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DEVELOPMENTAL INSTABILITY IN GERMAN IRIS FLOWER AS A POTENTIAL BIOMONITORING METHOD

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Abstract: In light of the increasing need for appropriate, cost-effective detection methods of anthropogenic pollution, we evaluated the biomonitoring potential of flower developmental instability (DI) on a widely planted decorative species, *Iris germanica*, under *in situ* conditions. DI was measured by fluctuating and radial asymmetries of parts of *Iris germanica* perianth (810 fall lengths and widths), from clones already growing in two distinct types of habitats with contrasting levels of anthropogenic pollution: in unpolluted (rural) areas, Novi Banovci, Stari Banovci and Belegiš (flowers from 137 clones sampled), and in a polluted (urban) Belgrade metropolitan area (flowers from 133 clones sampled). Our results revealed significantly higher flower radial asymmetry in the polluted habitats compared to unpolluted ones (for three out of four univariate indices, as well as both multivariate ones), but failed to detect a similar effect on fluctuating asymmetry indices. The results of our study therefore demonstrate the potential of DI (when estimated by flower radial asymmetry) in *Iris germanica* as a cost-effective biomonitoring method for *in situ* pollution detection based on readily measurable flower parts and moderate sample sizes.

Key words: biomonitoring; *Iris germanica*; developmental instability; fluctuating asymmetry; radial asymmetry

INTRODUCTION

Environmental anthropogenic pollution exerts significant harmful effects on many living organisms and is a major problem in urban environments [1,2]. A broad spectrum of pollutants is released from many different sources located in urban areas from industry, power plants and intense vehicular road traffic in particular [3,4].

The systematic detection, quantification and monitoring of anthropogenic pollution is a challenge for environmental science [5-7]. Biological monitoring (biomonitoring) is "the planned, systematic use of organisms to determine environmental quality" [8], or a "specific problem designed to provide information on the characteristic of the problem and changes in these over the course of time" [9]. To date, various biological methods have been applied to detect and evaluate the effects of environmental disturbances on populations by typically examining body size and growth rate as indirect measures of fitness [6,10,11]. These methods are often expensive, time-consuming and usually detect a change when it is impossible to reverse ongoing processes. An alternative biomonitoring method is developmental instability (DI) estimation, defined as a measure of the individual's capacity to maintain developmental precision during ontogeny [12]. Any deviation from perfect symmetry is believed to reflect the inability to buffer development against random cellular processes that separately affect each organ side or part [13]. Because the majority of DI studies has involved animals, bilateral symmetry is the most utilized; however, all organism-wide symmetries could also reflect DI [14,15]. DI can be influenced by extrinsic (environmental) or intrinsic (genetic) stress [16-18]. The general assumption is that the level of DI should increase with stress intensity, and animal studies have shown that DI of sexual traits is particularly sensitive to the effects of environmental stress [19].

Flowers are complex reproductive plant structures that have evolved in close co-adaptation with pollinators [20-22]. In entomophilous plants, symmetrical phenotypes are maintained by selection because they perform better than asymmetrical ones [23-29]. Angiosperm flowers generally display two main symmetry types: actinomorphy (radial symmetry) and

zygomorphy (bilateral symmetry). Symmetry is generally defined as overall symmetry characterized by the perianth reflecting perception by the human eye [30].

Measurements of the left and the right side of a bilaterally symmetrical structure (fluctuating asymmetry, FA), or measurements of homologous radially symmetrical structures (radial asymmetry, RA), both represent repeated measures of the same genetically identical developmental process under uniform (or very similar) environmental conditions. The left and right sides of each floral organ, as well as homologous repeated floral parts, can therefore exhibit different morphology due to DI after all genetic and environmental effects on trait size are eliminated [31-33].

Correlations between DI estimates of vegetative and reproductive plant organs and various types of stresses are inconsistent in the literature. In some studies, flowers and leaves [34], as well as shoots and flowers [35], showed similar patterns in response to environmental stress, i.e. a higher level of asymmetry in stressful environments. Other studies have presented different results, with reproductive features being more developmentally stable than vegetative ones [36-38].

Flower petals are the most important visual attractants, and insects and other pollinators show preference for large and symmetrical flowers [26,39]. Floral traits within taxa display constancy within the limits of ecological tolerance [40,41], presumably due to the high homeostatic control of floral structures [36]. The greater canalization of floral organs compared to vegetative ones is a result of severe stabilizing selection i.e. selection against asymmetry [26,27]. Flowers are under strong directional selection and an increase in their size can result in an increased level of DI and therefore increased production of asymmetric phenotypes [23,25-27,29,39]. Therefore, petal asymmetry measurements (DI measurements) could be a comprehensive tool in biomonitoring [24,42].

Iris is the largest and most complex genus of the Iridaceae family, which is characterized by extreme diversity of more than 300 species. The range of the genus extends to all of the continents of the Northern Hemisphere [43]. *Iris germanica* is a European hybrid with a broad distribution, ranging from Central Europe, the Mediterranean, Balkans and Asia Minor [44,45]. German iris plants grow up to 120 cm in height, and have roots up to 10 cm in depth. It blooms mostly in May-August, the perianth color is blue, violet, yellow, brown or white, with various patterns of pigment distribution [46]. This species has been extensively developed as an ornamental plant [47,48]. In addition to its ornamental value, German iris rhizomes contain an essential oil often used in cosmetic and perfume industries [49,50]. The German iris flower is an actinomorphic or radially symmetrical type of flower exhibiting multiple planes of symmetry [15,51,52]. The structure of the flower allows for insect-based pollination, with the nectar-searching insect entering the so-called pollinator tunnels. Every flower is composed of three pollinator tunnels, and every tunnel consists of three floral organ parts: the outer petal ("the fall") on the bottom, the female sex organ ("the style") and the male sex organs ("the stamens") on the upper part. Above every tunnel is the fourth floral organ, the inner petal ("the standard").

In this preliminary study, we examined whether the FA and RA of the widely planted species *Iris germanica* (German iris) increase significantly with anthropogenic pollution in heavily polluted urban habitats, and if these developmental instability measures (and which measure, in particular) could serve as a comprehensive biomonitoring tool. To that end we (i) examined whether the flower traits of the German iris had a significantly different level of DI, as estimated by different FA and RA indices in polluted habitats in comparison to unpolluted habitats, and (ii) evaluated whether the flower traits FA and RA in this species could serve as an efficient *in situ* indicator of anthropogenic urban pollution.

MATERIALS AND METHODS

Study sites

The unpolluted habitats covered a series of rural municipalities distributed on the right bank of the Danube, with no industrial facilities and with low-volume traffic. They included Stari Banovci, Novi Banovci and Belegiš (Figs. 1a and b). These villages are about 30 km from polluted habitats that are located in the wider area of Belgrade (Figs. 1a and c), which has a popula-

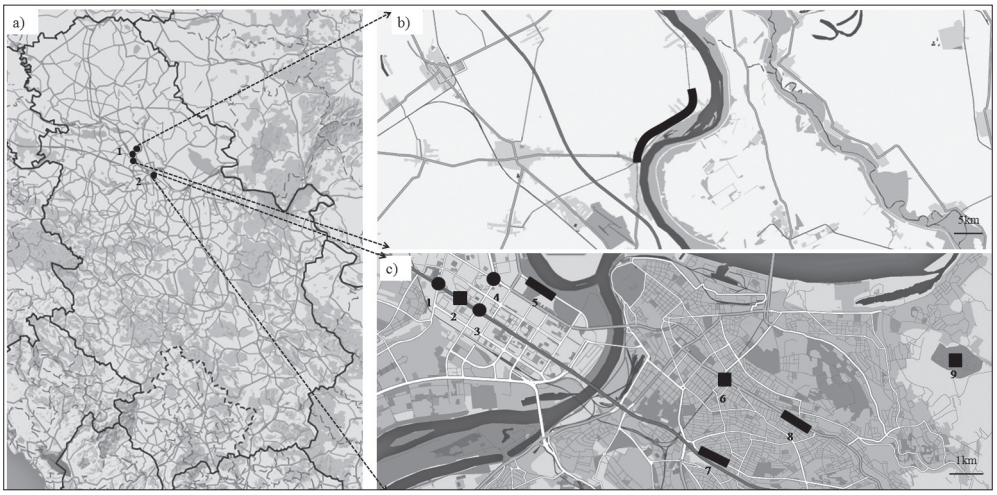

Fig. 1. The examined habitats. **a** – unpolluted (1) and polluted (2) habitats; **b** – unpolluted habitats (Novi Banovci, Stari Banovci, Belegiš); **c** – polluted habitats (Belgrade); intersections (•) of: Tošin Bunar St. and E 70 (1); Omladinskih brigada St. and E 70 (3); Blvd. Mihajla Pupina roundabout (4); median divider islands (▮) at Blvd. Nikole Tesle (5); Ustanička St. (7), Blvd. Kralja Aleksandra (8); street planters (▪) at the intersection of Narodnih heroja St. and E 70 (2); Blvd. Kralja Aleksandra and Ruzveltova St. (6); Lešće Cemetery (9).

tion of around 1.6 million. Belgrade is known for its air-polluting industry and high-volume traffic, with a less modern vehicle fleet with leaded gasoline still in use [53]. The annual averages for air pollutants in Belgrade in 2014 were: 21 μg/m^3 SO_2, 43 μg/m^3 NO_2, 1 μg/m^{3}CO and PM_{10} (particulate matter) 29 μg/m^3 [54], while soil analyses showed that various pollutants, including Cd, Cu, Hg, Pb, Zn and especially Ni, exceeded threshold values [55]. Both Belgrade and rural municipalities are characterized by a moderate continental climate with fairly cold winters and warm summers, with an average annual air temperature of 11.70°C [56]. We sampled German Iris flowers from 137 clones in the unpolluted and 133 clones in the polluted habitats.

Sampling design

In rural municipalities, German Iris is planted in individual home gardens. In the unpolluted habitats we collected one flower per individual household (home garden), along a 10-km long transect (Fig. 1b). In polluted urban habitats, German Iris is widely planted around major city roads in street planters and in divider islands, areas that separate opposing lanes of traffic. We sampled individual flowers from nine specific locations, mostly on main roads (Fig. 1c). From median divider islands, we collected individual German Iris flowers that were separated by not less than 10 m, and one flower per street planter. The sampling was designed to minimize the possibility of sampling genetically identical individuals (clones). To reduce variability in the analysis, we collected fully developed and undamaged individual flowers.

Examination of floral organs

Floral organs were cut at the perianth base and placed between two glass plates, flattened and embedded in glycerol to preserve the original shape and maintain the same focal length, and scanned with a Canon CanoScan 5200F at a resolution of 200 ppi. We measured the distance from the base of the fall to the top through the mid-axis (the fall length or FL), the distance between the left and right margins at the top of the beard (the fall width or FW), as well as the distance from the midrib to the left and right margins at the top of the beard (L – left; R – right). Interlandmark distances were calculated using the TMorphGen6 program (Sheets HD, 2000. Available at: http://www3.canisius.edu/~sheets/morphsoft.html) from the landmark coordinate data previously obtained by thetpsDIG 2.16 program (Rohlf FJ, 2010. Available at: http://life.bio.sunysb.edu/morph/). The two series of measurements were taken on different dates and in a random order to reduce bias.

Statistical analyses

Statistical analyses of radial and fluctuating asymmetries were performed following Palmer and Strobeck's recommendations [17,18,57-59].

Radial asymmetry analyses

After outlier analysis that was followed by Grubb's test [18,60], we estimated RA as the deviation of the individual fall trait values from the flower average. Two univariate RA indices, the standard deviation (SD) and the coefficient of variation (CV) for two measured traits FL and FW, as presented in Table 1, were calculated. We also calculated multivariate indices, multivariate standard deviation (MVSD) and multivariate coefficient of variation (MVCV) in order to present the overall instabilities more accurately (Table 1). In order to test for differences in RA between habitat types, we performed analysis of variance (ANOVA) followed by Scheffé's test [61], using SAS 9.3 (Statistical Analysis System for Windows. SAS Institute, 2010). The Scheffé test is designed for all possible comparisons, including both pairwise comparisons and contrasts. This method maintains type I error at the chosen level (0.05) [61,62].

Fluctuating asymmetry analyses

After outlier analysis [18,60], we tested if the estimates of FA were significantly greater than the measurement error for each habitat and for each trait. To test for directional asymmetry (effect of the side), differences in size and shape (effect of the individual), and non-directional asymmetry (side x part (individual) interaction), we performed factorial analysis of variance (two-way mixed ANOVA), with the side (fixed) and individual (random) as the main factors [17,18,57]. Prior to the analyses of FA indices, we tested our data for normality (Kolmogorov-Smirnov test) [60] and for directional asymmetry (one-sample t-tests by comparing the mean (R-L) to zero) [58]. The level of FA can increase with increasing size of the measured structure [57,58]. To test for association between absolute asymmetry and character size for each habitat studied, we calculated non-parametric (Spearman and Kendall) and parametric (Pearson) correlation coefficients [60]. Based on the results of preliminary analyses, we selected two univariate asymmetry indices, FA8a and FA10a [16], which allowed for the measuring of FA with and without measurement error (FA8a and FA10a respectively) (Table 1). After ANOVA for habitat effect, the between-habitat differences in FA were detected by the Scheffé test [61].

Table 1. The description of radial (univariate and multivariate) and fluctuating (univariate) asymmetry indices [17] for German Iris flower traits - fall length (FL) and fall width (FW). The acronyms R and L stand for measures taken from the right and the left side from the midrib of the flower part (see Fig. 2), respectively.

Indices	Description
RA	
Univariate indices	
SD= $\sqrt{\square}\Sigma(Xi- \overline{X})^2/N$	Standard deviation shows the deviation magnitude of every single flower trait (FL and FW) from the average for the individual
CV= SD/ $\overline{X}$	Coefficient of variation is the ratio of the standard deviation of every single flower trait (FL and FW) and the mean for the individual
Multivariate indices	
MCSD= ∑SD/T	Combines SD from multiple traits (T; the number of traits per individual)
MCCV= ∑CV/T	Combines information of CV from T
FA	
Univariate indices	
FA8a= mean |lnR-lnL|	Expresses FA as a proportion of trait size with no ME correction
FA10a=0.798 $\sqrt{2}\sigma 2i$	Measures the magnitude of the total non-directional asymmetry for a trait after ME has been partitioned out
σ2i = (MSsj - MS)/M	MSsj and MSm from a side x part (individual) ANOVA

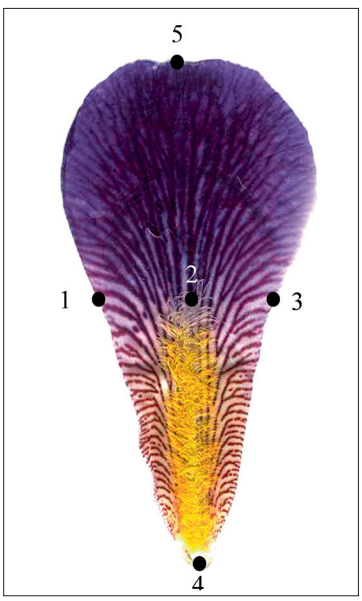

Fig. 2. The measured flower traits of German Iris. Fall length – FL (3-4); fall width – FW (1-3); left – L (1-2), right – R (2-3).

RESULTS AND DISCUSSION

The results of ANOVA for single trait RA indices (SD and CV) and multivariate RA indices (MCSD and MCCV) for both flower traits (FL and FW) (Fig. 2) are presented in Table 2. All indices, except FWCV, showed significant differences between the studied areas, with significantly higher values detected in the polluted habitats. Mean values for univariate and multivariate RA indices with standard errors and pairwise comparisons between habitats examined by Scheffe's test are presented in Fig. 3.

Before FA index estimation, factorial two-way mixed ANOVA with side (fixed) and individual (random) as the main factors, showed that for all traits and both studied habitats the between-sides variation, even after applying a sequential Bonferroni correction, was significantly (P<0.0001) higher than expected due to measurement error. The distributions of all traits appeared approximately normal and directional asymmetry was not detected. The results of non-parametric (Spearman and Kendall) and parametric (Pearson) correlation coefficients after applying a sequential Bonferroni correction for multiple tests, revealed size dependencies for FW in both examined habitats. Therefore, we used the logarithm transformed FA index – FA8a.

The ANOVA results for the single trait FA index (FA8a) and the results of the F test for FA index with measurement error correction (FA10a) are presented in Table 2. Neither of the FA indices showed significant differences between the studied habitats. Therefore, FA as a measure of flower DI did not possess potential as a biomonitoring method.

Organism-wide symmetry (or level of asymmetry as a measure of developmental instability) is

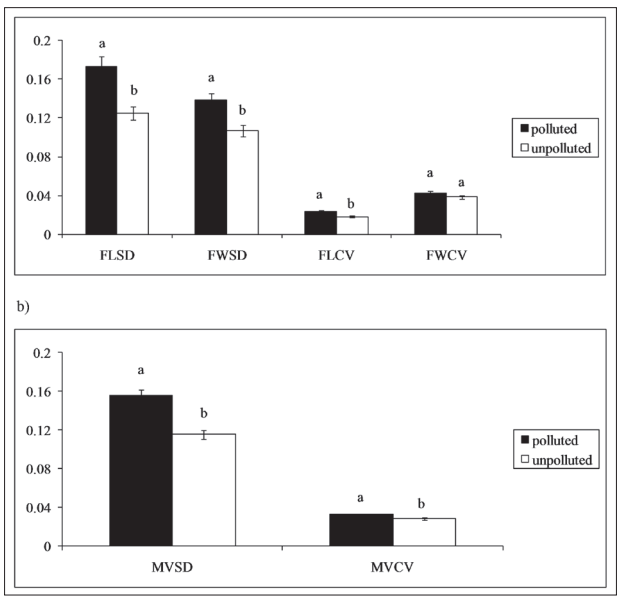

Fig. 3. Mean values and standard errors for radial asymmetry indices. Univariate (**a**) and multivariate (**b**) radial asymmetry indices for two traits (FW and FL) of German Iris flowers sampled from one polluted (black bars) and one unpolluted (white bars) site. The results of Scheffé's test are shown as letters above the histogram bars. Values with the same letter did not differ significantly according to Scheffé's test (P<0.05).

Table 2. The results of analyses of variance (ANOVA) for habitat type effect for univariate (SD and CV) and multivariate (MVSD and MVCD) RA indices for two German Iris flower traits (FL and FW), and for the univariate FA8a index for FW, and the results of the F-test for univariate FA10a index for FW. For acronyms, see Table 1.

Symmetry Type	Type of Analysis	Symmetry Index	Analysed Traits	Habitat df	Error df	MS x10^{-3}	Error MS x10^{-3}	F
RA	Univariate	SD	FL	1	268	19.063	1.245	15.31***
			FW	1	267	14.787	1.042	14.20***
		CV	FL	1	268	1.094	0.128	8.57**
			FW	1	267	0.903	0.516	1.75^{ns}
		SD	FL and FW	1	267	109.140	4.136	26.39****
		CV	FL and FW	1	267	1.202	0.186	6.48***
FA	Univariate	FA8a	FW	1	268	0.273	1.705	0.16^{ns}
		FA10a	FW					1.05^{ns}

ns-non significant,*P<0.05,**P<0.01,***P<0.001,****P<0.0001

known to respond to environmental variation, usually increasing with increased stress [16,65,66]. DI has been examined in the leaves, shoots and flowers of *Periploca laevigata* exposed to grazing disturbance [35]; in response to severe physiological stresses (high boron, high salt, low water, low light, low nutrients) in *Sinapsis arvensis* [37]; in the shoots and flowers of *Anthyllis cytisoides* under a precipitation gradient [64] and in the flowers and cotyledons in *Brassica cretica* under the effect of selfing and outcrossing [38]. In our study, plants in the heavily polluted habitats showed significantly higher floral radial asymmetry than plants from the unpolluted habitats (all indices, except FWCV), indicating lower developmental stability under anthropogenic pollution. Similar results were observed in *Cistus ladanifer* flowers [67], where the radial symmetry of petal length and width was significantly larger in the contact zone between serpentine and siliceous soils in the presence of increased concentrations of heavy metals, in particular Ni.

In species from the same genus, *Iris pumila*, which has an identical flower composition, both fluctuating and radial asymmetry differed significantly between studied sites – polluted (the Belgrade-Novi Sad motorway) and unpolluted (Deliblato Sands, a natural protected area). A significantly greater asymmetry (higher DI) was detected in plants originating from the polluted environment [68].

Fluctuating asymmetry analysis of the width of the fall of the German Iris in our study did not reveal significant differences between habitats with contrasting levels of pollution. In another *Iris pumila* DI analysis, the effect of light in the environment on FA was observed in natural habitats but not in a garden experiment [69,70].

The stability of any trait will depend on the ability of the organism to buffer environmental disturbances, so that characters that are directly related to individual fitness are expected have higher developmental stability, i.e. minimized phenotypic variation [63,71]. If developmental stability maintenance is costly, it can be expected that high symmetry will be preserved only for those traits (organs or organ parts) that contribute most to the fitness; thus, there may be a trade-off between allocations of resources and the symmetry of different traits [26,34].

It is well known that pollinators prefer larger and more symmetrical flowers because they have more pollinator rewards [67]. Floral symmetry usually refers to overall symmetry, the whole flower, the perianth, or just the corolla [72,73]. On the other hand, every single flower is composed of organs or groups of organs that could show partial functional independence and different symmetries in comparison to the flower as a whole [51]. The German Iris appears to be actinomorphic, but *sensu stricto* it may not actually be so. Overall, a radially symmetrical flower is composed of three separate bilaterally symmetrical units, three pollinator landing sites, i.e. pollinator tunnels, formed of the fall on the bottom and the style with the stamens on the upper part. This type of flower composition provides a new context to visual presentation of the flower and it therefore has ecological consequences because pollinators align themselves according to pollinator tunnels that represent actual nectar guides rather than to the whole Iris flower. Also, according to visual information, bilateral symmetry could provide "much greater possibilities for the transmission of visually mediated information than radial symmetry", or more signals and more information to the perceiving eye of the insect (i.e. greater landing precision) [74].

When the relative asymmetry in radial and bilateral flowers was compared, it was found that bilateral species display significantly lower levels of corolla asymmetry [26,51,73]. Bilaterally symmetrical traits are therefore more canalized than radially symmetrical traits and will provide greater developmental stability. The homeostatic mechanisms that regulate FA in the German Iris probably restore developmental stability, unlike the more complex RA-controlling mechanisms [75]. Symmetries of more complex traits, as deviations from radial, spiral, translational and fractal dimensions of plant structures [35], are therefore more sensitive indicators of stress when compared to FA, as was shown in our study, because such traits are controlled by a higher organized processes [19,76].

In conclusion, our study showed that developmental instability in the German Iris, estimated by flower radial asymmetry, demonstrated potential as a biomonitoring tool that is cost-effective and could be used for *in situ* detection of anthropogenic urban pollution. SD and multivariate indices appeared to be the most appropriate for biomonitoring application.

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