

## Asymmetry in the common wall lizard *Podarcis muralis* under different levels of urbanization: the effect of trait and FA index selection

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**Abstract:** The use of fluctuating asymmetry (FA) as a measure of developmental instability and its relationship to stress and fitness is highly controversial. We examined whether the selection of different FA indices and traits influences the results of FA analysis. We chose four meristic traits and three FA indices (two single-trait and two multiple-trait indices) to assess FA levels in the common wall lizard (*Podarcis muralis*) from three different habitat types (urban, suburban and natural). Urbanization has already been linked to developmental instability in *P. muralis*. We therefore expected to detect different FA levels among the habitats. However, we also wanted to see whether we obtained the same patterns using different indices and traits. Our results showed that different traits can yield different FA patterns between habitats. The only statistically significant difference between habitats was detected for the FA<sub>2</sub> index in femoral pores. The highest level of FA was detected in the urban population, while the lowest level was in the natural population. It is clear that caution must be exerted when deciding on which traits and indices are to be used for FA analysis.

**Keywords:** developmental instability; stress; fluctuating asymmetry; Lacertidae; meristic traits

### INTRODUCTION

One of the biggest challenges in conservation biology is how to identify threatened populations before stress causes irreversible negative effects. The level of developmental instability could be a simple and early indicator of populations subjected to stress. Developmental instability is the opposite of developmental stability – a process that buffers the effects of environmental perturbations on the ontogenetic development of an organism and thus maintains it along the trajectory within the same environment [1]. The quantification of the degree of developmental stability is challenging because the lack of within-individual variation caused by perturbation has to be measured. Therefore, developmental instability is used as a surrogate for measuring developmental stability [2]. In order to use developmental instability as an efficient early indicator of negative effects of stress on the organism, we assumed that (i) increasing levels of stress are accompanied by increasing levels of developmental instability, and that

(ii) stress-induced changes as a result of developmental instability are first detected in behavioral or external morphology traits rather than in more direct fitness components (e.g. fecundity, survival) [3].

The most widely used morphological measure of developmental instability is fluctuating asymmetry (FA) [4]. FA refers to subtle differences in phenotypic value between characters on serially homologous structures or non-directional differences between the left and right sides of paired bilateral structures [5,6]. The underlying assumption of FA is that both sides of a bilaterally symmetric organism are under the influence of the same genes and environmental conditions, and any developmental dissimilarity between them (developmental noise) must be a consequence of perturbations of the normal course of development [7,8].

FA has already been used to describe influences of different environmental stressors in lacertid lizards, i.e. pollution and urbanization [9-11], population iso-

lation [12,13], habitat fragmentation [14], incubation temperature [15], sexual selection [16], and locomotor performance [17,18]. These studies did not yield consistent results: some indicated that a stressful environment induces higher levels of FA, while others did not [9,14]. In addition, a literature survey for studies involving lizards as a subject species showed that different methodological approaches were used for the estimation of FA levels; some authors compared FA levels between target groups using several morphological traits [9,11,13,15] while others used just one trait [14,19-21]. As the FA indices can be calculated in many different ways [22], a literature survey showed the use of various FA indices in lizards, some were corrected or not for trait size, some were calculated for only one trait and others described multiple traits [11,13,19].

The main goal of this study was to test whether differences in the methodological approach (use of different traits and different FA indices) affect the assessment of the stress-induced developmental instability in the common wall lizard, *Podarcis muralis*. As a source of stress, we chose urbanization level and tested the FA levels in natural, suburban and urban populations. The common wall lizard is a good model species for such studies as recent research already established a link between urbanization and FA, with higher levels of FA observed in urban habitats [9-11]. This species, like all lacertid lizards, is abundant in suitable habitats [23], including urbanized areas [24], with specimens showing strong site fidelity, small home ranges, and they are usually generalist predators [25,26]. In addition, they have various quantitative traits as measurable (metrical) characteristics (e.g. limb and head measurements) and numerous countable (meristic) characters (e.g. different types of head scales, femoral pores) [27], which are appropriate for testing FA.

We hypothesized that: (i) the higher level of urbanization is accompanied by a higher level of developmental instability, with natural populations having the lowest FA, and urban populations possessing the highest FA; (ii) the patterns of the developmental instability resulting from urbanization as a stressor are the same regardless of the morphological trait tested, as it is expected that an individual that is more asymmetrical for one trait is more asymmetric for other traits as well; (iii) the patterns of the developmental instability resulting from urbanization as a stressor

will be the same, regardless of the type of FA index used to assess the levels of stress.

## MATERIALS AND METHODS

### Ethics statement

The collection of *P. muralis* adults from a natural population was approved by the Ministry of Environmental Protection of the Republic of Serbia (Permit No. 021-01-5/11/2017-09).

### Study species

The common wall lizard (*Podarcis muralis*) is a small lacertid species found in Europe, with the largest distribution range among *Podarcis* lizards [28], and a maximum life span of five years [29]. The common wall lizard is also one of the species that has the widest distribution on the Balkan Peninsula. It is even adapted to human-made habitats [30] and therefore could be used as a potential bioindicator. This lacertid lizard inhabits sunny, dry and stony habitats, but in the southern part of its distribution it can be also found in shady and humid areas [31]. *P. muralis* shows a high tolerance toward a broad range of habitats and therefore is considered as not threatened by both national [32] and global standards [33]. However, some recent studies have indicated that urbanization could have a negative effect on the developmental stability of the common wall lizard [9;10].

### Study area

All specimens were collected in the northern part of Serbia, Vojvodina, during 2017 and 2018 by noosing. This is an area where urbanization and agricultural practice have decimated almost all natural habitats of the common wall lizard. However, this species is widespread in Vojvodina, so it is important to establish how it copes with increased anthropogenic pressure. In total, we captured 184 specimens (96 females and 88 males) from different sites: three urban, three suburban and one natural (from 24 to 28 specimens per locality). Sites were classified into three categories according to McKinney [34]: urban (>50% impervious surface), suburban (20-50% impervious surface),

natural (no impervious surface). For urban (U) locations we chose three cities (Kikinda [N 45.827238; E 20.458923], Zrenjanin [N 45.383182; E 20.376970] and Kovin [N 44.740804; E 20.969798]) that are about 60km apart. Suburban (SU) populations were collected from three villages (Banatsko Veliko Selo [N 45.827848; E 20.589077], Ečka [N 45.324564; E 20.469495] and Banatski Brestovac [N 44.730363; E 20.805078]). Each village is approximately 15km from the city. The natural population (N) was sampled on Mt. Fruška Gora [N 45.161338; E 19.701357], one of the only two remaining natural habitats for the common wall lizard in Vojvodina [35]. The other natural habitat is Vršački Breg, but it was not possible to collect a sample due to the extremely small population density.

### Trait quantification

Four morphological traits were selected for this analysis (as they can be easily and rapidly quantified and they show extensive variability in *Podarcis* populations): supraciliar plates (SP), sublabial plates (SL), subdigital lamellae of fourth hindlimb toe (D4) and femoral pores (FP) [36] (Supplementary Fig. S1). In addition, these traits were chosen according to the literature on stress-induced developmental instability in lizards [9,13]. High-resolution photographs were taken using a Nikon D50 camera with a 1.2-million-pixel resolution and equipped with a Nikon 60 mm AF-S Micro lens. The number of plates, pores, and lamellae were counted twice with several days between counts by the same person (MM). Adults and subadults were scored together, as the scalation pattern did not show ontogenetic variation [37]. In addition, the size of all specimens was measured (snout-vent length) using a digital caliper.

### FA quantification

In order to test if the use of different FA indices will affect the assessment of stress-induced developmental instability, we chose the most often-used FA indices in lizard literature: (i)  $FA_1$  index uncorrected for trait size:  $\text{mean}|R-L|$ ; (ii)  $FA_2$  index corrected for trait size by the individual:  $[(|R-L|)/((R+L)/2)]$ ; (iii)  $FA_{14}$  composite index:  $\sum[|FA_{ij}|/\text{mean}|FA_j|]/Nt$ , where  $FA_{ij}$  is the deviation from symmetry of trait  $j$  in individual  $i$ , and  $\text{mean}|FA_j|$  is the average absolute deviation from symmetry of trait  $j$  for the entire sample, and  $Nt$  is the

number of traits per individual; (iv)  $FA_{17}$  composite index:  $\sum|\ln(R_j)-\ln(L_j)|/T$ , where  $R_j$  and  $L_j$  are measurements of the R and L sides for trait  $j$ , and  $T$  is the number of traits per individual [22]. To account for measurement error, we used the average trait values of two separate counts. In summary, for each individual, we calculated (i) the FA value ( $FA_1$ ,  $FA_2$ ) for each trait separately, and (ii) the composite FA value ( $FA_{14}$ ,  $FA_{17}$ ) for the combination of all four traits.

### Statistical analysis

The correct estimation of FA requires testing whether: (i) the FA variance in the sample is biologically relevant or the result of developmental noise; (ii) the FA variance in the sample is significantly larger than the measurement error; (iii) whether directional asymmetry (DA) affects the observed FA patterns; (iv) whether antisymmetry affects the observed FA patterns [22]. As we did not have a measurement error in our sample (two individual counts of meristic traits were identical), we confirmed significant FA (biological relevance) by comparing the hypothetical zero value with absolute left side-right side values by trait and group, using the permutation t-test. The absence of DA was confirmed by comparing the hypothetical zero value with left side-right side values by trait and group with the permutation t-test [38]. The only trait affected by DA was the number of subdigital lamellae of the fourth hind limb toe, but not for all groups (just for suburban and urban), so that the interpretation of the results for this trait should be undertaken with some caution. The presence of antisymmetry was excluded after visual inspection of the distributions of the left-right side values for every trait for every group separately.

Permutation-based p-values were used in all subsequent analyses, as a preliminary Kolmogorov-Smirnov test showed the absence of normal distribution in all FA values. A preliminary test also showed an absence of correlation of FA values with individual size, so that correction for size was not performed. The effect of sex on FA values was not confirmed and thus sexes were pooled prior to further analyses.

To test if the use of different traits and FA indices affected the assessment of the stress-induced developmental instability in the common wall lizard, we performed several permutational ANOVA tests with

habitat (U, SU, N) as a factor, and as dependent variables (i) individual  $FA_1$  values for each trait separately; (ii) individual  $FA_2$  values for each trait separately; (iii) individual  $FA_{14}$  values for the combination of traits; (iv) individual  $FA_{17}$  values for the combination of traits. All analyses were performed using Statistica 10 (StatSoft Inc., 2011) and R software v. 3.2.0 (packages *lmp*, *predictmeans*, *perm.t.test*) (R Core Team, 2015).

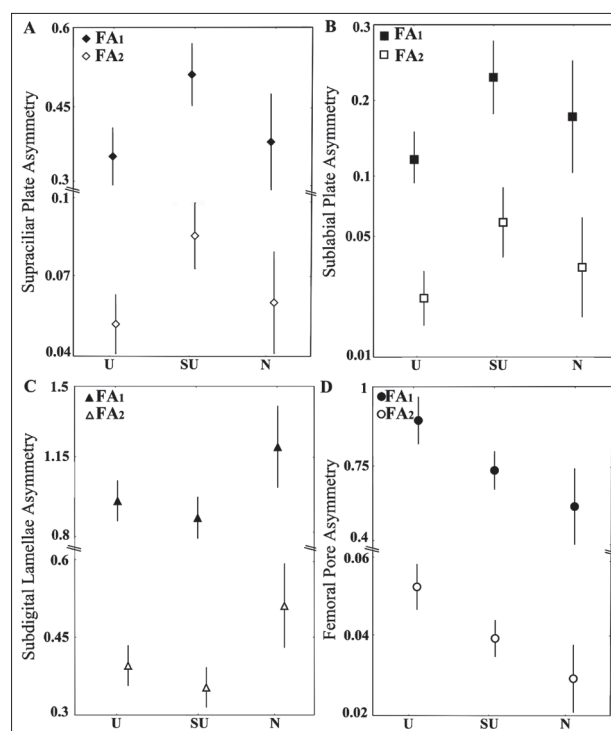
## RESULTS

Out of 188 specimens, 93.1% had asymmetry in at least one of four traits, 65.4% had asymmetry in D4, following 58.0% in FP, 38.8% in SP, with a minimum asymmetry presence of 17.5% in SL. Patterns of FA in all four traits are shown in Fig. 1. For the four analyzed traits, the only statistically significant difference between habitats was detected for femoral pores. The highest level of FA was detected in the urban population, then in the suburban, while the lowest level was observed in the natural population. When comparing the results obtained with different FA indices, a similar pattern of FA levels across different habitat types was observed (Fig. 1). According to  $FA_1$ , there was no significant effect of habitat on FA levels for all traits. The  $FA_2$  index showed a statistically significant difference between habitats for FP ( $P=0.0246$ ). With regard to FP, *post hoc* analyses showed a significant difference between natural and suburban ( $P=0.019$ ) and natural and urban ( $P=0.015$ ) populations. Finally, the effect of habitat was insignificant for both composite indices ( $FA_{14}$  and  $FA_{17}$ ).

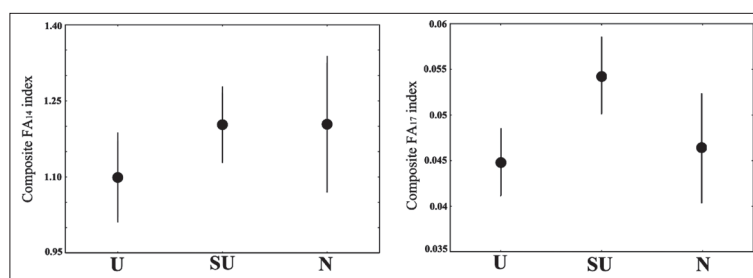
The patterns of FA differences between habitats revealed by composite indices are shown in Fig. 2, and here we can see that the patterns are similar.

## DISCUSSION

Our study provides the first insight into the patterns of FA in common wall lizards in Vojvodina as a result of developmental instability induced by different levels of urbanization. Our results showed the existence of FA in the number of supraciliar plates, sublabial



**Fig. 1.** The pattern of FA differences between habitat types (U – urban, SU – suburban, N– natural) in all four morphometric traits with indices  $FA_1$  and  $FA_2$ . **A**– Supraciliar plates; **B**– Sublabial plates; **C** –Subdigital lamellae of the fourth hindlimb toe; **D** – Femoral pores.



**Fig. 2.** The pattern of FA differences between habitat types revealed by the composite index between habitat types (U – urban, SU – suburban, N– natural).

plates, femoral pores and subdigital lamellae of fourth hindlimb toes in the common wall lizard. However, conclusions regarding the differential effects of urban, suburban or natural environments on developmental instability are highly dependent on the trait and the FA index used in the analyses.

We detected FA in populations of all three habitat types, but not all analyzed traits exhibited the same level of FA. For supraciliar plates and sublabial plates, we detected the same pattern of FA levels between

habitat types, while somewhat different patterns were observed for subdigital lamellae of fourth hindlimb toes and femoral pores. More importantly, FA levels of supraciliar plates, sublabial plates and subdigital lamellae of fourth hindlimb toes were not statistically different among habitat types. The expected statistically significant increase in FA in urban populations of the common wall lizard compared to suburban and natural ones was confirmed only for the number of femoral pores, and only for the  $FA_2$  index.

In the literature on FA and environmental stress, many authors analyzed the level of FA in a single trait. Almost always that trait was femoral pores [14,16,19,20,21]. Femoral pores, aside from being easy to count, are under strong sexual selection and have an influence on pheromone secretion; thus it is assumed that males with lower femoral pore FA are more likely to be successful in mating [21]. Also, there is a tendency in specimens that exhibit lower FA in femoral pores to also have lower FA levels in other traits [16]. However, in the majority of papers, FA levels were evaluated for several morphological traits [9,11,13,15]. The observed FA pattern for FP was in agreement with previous findings [9]. The authors examined a wide area of the city of Niš, and reported significant differences in the FA of supraciliar plates, subdigital lamellae of fourth hindlimb toes and femoral pores between urban and natural habitats, where the urban population exhibited higher FA. Lazić et al. [10] corroborated these results with their paper on the FA of pileus shape where they again discovered higher FA in urban than in natural populations.

On the other hand, a discrepancy between the patterns of different morphological traits was similar to the results of Sacchi et al. [11]. In their research, the authors incorporated site replication, as well as three different habitat types (natural, suburban and urban). They used four meristic traits: supraciliar plates, femoral pores, ventral scales and pileus shape, and detected different patterns of FA in different traits. For ventral scales, the highest FA was in the natural population, then in the urban, and it was lowest in the suburban population. The same pattern was observed for femoral pores. A completely different pattern was observed in supraciliar plates: the highest FA was in the suburban, then in the natural and urban populations. Importantly, a significant difference in FA between habitats was

detected only for femoral pores, while the difference for supraciliar plates and ventral scales was insignificant. The results for pileus shape were similar to those previously reported [10], where the highest FA was detected in the urban habitat and the difference between habitats was statistically significant. Dissimilar patterns of FA in different traits may be due to differential susceptibility to environmental stress, stress buffering capacity and different ontogenetic patterns and timing of developmental asymmetry [39].

Our study provides insight into the use of FA as a measure of developmental instability from one more angle. It is an addition to the ongoing debate regarding the choice of appropriate traits that can be affected by stress and for detecting developmental instability by FA [40]. Natural and sexual selection can impact morphological traits and can “confound” the effects on FA. Functional traits that strongly influence performance (e.g. reproductive success, survival, growth rate) [41] and individual fitness [42], are under the strong influence of selection. Strong nonlinear selection acts against asymmetry in functional traits [43] and should be avoided in FA/developmental instability studies. Some studies promote the use of functional traits [44], while others confirm that traits relaxed for functionality selection have higher FA and are therefore more appropriate to use [41]. A consensus about this issue still cannot be fully established as some analyses have showed that trait type does not have a predictive power in FA studies [45]. With regard to reptiles, contradictory results [11,46] point to the necessity of more elaborated studies, but the use of FA cannot be rejected as the latest long-term studies [47] and meta-analyses [48] have shown that ecological correlates and causes of asymmetry can be detected.

If we focus on results obtained from different FA indices, we can see that not all of them provide significant results. According to  $FA_1$ , femoral pores do not exhibit a statistically significant difference between the three habitat types, but according to  $FA_2$  they do. According to composite indices, we detected no significant results in FA levels between habitat types.

Concerning selection of the FA index, in most papers only one single-trait index was used (see Laia et al. [46] for the review of papers on FA in reptiles). Predominantly absolute (R-L) index ( $FA_1$  in this paper) was

used [14,20,49], but a several other indices were used in published papers. In some papers composite FA index was used [50]. Composite indices may give a more accurate picture about FA than single-trait indices because they are evaluated over several axes [51]. Sometimes a composite index can provide opposite results when compared to a single-trait index. In [13], the authors used one single-trait index and one composite index and obtained different results: even though a single-trait index showed a significant difference between populations, the composite FA index did not. To some extent our results are similar to this, as the composite indices showed an insignificant difference in FA between populations, however, one of two single-trait indices detected significant differences in femoral pores.

In general, the selection of a proper FA index is not an easy task as there are thirteen indices describing the level of FA in a sample, and each of them has some pros and cons [52]. A literature survey for lizards revealed an absence of consistency in the use of FA indices, as well as insufficient justification for their use. Our study confirms that selection of FA index can affect both the results and the conclusions.

## CONCLUSION

Our study showed that trait and FA index selection can affect conclusions about developmental instability induced by environmental stress. More work has to be done to establish an appropriate protocol for the use of FA as an indicator of stress. Work should be focused not just toward understanding whether some environmental feature causes stress and under which conditions this stress can be detected by examining FA levels, but also on which traits and asymmetry indices are used. Not all traits respond in the same manner to environmental stress, and some indices might not be equally sensitive to detect different levels of FA between populations.

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**Author contributions:** MM and TV collected the field data. MM prepared the dataset for the analysis. All authors discussed the results and contributed to the final manuscript.

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## Supplementary Data

### Supplementary Fig. S1.

Available at: [http://serbiosoc.org.rs/NewUploads/Uploads/Supplementary%20Fig.%20S1\\_Mirc%20et%20al\\_4044.pdf](http://serbiosoc.org.rs/NewUploads/Uploads/Supplementary%20Fig.%20S1_Mirc%20et%20al_4044.pdf)