Fluctuating asymmetry in Hungarian meadow viper (*Vipera ursinii rakosiensis*) populations compared with three closely related subspecies

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Abstract

Vipera ursinii rakosiensis is the most endangered venomous snake in Europe. Our information on the state of natural populations is somewhat limited apart from recent results indicating high genetic diversity in all of its three major areas in the Carpathian Basin. Developmental instability (DI) is a good indicator of environmental or internal perturbations an individual experiences during development, and fluctuating asymmetry (FA; random deviations from bilateral symmetry) is a good proxy for DI. We studied FA in head scale numbers in *V. u. rakosiensis* populations and contrasted it with the FA of other subspecies. The four *V. u. rakosiensis* populations showed similar FA levels. The FA in *V. u. rakosiensis* was higher than in the *V. u. moldavica* and *V. u. ursinii* populations, but similar to the *V. u. macrops* population. The (not exceptionally) high FA in *V. u. rakosiensis* might indicate external or internal stressors increasing DI. We suggest that FA should be monitored to get information on changes in DI.

Keywords: developmental instability, fluctuating asymmetry, reptile, snake

Asymmetry in bilateral traits is common in nature. The three main forms of asymmetry have been separated early (Van Valen 1962): (i) directional asymmetry is present when there is a consistent bias towards one side, (ii) antisymmetry is present when there is consistent asymmetry without one side being dominant, and (iii) fluctuating asymmetry (FA) consists of random deviations from perfect symmetry. Directional asymmetry and antisymmetry are results of normal development and typically occur as responses to natural selection (Palmer 1996, Graham et al. 1998). Fluctuating asymmetry, on the other hand, results from perturbed development (Palmer and Strobeck 1986; Leary and Allendorf 1989) and thus it was considered a good and easy to gather proxy for Developmental Instability (DI; Palmer and Strobeck 1986). Thanks to the compelling clarity of the theory and the relative ease of data collection, FA was proposed as a particularly promising tool for conservation biology and an important trait for evolutionary biology (Parsons 1992; Clarke 1995). This notion was strengthened by the accumulating evidence connecting FA with external or internal stressors (e.g. Bengtsson et al. 1985; Sarre 1996; Roldan et al. 1998; Siikamäki and Lammi 1998), demonstrating its link to fitness (Martín and López 2000, 2002; López et al. 2002) and occasionally proving its heritability (e.g. Loehr et al. 2012; but see Leamy 1997). Studying meristic traits that can be counted without inherent measurement error provides relatively easy opportunities to use FA as an indicator for conservation or to understand the evolutionary background of DI (Herczeg et al. 2005; Loehr et al. 2012; Trokovic et al. 2012; Mészáros et al. 2023). Most reptiles offer a set of easily recordable bilateral meristic traits (head scales) and thus they might be good models to study the evolutionary significance of FA, whether it can be used in conservational herpetology.

The Hungarian meadow viper (*Vipera ursinii rakosiensis*) is a lowland ecomorph subspecies of the *V. ursinii* complex (Ferchaud et al. 2012). It is considered the most threatened European venomous snake. It occurs only in three main areas in the Carpathian Basin: Hanság

(North-Western Hungary), Kiskunság (Central Hungary) and Transylvania (Romania). Due to the fragmented distribution, small local areas and low density, *V. u. rakosiensis* was on the verge of extinction (Újváry et al. 2002; Rehák 2004). Thanks to a cooperative conservation effort culminating in a Species Conservation Plan including captive breeding, rearing and reintroduction together with habitat restoration, the situation is more promising currently (Halpern et al. 2024). However, despite the continuous efforts in the last decades, including permanent field monitoring, the state of the natural populations in terms of, e.g., reproductive output, age distribution, operative sex ratio, growth patterns or body condition is yet unknown, or evaluations are based on anecdotal evidence. The reasons behind it are likely to be the highly cryptic pattern and behaviour of the subspecies, resulting in low chances of observation and potentially affecting the observation probability of individuals of different size, sex, and state (e.g., gravid *vs.* nongravid) and conservation priorities of minimising stress and disturbance *via* forbidding specimen handling and measurements in the field. The genetic results are also somewhat mixed. Újváry et al. (2002) reported low genetic variation (especially within-population) in major histocompatibility class I loci compared to the closely related *V. renardi*, while recently, Vörös et al. (2022) showed that microsatellite-marker-based neutral genetic diversity was surprisingly high.

In the present paper, we estimated the FA of bilateral head scale characters of four populations of *V. u. rakosiensis* from Hanság, Kiskunság and Transylvania. Note that we identify every isolated occurrence within a region as a separate population, because these sites are separated by areas that are not suitable for *V. u. rakosiensis* (Halpern et al. 2024). However, a recent study based on neutral microsatellite loci diversity concluded (based on low F_{ST} values) that genetic isolation between *V. u. rakosiensis* populations typically exists, but is not strong, indicating relatively recent fragmentation (Vörös et al. 2022). Historically, the species and subspecies of the *Vipera ursinii* complex have been grouped according to the

altitudinal differences between populations, distinguishing between lowland and montane taxa. This grouping between the taxa was also supported by the phylogenetic relationship based on mitochondrial DNA (Ferchaud et al. 2012). First, we aimed to compare FA between the populations to see whether any of them were particularly affected by external or internal stressors. Second, we compared FA in *V. u. rakosiensis* to two large populations of the other lowland ecomorph subspecies, *V. u. moldavica* (genetically the closest relative to *V. u. rakosiensis*; Ferchaud et al. 2012; Zinenko et al. 2015), and two montane ecomorph subspecies, *V. u. ursinii* and *V. u. macrops*, to see whether *V. u. rakosiensis* populations were under larger stress than the other subspecies. Finally, we tested whether FA levels were negatively correlated with body length (≈ age), which would have indicated negative survival selection on FA (and the underlying DI) (Forsman et al. 1994) and differed between sexes, which would indicate sexual differences in developmental sensitivity to stress. The Periteaşca population of *V. u. moldavica* occurs on Grindul Perişor, in the Romanian Danube-Delta, with 1,800 ha of continuous habitat with low levels of human activities and related disturbance, while the Sfântu Gheorghe population occurs on Grindul Sărături with over 1,000 ha of habitats, with increasing human effects in the surroundings of the village and presence of low numbers of extensively herded cattle and horses (Halpern et al. 2007). In both habitats, the population sizes may exceed well over 1,000 individuals. However, the mountain populations are found in more restricted habitats. The Orgeas population of *V. u. ursinii* inhabits mountain meadows of Haute-Provence in France, where nature conservation attempted the enlargement of available grasslands by the removal of non-native pine forests, connecting a few hectare patches to 10-20 ha of connected habitats (Lyet 2008, Lyet et al. 2013). Their numbers are unlikely to exceed a few hundred individuals. The Veliki Stirovac population of *V. u. macrops* in Paklenica National Park, Croatia, also occurs on 10-20 ha (Burić 2012, Lukač et

105 al. 2016). Accordingly, we predicted higher FA levels in the smaller and more isolated
106 montane populations compared to the larger lowland populations.

107 Samples were collected during field trips of consecutive LIFE-projects over the years 1999-
108 2021, which we summarised in Table 1 and Fig. 1. Only data that were collected before the
109 reintroduction efforts of the conservation project started were used in the analysis to avoid the
110 confounding effects of the captive-bred individuals. Vipers observed during field visits were
111 always photographed and sexed, and in some of the cases, total length was also measured.
112 Head scalation was assessed by counting scales of certain scale groups on the photographs for
113 both individual identification and estimating FA. Scale groups were used as described by
114 Nilson & Andrén (2001). Out of the described scale characters, we used the following
115 bilateral scale groups for estimating FA: *Intersupraoculare, Circumoculare* and *Lorealia* (Fig.
116 2).

117 To test whether FA differs across populations, also whether sex- and size differences affect
118 this divergence, we analysed the so-called composite standardised relative asymmetry
119 (CSRA) index (cf. Trokovic et al. 2012). Briefly, CSRA describes the individual-level
120 asymmetry considering every trait with equal weight; for more information on how to
121 calculate CSRA, see the supplementary text. As CSRA showed a continuous, but right-
122 skewed distribution, we built a generalized linear model (GLM) with Gamma distribution. In
123 this model, CSRA was the response variable, and population, sex, and their interaction as
124 fixed factors. A second GLM was fitted on a restricted database where we had information on
125 individual size (N = 321). This second GLM was built similarly to the first one, but
126 population, body length, and their interaction were fitted as fixed effects. In the second model,
127 we pooled the sexes due to sample size restrictions. We simplified our models by inspecting
128 parameter estimates of interactions and main effects, removing the least significant effects
129 until we got the minimal adequate model. We extracted the model's estimated marginal

means using the *emmeans* package (Lenth 2019). Pairwise comparisons between populations were conducted using estimated marginal means, with p-values adjusted for multiple comparisons using the False Discovery Rate (FDR) correction. Fixed effects were tested by Wald's chi-squared tests. All models were built using the R package *lme4* (Bates et al. 2015).

Our GLMs indicated significant CSRA differences across populations (Fig. 1); while neither sex, nor population × sex interaction affected FA (population: $\chi^2 = 22.52$, df = 7, P = 0.002; sex: $\chi^2 = 0.36$, df = 1, P = 0.55; population × sex: $\chi^2 = 3.96$, df = 7, P = 0.79). Similarly, neither body length, nor population × body length interaction affected CSRA (size: $\chi^2 = 1.59$, df = 1, P = 0.21; population: $\chi^2 = 17.83$, df = 7, P = 0.01; population × size: $\chi^2 = 5.26$, df = 7, P = 0.63). Although 'population' had a significant effect, pairwise comparisons revealed that the studied four *V. u. rakosiensis* populations from the three regions did not differ systematically in FA levels. Hence, we found no sign of differences between populations or regions in the estimated level of DI. This result, together with the generally high genetic variation in all populations (Vörös et al. 2022), suggests that there is no known *V. u. rakosiensis* population that is particularly ran-down compared to the others. However, this does not mean automatically that all populations are in equal shape, since the alternative explanation about a rapid and relatively recent habitat deterioration and fragmentation that is not yet detectable *via* genetics or DI cannot be excluded.

The comparison between *V. u. rakosiensis* and other subspecies did not provide a clear-cut picture. When compared to the large populations of the genetically closest and also lowland-inhabiting *V. u. moldavica* (Ferchaud et al. 2012; Zinenko et al. 2015, Freitas et al. 2020), FA levels of *V. u. rakosiensis* were higher, which may be explained by the difference in land-use and effective population size. *V. u. moldavica* habitats in the Danube Delta are not subject to agricultural use; however, *V. u. rakosiensis* habitats are managed to a certain extent, which causes a higher level of recurring disturbance. This is similar to the findings of Buldain et al.

(2024) in relation to agricultural use and FA in *V. aspis* populations. This would suggest that the external (e.g., habitat quality) or internal (e.g., inbreeding) stressors are generally higher in the studied *V. u. rakosiensis* populations than in the studied large *V. u. moldavica* population. However, when comparing with the montane subspecies, we found that *V. u. macrops* had similar FA to *V. u. rakosiensis*, but higher than *V. u. moldavica* and *V. u. ursinii*. The latter had similar FA to *V. u. moldavica*, somewhat lower than *V. u. rakosiensis*. It suggests that all *V. u. rakosiensis* populations have high FA levels within the species, but these levels are not extreme within the studied subspecies. We note that *V. u. macrops* also inhabits relatively small, fragmented, altered and isolated environments (Jelic 2012), and thus the relatively high FA level of the sampled population at Veliki Stirovac is not surprising, since we consider this population limited in extent. We must also note that our sample sizes were sometimes small; hence, the chance that we could detect small differences is low.

One must separate the meaning of FA in functional (directly linked to fitness) and quasi-neutral (with no straightforward function) traits. Asymmetry in functional traits might translate to direct function loss and thus direct fitness loss too. Such examples can be seen in *Psammodromus algirus,* where hindlimb asymmetry was negatively associated with locomotor performance, which might be selected against by survival selection (Martín et al. 2001) or in *Iberolacerta cyreni*, where males with higher femoral pore FA were less attractive to females, which might be selected against by intersexual selection (Martín and López 2000). Head scale asymmetry, on the other hand, is unlikely to affect any functions directly (note that none of our sampled vipers were malformed), and thus it should be seen only as an indicator of DI. Therefore, we cannot propose direct fitness links of the relatively high head scale FA seen in the studied *V. u. rakosiensis* populations or the *V. u. macrops* population. Since scale determination is completed during embryogenesis (Maderson 1965), any correlation between body length (≈ age) and FA levels would indicate survival selection

acting on individuals with different FA (Forsman et al. 1994), even though most probably the trait under selection would not be head scale asymmetry *per se*, but DI indicated by FA in general. The fact that we did not find correlations between body length and FA in our sample suggests that there is no survival selection acting against individuals with higher head scale FA, and thus the detected FA levels are not indicating direct conservation concerns.

Taken together, we found that head scale FA of *V. u. rakosiensis* was uniformly high within the studied *V. ursinii* populations, but cannot be considered as extreme. In the lack of true head malformations and any indication of fitness loss, we conclude that the patterns revealed here do indicate some sort of higher-than-average levels of external or internal stress in *V. u. rakosiensis* within the studied subspecies, but not to the extent that need immediate (extra) targeted actions in any of the populations. However, we recommend collecting data with a sufficient sample size in the future (needs dorsal and two lateral head photographs of the encountered snakes) from which FA can be estimated, so that temporal trends can be established. It can be particularly interesting in assessing the effects of the yearly and increasingly intensive reintroductions of captive-bred vipers (note that we only analysed individuals from the natural populations measured before reintroductions) to some of the populations (Halpern et al. 2024).

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Data availability

Data used for the statistical analysis is available from Figshare Digital Repository doi: 10.6084/m9.figshare.29382110. The DOI becomes active upon acceptance of the manuscript.

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321 Table 1. Summary table for the sampled *Vipera ursinii* subspecies by collection sites and
322 sample size. An asterisk (*) indicates the undetermined sex for seven individuals in the V.
323 Stirovac population.

Subspecies	**Country**	**Locality**	**N_{total}**	**N_{male}**	**N_{female}**
V. u. rakosiensis	Romania	Csengerpuszta	65	25	40
	Hungary	Bugac	95	41	54
	Hungary	Peszéradacs	81	33	48
	Hungary	Hanság	45	25	20
V. u. moldavica	Romania	Periteasca	57	32	25
	Romania	S. Gheorghe	41	21	20
V. u. macrops	Croatia	V. Stirovac	23*	10	6
V. u. ursinii	France	Orgeas	16	5	11
Total			424	193	224

324

Figure legends

Fig. 1. The studied populations of *V. ursinii* complex (see Table 1) and the differences in composite standardized relative asymmetry (CSRA) across the studied populations and subspecies of *V. ursinii* complex. Back-transformed estimated marginal means and 85% confidence intervals (CI) are shown. Note that lack of overlap in 83–84% CIs is analogous to a *P* value < 0.05 (Payton et al. 2003)

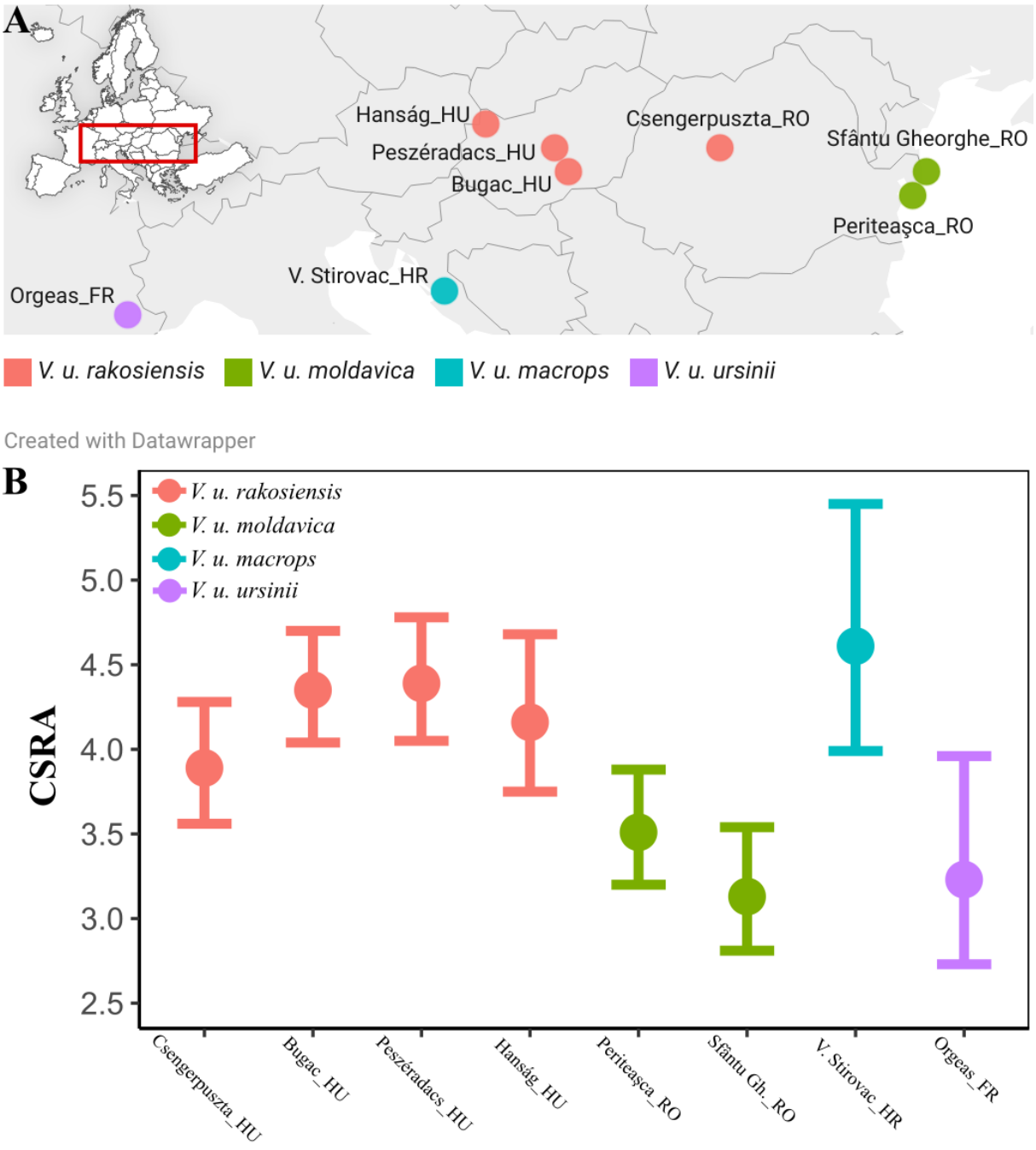

Fig. 2. Bilateral head scale groups of *V. ursinii* individuals, counted from photographs and used for the analysis of fluctuating asymmetry. Blue: *Intersupraoculare,* Pink: *Circumoculare* and Green: *Lorealia*