice as high as those outside the irrigation season. During the winter period (low water season), the treated wastewater may exceed the water quantities measured in the section of the river before the discharge of WTP Stara Zagora. This pronounced seasonality of the river outflow is also confirmed by the large fluctuation in the measured values of TN, TP, BOD_5 and COD indicators in the waters of the Sazliyka River. They strongly correlate with the sampling time – high values outside the irrigation season and lower values in the irrigation season (for details see Zhelev *et al.*, 2019). The above facts show that with the so set water purification degree WTP Stara Zagora is unable to reach the standards for high water quality of inland surface water for the TP < 0.150 mg/l, TN < 0.07 mg/l, BOD_5< 2 mg/l, COD – 25 mg/l. The treated wastewater from WTP Stara Zagora constitute one fourth from the resource of the Sazliyka River. Due to the lack of other significant wastewater emitters, it can be said with a high degree of reliability that the main pollution of the river in this section is from domestic wastewater coming from the big city. In the section after the town of Radnevo, the Sazliyka River crosses the territory of the coal-mining complex Maritsa East. In the region of the town of Galabovo a part of river water is used for industrial purposes; it is diverted to TPS Brikel (200MW) and then it enters the

Rozov Kladenets Dam (3.6 km^2). Since 2011 there has been a newly built TPS in the region: AES Galabovo (670MW). The Rozov Kladenets Dam has been built for the purposes of the neighboring TPS Brikel and AES Galabovo, where lignite coal is burnt. Industrial effluents, that are used to cool the turbines, enter the dam; it is in direct connection with the Sazliyka River. Besides, this river section is the place of wastewater discharge from the lake-precipitator of TPS Brikel and AES Galabovo. In comparison with the data from the physicochemical analyses conducted on Sazliyka River in the period 2001-2012 (see Zhelev *et al.*, 2013, 2015), the new physicochemical analyses, in the period 2012-2017 (see Zhelev *et al.*, 2019), do not give any reason to state that the state of the river has improved with the commissioning of a wastewater treatment plant after the town of Stara Zagora.

The results of research conducted with the other species tailless anuran – the green toad *B. viridis* performed in urbanized area – the town of Plovdiv are presented in Table 3. The results of this study show low levels of fluctuating asymmetry for *B. viridis* individuals from both sites: 0.27 (the Halm"Bunardzhik" Hill) and 0.25 (the "Mladezhki Halm" Hill), respectively. Low asymmetry levels reported for *B. viridis* individuals from both sites indicate that their early stages of development have occurred under good environmental conditions with minimal risk of disturbances in their developmental stability. This means that the environmental conditions at both sites were good at the time of the study (for details see Zhelev *et al.*, 2021).

Conclusions

1. The results of our investigations conducted over a period of 9 years, show that changes in asymmetry levels in meristic morphological traits in adult and sexually mature representatives of *P. ridibundus* are a very good bioindication method, supplementing physicochemical analysis data, while providing an independent, parallel assessment of the state of the living environment. In addition, the method is easy to implement, it does not involve expensive equipment and does not require the killing of the test animals. It can be applied at any point in the area inhabited by the species, even in protected areas because frogs are returned to nature.

2. The results of the analysis of fluctuating asymmetry levels in our researches in the 2009-2017 periods support the potential for the use of green toads in bioindication analyses. This reveals prospects for the search for new indicator species and the widening of the possibilities for the practical introduction of the method into bio-indication experiments.

Acknowledgments

This research was supported by the National program "Young scientists and Postdoctoral candidates" funded by the Bulgarian Ministry of Education and Science.

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Received: 01.08.2021
Accepted: 01.09.2021