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Fluctuating Asymmetry in Meristic Morphological Traits of *Bufotes viridis* (Laurenti, 1768) (Anura: Bufonidae): Application for Assessing Environmental Quality of Two Semi-natural Habitats in Plovdiv City, Bulgaria

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Abstract: Fluctuating asymmetry is a result of the organism's inability to develop in a certain way and exhibits disruptions in its early-stage developmental stability. The analysis of fluctuating asymmetry levels in anuran populations allows for a parallel assessment of the quality of the living environment. Meristic traits of anurans, such as colour marks (stripes and spots) on the backside of the body and limbs, are suitable for measuring levels of fluctuating asymmetry. The current study applies the method of fluctuating asymmetry in meristic morphological traits in adult individuals of *B. viridis* as a biomarker for assessment of the environmental quality of two semi-natural habitats – Halm Bunardzhik Hill (site 1) and Mladezhki Halm Hill (site 2), located in a highly urbanised area of Plovdiv City, Bulgaria. This study recorded low levels of fluctuating asymmetry for individuals of *B. viridis* from both sites: 0.27 (site 1) and 0.25 (site 2) for both studied hills, respectively. The low asymmetry levels reported for individuals of *B. viridis* from both sites indicated that their early stages of development had occurred under good environmental conditions, with a minimum risk of disturbances in their developmental stability. These results suggested that the environmental conditions at both sites were good at the time of the study.

Key words: green toad, fluctuating asymmetry, developmental stability, urban area, south Bulgaria

Introduction

Fluctuating asymmetry is a result of the organism's inability to develop in a certain way and indicates disruptions in its developmental stability (morphogenetic homeostasis) occurring in the early stages of development (Van Valen 1962, 1978). Genetic (Frankham 2005, Garrido & Pérez-Mellado 2014) and environmental stress factors (Zakharov & Graham 1992, Wright & Zamudio 2002, Schmeller et al. 2011) can cause ontogenetic disturbances in the developmental stability of the organism. They depend on the dynamic balance between two opposing forces: developmental stability and developmental noise (Palmer 1994, 1996). When developmental noise is higher than developmental stability, in the developing organism, small random differences may occur in cellular, biochemical and physiological processes (McAdams

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& Arkin 1999). These disturbances, which are not necessarily genetically determined and apparently do not influence individuals' vitality, could lead to the development of the phenotype characteristics fluctuating above the level of natural variations (Palmer & Strobeck 1986, Van Dongen & Lens 2000). The manifestation of phenotypic differences is a reflection of a certain level of developmental disturbances. They occur in the first generation developing under suboptimal conditions and disappear when the optimal conditions are restored, i.e., they are reversible. Currently, it is widely assumed that, among the three types of asymmetry (fluctuating asymmetry, directional asymmetry and antisymmetry), fluctuating asymmetry is the most appropriate to describe violations in developmental stability of individuals in "wild populations" (Cánovas et al. 2015, Lajus et al. 2015, Peligro & Jumawan 2016, Shadrina & Vol'pert 2016, Coda et al. 2017, Zhelev et al. 2019). Concomitantly, the analysis of levels of fluctuating asymmetry in these populations allows for a parallel assessment of the quality of the living environment. Meristic traits of anurans, such as colour marks (stripes and spots) on the backside of the body and limbs, are suitable traits for measuring the levels of fluctuating asymmetry. Although most of the studies dealing with measuring the levels of fluctuating asymmetry with test-subjects anurans are conducted with aquatic frogs (family Ranidae), there is enough evidence that toads can be suitable test-subjects for bioindication analyses as well (Chikin 2001, Peskova et al. 2011, Zhelev et al. 2014, Guo et al. 2017).

Analysis of fluctuating asymmetry conducted in populations of European green frogs (*Pelophylax* kl. *esculentus* Linnaeus, 1758) from different parts in their Eurasian geographical range demonstrate that fluctuating asymmetry levels can be high in anthropogenically contaminated habitats and low in less disrupted habitats. High fluctuating asymmetry (FAMI index) values were recorded in populations of the marsh frog inhabiting areas with contamination with heavy metals (Loginov & Gelashvili 2005, Nikashin 2005, Fomin 2006), pesticides pollution (Ustyuzhanina & Streltsov 2001), nutrients and phosphates (Peskova et al. 2011, Vasilev & Vasileva 2009) and wastewaters from chemical enterprises (Chubinishvili 1998, Spirina 2009). Our long-standing studies conducted with the marsh frog *Pelophylax ridibundus* (Pallas, 1771) on the territory of Bulgaria confirmed these results: high levels of fluctuating asymmetry (FAMI index) were found in populations of *P. ridibundus* in polluted habitats with domestic sewage pollution (Zhelev et al. 2019), heavy metal pollution (Zhelev et al. 2015) and pesticides (Zhelev et al. 2017). Data on fluctuating asymmetry levels in anurans inhabiting urbanised areas are scarce (Chubinishvili 2001, Guo et al. 2017).

The green toad (*Bufotes viridis*) was chosen as a test object because this anuran species is commonly found in these habitats. It is not as firmly attached to water basins like frogs and adult individuals can endure long periods without water (Mollov 2019). Nevertheless, the larvae develop entirely in water. In addition, since fluctuating asymmetry levels reflect precisely disturbances occurring in the early stages of development, the species can be used for bioindication analyses. This was confirmed in our previous study conducted in a heavy metal contaminated reservoir used for breeding by *P. ridibundus* and *B. viridis,* where we recorded even higher levels of fluctuating asymmetry in adult individuals of *B. viridis* than of *P. ridibundus* (Zhelev et al. 2014).

The study aims to apply the method of fluctuating asymmetry in meristic morphological traits in adult individuals of *B. viridis* as a biomarker for assessment of the environmental quality of two semi-natural habitats, located in a highly urbanised area – the city of Plovdiv in south Bulgaria.

Materials and Methods

Sampling area

Natural Monument Halm Bunardzhik Hill (Site 1) is situated on 22.0 hectares, with peak altitude at 265 m a.s.l. and Natural Monument Mladezki Halm Hill (Site 2) – on 36.2 hectares with the highest point at 285.5 m a.s.l. Both sites are located in the centre of the city (Fig. 1) and, by origin, are syenite hills formed during the Palaeogene (Mollov 2019). Both hills are declared as protected territories by the Bulgarian Ministry of Environment and Waters (MOEW), aiming to conserve the natural landscape and the unique geomorphologic formations. Despite the restrictions on public and business activities, they are subject to tourism and recreation and, especially, during the weekends, a significant flow of people visits them. Their territory is relatively small and there is no permanent water source, which is a problem for the direct physicochemical analyses of temporary water bodies (formed mainly in spring and summer after heavy rains).

Capturing toads and identification of fluctuating asymmetry

The fluctuating asymmetry method in individuals of *B. viridis* on these hills was applied as a biomarker for assessing environmental quality. The advantage of the method is that it does not require killing ani-

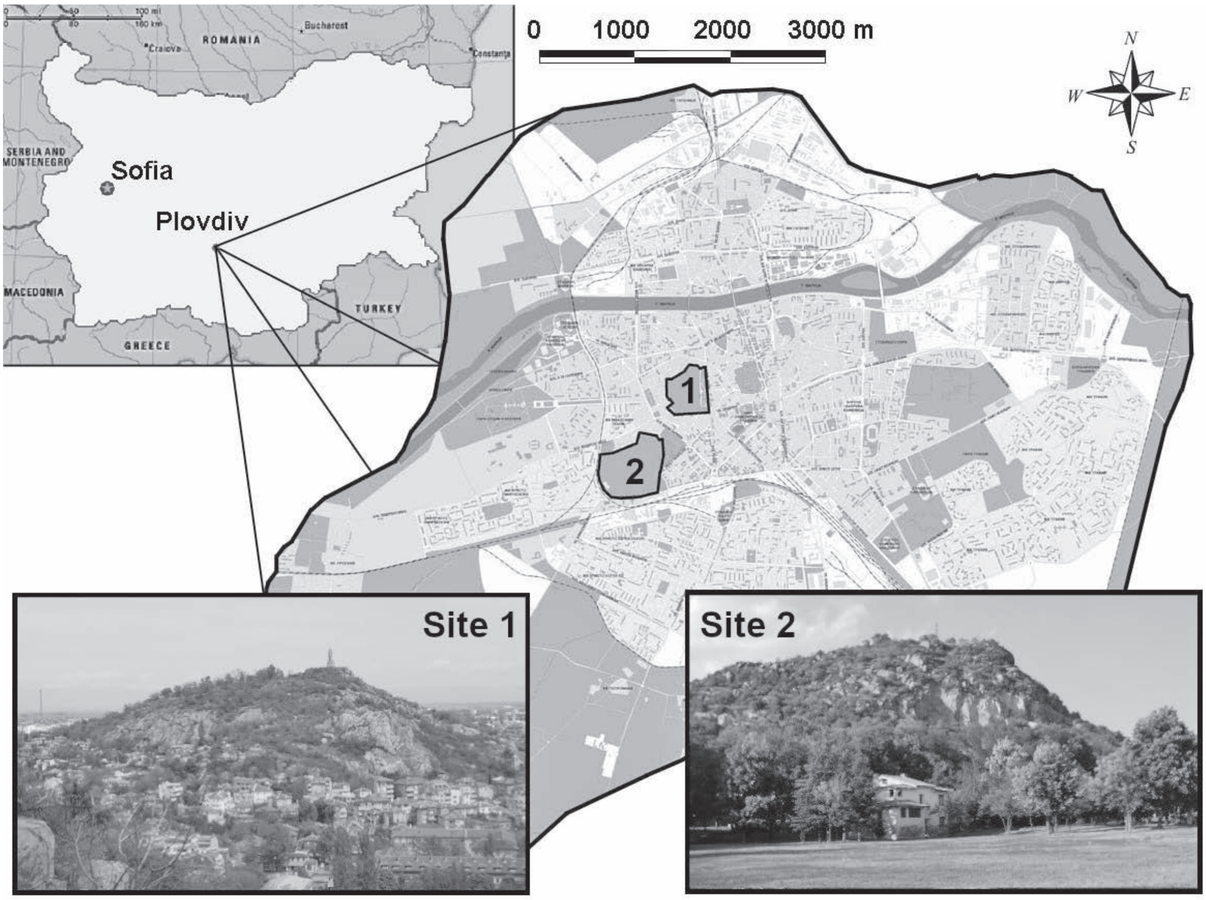

Fig. 1. Indicative map of the sites of the Plovdiv city where individuals of *B. viridis* were captured in 2017. Site 1 – Halm Bunardzhik Hill, Site 2 – Mladezki Halm Hill.

mals and according to the methodologies proposed by Zakharov et al. (2000a, 2000b), a minimum of 20 adults is sufficient for the tests. The methodology even allows work with an assembly group of males and females as fluctuating asymmetry levels do not show differences between sexes (Zhelev et al. 2015, 2017, 2019). The toads, 30 males and 30 females from each site, sexually mature at SVL > 60.0 mm (after Bannikov et al. 1977), were captured in April 2017, at night. For each individual, we recorded SVL using a digital calliper with an accuracy of 0.1 mm (KERN EMB 600–2, Germany). The analyses were done with live animals, on the next day after their collection. After the analyses, the toads were released back in their natural habitats.

As a method for assessing the developmental stability, we used fluctuating asymmetry in 11 meristic morphological traits as suggested by Peskova (2006) and Peskova et al. (2011) and successfully applied in our previous works on this frog species (Zhelev et al. 2014, Zhelev 2016). These traits were: 1 – number of stripes on the dorsal side of the thigh (in both legs); 2 – number of spots on the dorsal side of the thigh (femur); 3 – number of stripes on the dorsal side of the shank (crus); 4 – number of spots on the dorsal side of the limb; 5 – number of stripes on the foot (pes); 6 – number of spots on the foot; 7 – number of spots on the back (dorsum); 8 – number of stripes on the dorsal side of the forearm; 9 – number of spots on the dorsal side of the forearm; 10 – number of stripes on the dorsal side of the wrist; and 11 – number of spots on the dorsal side of the wrist.

For each individual, we identified the number of asymmetrical traits by the degree of their expression on the left and right side of the body. All the 11 meristic traits in *B. viridis* are present on both sides of the body; they are distinct and, therefore, the possibility for subjective mistakes is negligible. Various variants of this methodology, mainly concerning the number of characters used, have been applied to identify fluctuating asymmetry levels in other anurans species of the genus *Bufo* (Chikin 2001, Peskova et al. 2011, Guo et al. 2017). The level of asymmetric manifestation for each of the ten traits was recorded for each individual; it may vary from

Table 1. The FAMI values (means ± standard errors) of asymmetric individuals, the number of individuals asymmetric on any trait (%) and results from analyses of the effect of the habitat (site) on the rate of observed asymmetries in the studied individuals of *B. viridis.* **Legend:** n – number of individuals; * – the degree shows the number of asymmetric traits; meristic traits: 1 – number of stripes on the dorsal side of the thigh (in the both thigh), 2 – number of spots on the dorsal side of the thigh (femur), 3 – number of stripes on the dorsal side of the shank (crus), 4 – number of spots on the dorsal side of the shank, 5 – number of stripes on the foot (pes), 6 – number of spots on the foot, 7 – number of spots on the back (dorsum), 8 – number of stripes on the dorsal side of the forearm, 9 – number of spots on the dorsal side of the forearm, 10 – number of stripes on the dorsal side of the wrist, 11 – number of spots on the dorsal side of the wrist.

Sites	Asymmetric individuals	Meristic morphological traits (1 to 11)										
		1	2	3	4	5	6	7	8	9	10	11
Site 1 n=60	27^{2*} (45%)	n = 34 (57%)	n = 31 (52%)	n = 36 (60%)	n = 35 (58%)	n = 2 (3%)	n = 3 (5%)	n = 2 (3%)	n = 4 (7%)	n = 2 (3%)	n = 4 (7%)	n = 2 (3%)
	26^{3} (43%)											
	7^{4} (12%)											
	FAMI (0.27 ± 0.008); Grade scale (1)											
Site 2 n=60	35^{2} (58%)	n = 32 (53%)	n = 27 (45%)	n = 30 (50%)	n = 30 (50%)	n = 3 (5%)	n = 3 (5%)	n = 1 (2%)	n = 2 (3%)	n = 2 (3%)	n = 3 (5%)	n = 3 (5%)
	22^{3} (37%)											
	3^{4} (5%)											
	FAMI (0.25 ± 0.007); Grade scale (1)											

GLM model		Log of odds	Odds	Std. Error	Z value	P-value
	Intercept	-1.0116	0.36	0.0923	-10.958	< 0.001
	Site 2	-0.1048	0.90	0.1322	-0.793	0.428

0 (no asymmetry) to 1 (all the traits are asymmetric). It is possible that some of the traits do not express asymmetry, but only in very rare cases, all 11 traits can be bilateral (Zakharov et al. 2000a, 2000b). The fluctuating asymmetry was defined by the index frequency of asymmetric manifestation of an 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; *n* is the number of individuals in the sample (Palmer 1994, Zakharov et al. 2001). Based on the observed mean values of FAMI, 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, Zhelev et al. 2015, 2017, 2019) and for *B. viridis* (Chikin 2001, Peskova et al. 2011, Guo et al. 2017, Zhelev et al. 2014). 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: minimum 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); FAMI ≥ 0.71 – grade 5: a critical condition of organisms (very heavily polluted water basin).

Statistical analyses

Mathematical processing of the data was carried out using standard statistical procedures, using the statistical package R-3.1.2 (R Development Core Team 2015). In order to examine the odds for observing asymmetry in the studied traits in the individuals from each habitat (site), a GLM model using logit link function was fitted to the FAMI data (Molenberghs 2003).

Results

The SVL parameter varied in individuals from site 1 in the range of 60.19–74.27 mm (60.19–74.27 mm for females and 61.17–72.31 mm for males) and in those from site 2 in the range of 61.04–74.52 mm (61.04–74.15 mm for females and 62.47–74.52 mm for males).

According to the results of the GLM model, there was no difference in the chance of observing asymmetries between the sexes. Therefore, the data were analysed together (regardless of sex). The mean values of FAMI in individuals of the green toad from the two studied habitats in the Plovdiv city were 0.27 and 0.25 from site 1 and site 2, respectively (Table 1). From both sites, we recorded low levels of asymmetry: 88% of individuals from site 1 and 95% of those from site 2 had two and three asymmetric traits. Only 12% of the toads from site 1 and 5% of these from site 2 were with four asym-

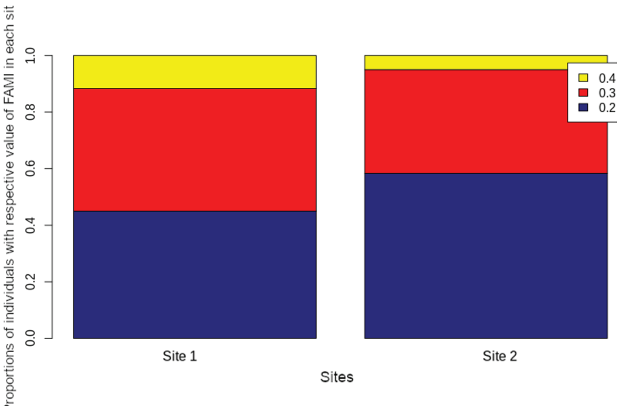

Fig. 2. GLM model: visualisation of values of the FAMI index (frequency of asymmetric manifestation of an individual) in individuals of *B. viridis* from the studied sites in the Plovdiv city.

metric traits out of eleven (Fig. 2). The traits 1, 2, 3 and 4 were the most asymmetric in the individuals of the two compared groups. At the same time, for the toads from both sites, we recorded the lowest levels of asymmetry for traits 5, 6, 7, 9 and 11. The results from the GLM model showed that the chance for no asymmetry observation in the studied traits in individuals of *B. viridis* from both sites was bigger than the opportunity to observe asymmetry (the Intercept < 1, see Table 1).

Discussion

Meristic traits of anurans, such as colour marks (stripes and spots) on the dorsal side of the body and limbs, react to the changes in the environment and are proven markers to evaluate the levels of fluctuating asymmetry (Zakharov et al. 2000a, 2000b). Aquatic frogs, such as the European green frogs *Pelophylax esculentus* complex, are suitable test-subjects because they spend most of their life in the water and are in direct contact with various stress-agents, such as toxic substances with an anthropogenic origin. In addition, unlike toads, they are firmly attached to the water basin in which their larval development has taken place. It is not surprising, therefore, that most bioindication analyses using fluctuating asymmetry as a marker for assessing developmental stability have been explicitly directed to representatives of this group (Chubinishvili 1997, Peskova & Zhukova 2007, Spirina 2009, Zhelev et al. 2019). Green toads, unlike water frogs, are less dependent on the water basin and after finishing their larval development, they leave the water and return later only briefly for breeding. However, studies on syntopically inhabited ponds during the breeding season (when disturbances in developmental stability occur) with representatives of *Pelophylax* kl. *esculentus* have shown good results and indicate that green toads can also be good bioindicators (Chikin 2001, Peskova et al. 2011, Zhelev et al. 2014, Guo et al. 2017). However, terrestrial life and little connection to water bodies in adult green toads impose some limitations on their use as test objects, especially in long-term analyses that track fluctuating asymmetry levels in the same population.

Our long-term analyses conducted with *P. ridibundus* as a test subject in various freshwater ecosystems in Bulgaria have generally shown high FAMI values in populations in conditions of environmental stress. It is also essential that fluctuating asymmetry values are not affected by the type of toxicants but only by their levels. In a population inhabiting a rice field that is heavily contaminated with pesticides, the mean FAMI values were 0.71 ± 0.01 (Zhelev et al. 2017). In water bodies contaminated with domestic sewage pollution, the FAMI ranged from 0.67 ± 0.001 to 0.84 ± 0.014 (Zhelev et al. 2019). In water basins contaminated with heavy metals, the values were from 0.73 ± 0.01 to 0.76 ± 0.01 (Zhelev et al. 2015). All these FAMI values corresponded to a 4-grade scale (heavily polluted water basin) and a 5-grade scale (very heavily polluted water basin). In the same studies, relatively clean water bodies were used as controls (all parameters monitored by parallel physicochemical analyses) and the reported FAMI indices were in the range of 0.37 ± 0.01 to 0.39 ± 0.009, which corresponded to a 1-grade scale (clean water basin). In our study, for a three-year period in populations of *P. ridibundus* and *B. viridis* inhabiting the region of the lead and zinc plant Kardzhali, had mean values of FAMI index from 0.53 ± 0.03 to 0.63 ± 0.02 for the *P. ridibundus* and 0.57 ± 0.03 to 0.68 ± 0.03 for *B. viridis* (Zhelev et al. 2014). These results strongly demonstrate the potential for the direct application of fluctuating asymmetry in bioindication analyses. The present study is an illustration of this possibility: based on the levels of fluctuating asymmetry in individuals of *B. viridis* inhabiting the two tested sites, we evaluated their living environment.

The low asymmetry levels reported for individuals of *B. viridis* 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. The results of the GLM model confirm that the chance of not observing asymmetry in individuals of the green toad from both sites is greater than the chance of doing so. This means that the environmental conditions at both sites were good at the time of the study. Although both protected areas are located in a highly urbanised area, the results show that they are free of anthropogenic stressors and that the biota is in balance with the environment. Although we have not explored directly the environmental factors, based on the results of the fluctuating asymmetry analysis on the individuals of *B. viridis*, we believe that the hills are unique "islands" that have preserved flora and fauna in the very centre of the big city. The results of the analysis of fluctuating asymmetry levels in this study 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 bioindication experiments.

In particular, because *B. viridis* is a species with a wide range of distribution, the practical application of the fluctuating asymmetry method in meristic morphological traits as a biomarker for assessing environmental quality illustrated in the present study can be directly applied at different points in the species' range. The method is ethical, relatively easy to implement and does not require expensive equipment. It can also be used with other types of anurans, including those protected by national laws and international conventions, especially where physicochemical analyses cannot be performed directly for one reason or another.

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