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# Effects of Environmental Disturbance on Phenotypic Variation: An Integrated Assessment of Canalization, Developmental Stability, Modularity, and Allometry in Lizard Head Shape

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abstract: When populations experience suboptimal conditions, the mechanisms involved in the regulation of phenotypic variation can be challenged, resulting in increased phenotypic variance. This kind of disturbance can be diagnosed by using morphometric tools to study morphological patterns at different hierarchical levels and evaluate canalization, developmental stability, integration, modularity, and allometry. We assess the effect of urbanization on phenotypic variation in the common wall lizard (*Podarcis muralis*) by using geometric morphometrics to assess disturbance to head shape development. The head shapes of urban lizards were more variable and less symmetric, suggesting that urban living is more likely to disturb development. Head shape variation was congruent within and across individuals, which indicated that canalization and developmental stability are two related phenomena in these organisms. Furthermore, urban lizards exhibited smaller mean head sizes, divergent size-shape allometries, and increased deviation from within-group allometric lines. This suggests that mechanisms regulating head shape allometry may also be disrupted. The integrated evaluation of several measures of developmental instability at different hierarchical levels, which provided in this case congruent results, can be a powerful methodological guide for future studies, as it enhances the detection of environmental disturbances on phenotypic variation and aids biological interpretation of the results.

*Keywords:* urbanization, temperature, contamination, geometric morphometrics, *Podarcis.*

## Introduction

Phenotypic variation is determined by the balance between two sets of proximate causes: while mutations, environ-

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mental conditions experienced during development, and errors in development tend to increase it, selection and regulatory developmental mechanisms tend to constrain it (Debat and David 2001). When populations experience novel, usually unfavorable environmental conditions, an increase in phenotypic variation generally follows (Hoffmann and Parsons 1997; Hoffmann and Hercus 2000). This increase can be a result of newly revealed genetic variation or of impairing the mechanisms that regulate development, particularly for morphological traits (Badyaev et al. 2005).

Canalization and developmental stability (DS) are important developmental organismal properties, and they are ultimately involved in determining the observed degree of phenotypic variation (Willmore et al. 2007). Canalization (Waddington 1942, 1952; Schmalhausen 1949) refers to the capability of organisms to follow a predetermined developmental pathway in spite of environmental and genetic perturbations (Zakharov 1992). As such, canalization suppresses phenotypic variation (Willmore et al. 2007). Similarly, DS refers to the tendency to follow the same predetermined developmental pathway, under the same conditions (Willmore et al. 2007). Although both suppress phenotypic variation, canalization and DS act on different levels. While canalization limits variation between individuals, DS limits the variation within individuals. Consequently, canalization is most frequently measured as phenotypic variation among individuals, while fluctuating asymmetry (FA; Van Valen 1962) is most frequently used as a measure of DS (Palmer 1986; Klingenberg and McIntyre 1998; Debat and David 2001). Because both canalization and DS are involved in limiting phenotypic var-

iation, many authors have addressed the question of whether they are governed by the same or partially overlapping genetic and molecular mechanisms (Klingenberg and Nijhout 1999; Klingenberg 2003*b*). In this context, a high congruence of variance components associated with among-individual variation and FA may suggest common underlying systems (Klingenberg and McIntyre 1998).

Under stressful situations (considered here as environmental conditions in which homeostasis may be disrupted, ultimately causing a decline in individual fitness), the efficacy of the mechanisms underlying DS and canalization may be reduced and cause an increase in phenotypic variation within and among individuals (Scharloo 1991; Parsons 1997; Hoffmann and Hercus 2000). In many species, increased levels of FA were found in populations exposed to different kinds of environmental stressors (Badyaev et al. 2000; Lazić et al. 2013 and references therein), suggesting that DS may be unsettled under such conditions. However, studies reporting opposite results are not rare (Clarke 1995, 1998). Similarly, under stressful conditions canalization can become less stringent, allowing the expression of cryptic genetic variation, undetected under optimal conditions (Rutherford 2000).

Another organismal property related to how phenotypic variation is structured is that of morphological integration (Hallgrímsson et al. 2002). Integration is the interdependence between two or more traits due to shared developmental pathways or function (Klingenberg 2008). Closely linked to morphological integration is the concept of modularity. A module is understood as a group of characters that are internally more integrated in comparison to other modules (Klingenberg 2008). Integration can also contribute to DS, as higher phenotypic stability is expected in traits that are strongly integrated (Badyaev and Foresman 2000). Studies investigating the relationship between environmental perturbations and morphological integration have also provided contradictory results. In some cases, exposure to environmental stress has been associated with increased morphological integration (Klingenberg et al. 2001; Badyaev and Foresman 2004; Gonzalez et al. 2011*a*, 2011*b*). By contrast, other studies have reported a reduction in the degree of integration as a response to stress (Badyaev et al. 2005).

Allometry (shape variation associated with variation in size; Gould 1966) can also generate and limit morphological variation. As individuals become larger, their shape changes under specific allometric rules. For that reason, allometric growth is seen as a highly canalized process and a strong integrating agent (Klingenberg 2010). Environmental factors, such as temperature or nutrition, can directly affect growth, causing alterations to both ontogenetic and static allometric trajectories. Extrinsic causes can also affect allometry indirectly. For example, they may in-

terfere with the developmental mechanisms that coordinate the relationship between shape and size through the regulation of the secretion of growth factors or hormones (Shingleton 2007). Such direct and indirect disturbances to allometric trajectories could be manifested as higher deviations from the shape-size relationship, which could thus serve as an additional measure of developmental instability.

All the aforementioned organismal properties and the morphological patterns in which they are reflected (canalization, DS, integration, modularity, and allometry) are involved in determining the degree of phenotypic variation generated throughout development. Because environmental disturbances can interfere with development, increased variance across individuals and/or populations as well as increased FA are expected under stressful conditions. However, exactly how environmental perturbations contribute to increased phenotypic variation remains poorly understood. As such, the extent to which disturbances to development produce effects that are transversal to different levels of phenotypic variation remains unknown (but see Badyaev and Foresman 2000). Some studies have failed to detect associations between different indicators of disturbance (Hoffmann and Woods 2001), advising caution when using trait variation to assess stress effects. Furthermore, because many other factors can generate phenotypic variation (e.g., underlying genetic variation, phenotypic plasticity), there is a pitfall in directly associating increased variation with underlying causal factors. Instead, integrating the study of phenotypic variation at different hierarchical levels by simultaneously examining the effect of stressful conditions on several of the described developmental indicators would provide strong evidence for a significant contribution of environmental disturbance to increased phenotypic variation (Willmore et al. 2007).

Here, we examine the effect of human disturbance on phenotypic variation by assessing its effects on head shape in rural and urban populations of the common wall lizard (*Podarcis muralis*). The lizard head provides an excellent study system to test whether and how environmental stress may contribute to phenotypic variation through influences on developmental pathways. It is a developmentally complex, functionally integrated system that is involved in multiple highly relevant ecological and social activities, such as feeding (Herrel et al. 1999, 2001*a*), mating (Gvoždík and Van Damme 2003), habitat use (Kaliontzopoulou et al. 2010), territory acquisition, and defense (Husak et al. 2006*b*). As such, head shape is highly variable within and across populations and species (Kaliontzopoulou et al. 2010, 2012) and a potential target of selection. On the basis of the quantification of head shape in adult lizards through geometric morphometric techniques, we perform comparisons between rural and urban populations in

search of signs of impaired canalization and developmental instability in urban populations. For this purpose, we use a wide array of available methods to evaluate the hypothesis that head development is more disturbed in urban populations. Specifically, we integrate information across hierarchical levels of variation by assessing the following indicators: (1) degree of phenotypic variation across individuals; (2) phenotypic variation within individuals, as described by the degree and type of FA; (3) variation across modules, as measured by the level of head shape integration; (4) size-shape allometric variation; and (5) disturbances to allometric growth, as represented through the degree of deviation from size-shape allometries.

## Material and Methods

### Data Collection

We studied a total of 359 adult individuals of the common wall lizard (*Podarcis muralis*), collected by noosing (García-Muñoz and Sillero 2010) between mid-April and July 2012. The sample included lizards from five rural and five urban populations that had been previously studied to investigate the effects of human activities on meristic trait asymmetry (Lazić et al. 2013). All sampling sites were situated at a maximum distance of 30 km from the city of Niš (southern Serbia) to reduce the potential effects of geographic and genetic variation in our data. In urban sites, the lizards inhabited human-altered habitats and were directly exposed to higher temperatures (Unkašević and Tošić 2009) and considerable levels of urban contamination. High contamination with heavy metals, persistent organic pollutants, benzene, and polycyclic aromatic hydrocarbons has been detected in Niš (Škrbić et al. 2002; Nikić et al. 2009; Jovanović et al. 2011). In rural sites, the lizards also inhabited human-constructed habitats (stone walls, abandoned houses, etc.), as is usual for *P. muralis*, but, in contrast to urban sites, they were not exposed to human-induced pollution. All individuals were released in the capture sites after data collection.

To quantify variation in head size and shape, we used landmark-based geometric morphometrics (Bookstein 1997). Such techniques have been extensively used to quantify head shape variation in lacertids in recent years (see Kaliontzopoulou 2011 for a review), taking advantage of the intersections between the large scales covering the dorsal side of the head in these lizards to define accurate, operationally homologous landmark locations. Because these scales tightly correspond to the underlying cranial bones (Costantini et al. 2009) and develop in concert with them (Bellairs and Kamal 1981), their intersections provide a means of directly assessing morphological variation in this functionally important body structure. For this pur-

pose, we first took high-resolution photos of the dorsal side of the head of all individuals by means of a digital camera (Fuji Finepix S1600). Subsequently, one of us (M.M.L.) digitized 28 landmarks (fig. 1) using tpsDig2 (Rohlf 2005). Landmark recording was repeated twice for each animal to assess digitizing error. Specimens in which the landmarks could not be appropriately recorded due to any kind of damage in their scales were not considered in this study. Data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9jq7j (Lazić et al. 2014).

### Global Patterns of Shape Variation

To obtain shape variables from *X* and *Y* landmark coordinates, we first reflected all landmark configurations to obtain their mirror images (Klingenberg et al. 2002). Then we used a generalized Procrustes analysis to superimpose all configurations, thus removing the effects of position, orientation, and size (Rohlf and Slice 1990). For object symmetry, this procedure superimposes the original landmark configurations and their mirror images, thus allowing the separation of symmetric and asymmetric variation components (Mardia et al. 2000; Klingenberg et al. 2002).

To test for the presence of directional asymmetry (DA) and/or FA while accounting for measurement error, Procrustes ANOVA was performed on replicate measurements, following Klingenberg et al. (2002). Procrustes ANOVA is the extension for shape data of the classic two-way ANOVA model used in studies of asymmetry (Klingenberg and McIntyre 1998; Klingenberg et al. 2002), which includes individuals and sides as main effects as well
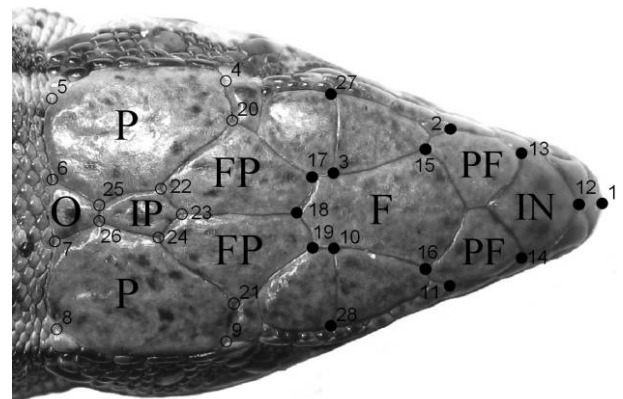


**Figure 1:** Landmarks used to quantify dorsal head shape. Landmarks with filled symbols correspond to module A, and landmarks with open symbols correspond to module B, used in tests of morphological integration. O = occipital scale, P = parietal scales, IP = interparietal scale, FP = frontoparietal scales, F = frontal scale, PF = prefrontal scales, IN = internasal scale.

as their interaction (Palmer and Strobeck 1986; Palmer 1994). In this extended model, the deviations from the mean landmark configuration are portioned into variation due to individuals, reflection (in the case of object symmetry; i.e., Klingenberg et al. 2002), individuals × reflection interaction, and measurement error. The effect of individuals represents the variation among individuals corrected for any asymmetry effects. The effect of reflection represents the variation due to differences between the right and left side of the body, thus providing an evaluation of DA. Finally, the individual × reflection interaction captures FA, that is, the variability of right-left differences among individuals (Klingenberg et al. 2002). This procedure was first performed on each of the populations separately to verify that significant asymmetry was observed in each population while accounting for digitizing error (table A1; tables A1, A2 are available online). In continuation, we repeated the analysis on the complete data set to describe global patterns of individual and asymmetric shape variation and calculate symmetric and asymmetric shape components with coherence across the entire sample. After Procrustes ANOVAs, the shape of each individual was represented by the mean of the two replicate landmark configurations for all subsequent analyses. Landmark reflection, Procrustes superimposition, and Procrustes ANOVAs to evaluate asymmetry patterns as well as extraction of the symmetric and asymmetric shape components based on the sums of squares and cross-product matrices for corresponding ANOVA effects (i.e., individual effect in the case of symmetric shape and individual × side in the case of FA) were performed using the bilat.symmetry function of the geomorph R package (Adams and Otarola-Castillo 2013).

After describing global patterns of symmetric and asymmetric head shape variation, we were interested in testing whether common developmental pathways generate such patterns or, alternatively, whether symmetric and asymmetric variation are controlled by different underlying mechanisms. For this purpose, we compared the variance-covariance (VCV) matrices corresponding to the individual and FA effects in the Procrustes ANOVA. Because the symmetric and asymmetric shape components of structures with object symmetry occupy complementary portions of the total space of multivariate shape variation, a direct comparison of the corresponding VCV matrices is not informative (Klingenberg et al. 2002). For this reason, comparison of VCV matrices was based on half configurations (only one side of the head) and included variation only in paired landmarks (Klingenberg et al. 2002). As a measure of the similarity between symmetric and asymmetric VCV matrices we used the correlation between matrices, and we evaluated its significance using a Mantel test of matrix association with 10,000 permutations (Mantel

1967; Manly 1991). Because of the nature of landmark-derived shape data, this Mantel test was adjusted to permute landmarks, not single variables, and included the diagonal elements of the VCV matrices in the calculation of correlations (Klingenberg and McIntyre 1998).

Because the correlation between VCV matrices for the symmetric and asymmetric shape components was significant (see "Results"), we further explored patterns of covariation based on comparisons of principal components (PCs; Klingenberg and McIntyre 1998; Klingenberg et al. 2001). For this purpose, we first calculated the observed PC vectors for each shape component (from the reduced VCV matrices including only the symmetric landmarks and half configurations) and calculated the angle between them (formula in Klingenberg et al. 2001, p. 13). The statistical significance of this angle was then evaluated by comparing it to the null distribution of angles generated by a bootstrapping procedure (with replacement) under the null hypothesis that PCs do not differ based on 10,000 permutations.

To gain further insight into the mechanisms underlying lizard head shape variation and because both symmetric and asymmetric components of variation were localized at the posterior region of the cranium (see "Results"; fig. 2), we conducted a modularity analysis on total head shape to test whether different areas of the landmark configuration correspond to different developmental modules (Klingenberg 2009). For this purpose, we divided landmark coordinates in two modules, determined by the shape variation patterns observed in previous analyses (see "Results"; fig. 2) and by the timing of ossification during head development. The first module (fig. 1, module A) corresponds to the anterior region of the lizard head, which ossifies relatively early during head development (Bellairs and Kamal 1981). The second module (fig. 1, module B) corresponds to the posterior region of the head, and it includes the frontoparietal, parietal, interparietal, and occipital scales, which are the last to ossify in lacertids (Barahona and Barbadillo 1998). To test the hypothesis that these two landmark subsets correspond to different modules, we used the RV coefficient (Escoufier 1973; Klingenberg 2009), which expresses the amount of covariation across modules scaled by the amount of within-module covariation in landmark coordinates (Klingenberg 2009). To evaluate the hypothesis of modularity and quantify the degree of integration between modules, the RV coefficient was compared with an empirical distribution of RV values generated by iteratively partitioning the landmark configuration into random modules of the same number of landmarks as the hypothesized modules A and B. This procedure was implemented using the function compare.modular.partitions of the R package geomorph (Adams and Otarola-Castillo 2013) based on 10,000
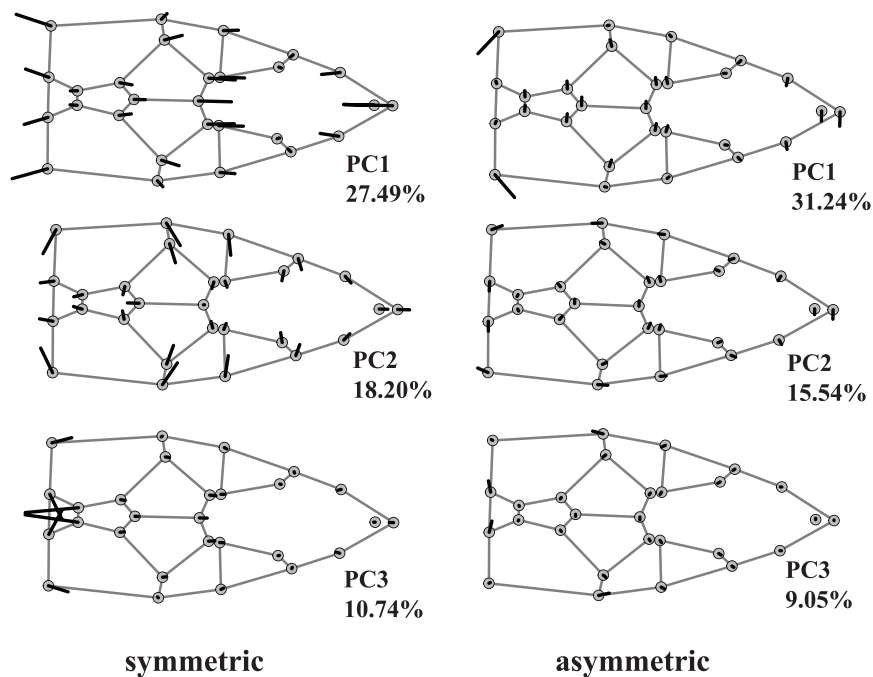
**Figure 2:** Patterns of shape variation as visualized through a principal component (PC) analysis of the sums of squares and cross-product matrices corresponding to Procrustes ANOVA effects for individual (symmetric shape component; *left*) and individual × side (fluctuating asymmetry; *right*). The figure shows the first three PCs of variation for each shape component. The percentages indicate the portion of shape variation explained by each PC. Shape changes are magnified fivefold compared with the actual data to enhance visualization.

permutations. Because allometric effects may have an influence on the observed integration between modules (Klingenberg 2009), we also repeated this analysis after removing allometric effects through a multivariate regression of shape coordinates on centroid size (CS).

### Comparisons between Urban and Rural Populations

After describing global patterns of shape variation, our main objective in this study was to examine how disturbances to development due to living in urban environments may be reflected in lizard head shape. To address this question, we first examined whether urban populations exhibited a higher total head shape variance than rural ones. We calculated shape variance as the trace of the within-population-type VCV matrix (Zelditch et al. 2004) and then compared variances across population types through a resampling procedure with 10,000 permutations. Furthermore, we tested whether symmetric and asymmetric head shape variation components were differently structured in the two population types. For this purpose, we examined the correlation between VCV matrices and the angle between PCs as described above but in this case examining matrix association across population types. These analyses were conducted separately for

the symmetric and asymmetric shape component and considering only the symmetric landmarks of a half configuration, as described above.

Furthermore, we tested whether urban populations exhibited a higher degree of FA. To assess the degree of individual asymmetry, we modified the multivariate generalization of the unsigned asymmetry index proposed by Klingenberg and McIntyre (1998) for paired structures to match the characteristics of shape data on structures with object symmetry, specifically by considering only the paired landmarks of a half configuration in our calculations. Using these landmarks, we first calculated the difference in landmark coordinates between original and reflected configurations for each individual. These correspond to the differences between the right and left side of the lizard head. To obtain an individual index of unsigned asymmetry, we considered the original-to-reflected difference in landmark coordinates as the reference and used the reflected-to-original differences whenever that was negative (see Klingenberg and McIntyre 1998 for mathematical procedures). This matrix of unsigned differences in landmark coordinates was then used to calculate individual unsigned FA indexes as the square root of sums of squared differences from all landmark coordinates. This procedure results in an index that represents an unsigned

Procrustes distance between the right and left side of the head of each individual, projected to tangent space.

To test whether size had an effect on the observed degree of shape asymmetry, we used regression analysis of the unsigned FA index on CS based on 10,000 permutations. As no effects of size on the degree of FA were observed ($R^2 = 0.0055$, $P = .1586$), we tested whether urban populations exhibited a higher degree of unsigned FA through a permutation ANOVA with 10,000 permutations on individual unsigned FA index values with sex, population, and population type as well as all interaction effects as factors. Note that although we were not directly interested in sexual dimorphism in this study, we always included sex as a predictor variable because sexual dimorphism is known to be an important contributor to head shape variation in *Podarcis* wall lizards (Kaliontzopoulou et al. 2008).

Furthermore, we tested the hypothesis that a lower degree of integration between modules could be responsible for the higher degree of head shape asymmetry observed in urban populations (see "Results"). Because morphological integration is highly dependent on developmental pathways, any perturbations during development that may cause increased asymmetry are prone to be manifested as a lower integration of modular structures (Klingenberg 2003*a*). We investigated whether urban populations exhibited lower integration between dorsal head regions by calculating the difference in RV coefficients between urban and rural populations ($RV_U - RV_R$). We then evaluated whether the RV coefficient was significantly higher in urban populations by comparing this index to a random distribution of differences in RV between populations obtained through 10,000 permutations of individuals across population types.

The last series of analyses conducted to evaluate the potential effect of urbanization on head shape development considered modifications in the observed trajectories of shape-size allometry. First, we tested for differences in head size through a permutation ANOVA with 10,000 permutations on CS with sex, population, and population type as well as all interaction effects as factors. This provided us with a summary image of the length of allometric trajectories, allowing us to test whether urban populations exhibit a delay or arrestment in head development. We then performed an ANCOVA on Procrustes residuals of symmetric head shape with CS as the covariate and sex and population type as factors to test for differences in allometric trajectories of shape between population types. To visualize the allometric relationship between head shape and CS across population types and sexes, we calculated the first PC of predicted values from the multivariate regression of shape on CS and plotted it against CS to obtain a view of allometric trends (Adams and Nistri 2010). Fur-thermore, we examined whether urban populations exhibited a higher deviation than rural ones from the shape-size allometric relationship. To the extent that the relationship between shape and size can be considered a result of developmental canalization (Klingenberg 2010), deviations from this relationship can be used as a measure of developmental instability. To quantify these deviations, we used the residuals from the linear model of head shape on CS. Because each sex and population type was shown to follow a different allometric trajectory (see "Results"), we used the within-group allometric line as a reference for these analyses. As such, our index of deviations from allometry is not influenced by differences in size, shape, or the relationship between them across population types. We then tested for differences in deviations from the group shape allometry using a permutation ANOVA with 10,000 permutations on individual deviations with sex, population type, and their interaction as factors.

All statistical analyses were conducted in RStudio (RStudio 2012) using the packages geomorph (Adams and Otarola-Castillo 2013) and vegan (Oksanen et al. 2013).

## Results

### Global Patterns of Variation

Procrustes ANOVA on the complete data set (table 1) as well as separate ANOVAs for each population (table A1) revealed statistically significant individual variation and variation due to DA and FA. Symmetric shape variation, as quantified by the sum of Procrustes distances on the component corresponding to the individual effect matrix of sums of squares and cross products, accounted for 73.38% of total shape variation, while the asymmetric shape component accounted for 26.62%. Shape variation across individuals, as visualized by a PC analysis of the matrix of sums of squares and cross products corresponding to the individual effect in the Procrustes ANOVA, revealed that most of the variability is contained in the distal part of the parietal region of the head for all three PCs (fig. 2). For symmetric shape, the first PC describes the displacement of landmarks along the anteroposterior axis

**Table 1:** Results from Procrustes ANOVA on deviations from the mean landmark configuration of the entire sample

|  | df | SS | *F* ratio | *P* |
|---|---|---|---|---|
| Individual | 9,308 | 1.226 | 7.346 | **<1 × 10⁻⁴** |
| Side | 26 | .008 | 17.238 | **<1 × 10⁻⁴** |
| Individual × side | 9,308 | .167 | 8.180 | **<1 × 10⁻⁴** |
| Error | 18,668 | .041 | | |

Note: Calculation of the error term is based on two replicates of landmark digitizing. Shown is Goodal's *F* ratio under an isotropic model of shape variation. Significant effects are highlighted in boldface type.

**Table 2:** Results from permutational ANOVA on unsigned Procrustes distance between the right and left side of the head, with sex, population type (urban vs. rural), and population as factors

|  | df | SS | F ratio | P |
|---|---|---|---|---|
| Sex | 1 | $1.95 \times 10^{-4}$ | 1.470 | .226 |
| Type | 1 | $1.56 \times 10^{-3}$ | 11.772 | **6 × 10⁻⁴** |
| Population | 8 | $1.93 \times 10^{-3}$ | 1.819 | .073 |
| Residuals | 348 | .046 |  |  |
| Total | 358 | .050 |  |  |

Note: Significant effects are highlighted in boldface type.

and is associated with the elongation of parietal and interparietal scales and shortening of the frontal part (scales F, PF, IN; see fig. 1) of the head. The second PC of symmetric shape variation is associated with the contraction of the head along the right-left axis, while the third PC describes changes in the area occupied by the occipital scale. Variation due to FA is mostly concentrated in the distal region of the head, while variation in other landmarks is spread more evenly across all three PCs.

The Mantel test of matrix association between VCV matrices for the symmetric and asymmetric components across the entire sample indicated common patterns of shape variation ($r = 0.56$, $P = .0031$). The same was true for the first PCs of symmetric and asymmetric shape, where no significant difference in their direction was observed (angle $= 69°$, $P = .5861$).

Modularity analysis indicated that the anterior and posterior region of the head correspond to independent, integrated modules (RV $= 0.52$, $P = .0304$). A slight decrease in the degree of integration was observed after removing allometric effects, but the significant association between modules persisted (RV $= 0.44$, $P = .0223$).

### Comparisons between Rural and Urban Populations

Urban populations exhibited significantly higher total head shape variance compared with rural ones ($V_U/V_R = 1.149$, $P = .0067$). Patterns of symmetric shape variation were similar between urban and rural populations, as indicated by the high and statistically significant correlation between the corresponding VCV matrices ($r = 0.951$, $P = .0001$). The same was true for the asymmetric shape component ($r = 0.916$, $P = .0001$). Moreover, the orientation of the first PCs of symmetric (angle $= 28°$, $P = .347$) and asymmetric ($\theta = 21°$, $P = .084$) shape did not differ significantly between rural and urban populations.

Significant differences in unsigned shape asymmetry were found between the two population types, where individuals from urban populations were more asymmetric in dorsal head shape. There were no differences in the degree of

unsigned asymmetry between sexes or populations within population types (table 2; fig. 3). Because one of the urban populations (A) showed a 10-fold increase in the degree of FA compared with other populations, we repeated the ANOVA without it, which had no effect on the observed patterns (table A2). The increase in head shape asymmetry observed in urban populations cannot be attributed to a decrease in integration between the anterior and posterior part of the head, as the comparison of RV coefficients between population types gave nonsignificant results, both when including ($RV_U - RV_R = -0.010$; $P = .381$) and when excluding ($RV_U - RV_R = -0.059$; $P = .132$) allometric effects.

### Allometric Patterns

ANOVA on CS revealed significant effects of sex, population, population type, and the sex × population interaction on head size (table 3). Males had larger heads than females in all analyzed populations, and individuals from rural areas tended to have larger heads than those from urban areas (fig. 4).

Multivariate regression of head shape on CS revealed a significant effect of sex and population type as well as a significant type × CS interaction, indicating different allometric slopes in the two population types (table 4; fig. 5a–5c). Moreover, urban populations exhibited a higher deviation from their groups' allometric line (table 5; fig. 5d).
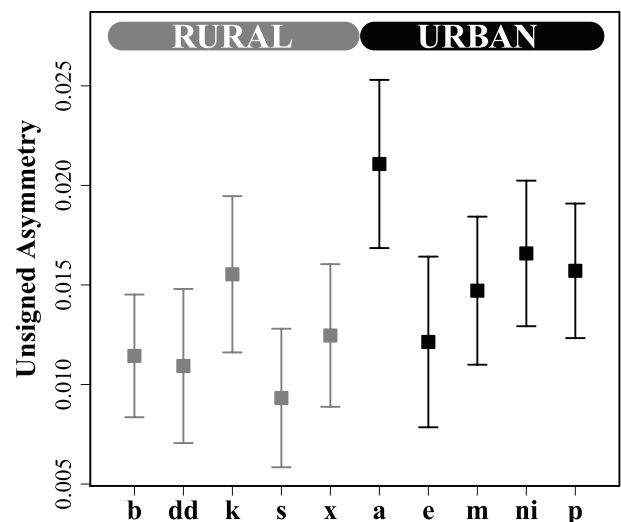


**Figure 3:** Mean degree of unsigned head shape fluctuating asymmetry in urban (black) and rural (gray) populations of *Podarcis muralis* from southern Serbia. Error bars show 95% confidence intervals.

**Table 3:** Results from permutational ANOVA on centroid size, with sex, population type, population and interactions as factors

|  | df | SS | *F* ratio | *P* |
|---|---|---|---|---|
| Sex | 1 | 1.531 | 214.744 | **$1 \times 10^{-4}$** |
| Type | 1 | .437 | 61.350 | **$1 \times 10^{-4}$** |
| Population | 8 | .844 | 14.793 | **$1 \times 10^{-4}$** |
| Sex × type | 1 | $2.07 \times 10^{-5}$ | .003 | .959 |
| Sex × population | 8 | .142 | 2.487 | **$9.1 \times 10^{-3}$** |
| Residuals | 339 | 2.417 | | |
| Total | 358 | 5.370 | | |

Note: Shown is Goodal's *F* ratio under an isotropic model of shape variation. Significant effects are highlighted in boldface type.

## Discussion

Understanding the processes involved in shaping the patterns of phenotypic variation observed in nature is a long-standing objective of evolutionary biology. This objective has become particularly relevant in recent years, in light of the increasing influence of human-mediated disturbance on ecosystems and biodiversity. The results obtained in this study reveal multifaceted effects of urbanization on phenotypic variation in the common wall lizard and provide evidence for the impairment of developmental buffering. The integrated assessment of various indexes that gauge the contribution of different developmental components that determine phenotypic variation reveal that lizard populations exposed to urbanization suffer impaired head development, with potential effects for individual fitness. However, our analyses also identify patterns of lizard head shape variation that are transversal to various hierarchical levels, pointing to mechanisms that apparently delimit phenotypic variation.

### Limits to Variation: Global Regulation, Integration, and Modularity

The analysis of head shape in individuals from rural and urban populations of the common wall lizard revealed extensive phenotypic variation that could be traced to different components, allowing an integrated view of the contribution of developmental buffering. Globally, our analyses sustain the view that the lizard head shape is a highly integrated structure, where variation within individuals, across individuals, and across populations follow similar directions in shape space. Indeed, a comparison of VCV matrices and PCs revealed congruent patterns of head shape variation within and across individuals as well as across different population types (see "Results"). This finding may be seen as support to the hypothesis that canalization and DS are two related phenomena. Similar results have been found across various animal taxa (Clarke 1993; Klingenberg and McIntyre 1998; Klingenberg et al. 2001;

Hallgrímsson et al. 2002; Willmore et al. 2005, 2006; Breuker et al. 2006), suggesting that both could depend on the same molecular and genetic processes, as predicted by theoretical models (Klingenberg and Nijhout 1999). Unfortunately, it is difficult to assess whether this is a common feature of the squamate cranium, as there are no other studies comparing these two sources of variability in this group. From other organisms, however, it is clear that, although canalization and DS buffer against perturbations that have different origins, mechanisms stabilizing one sort of perturbations can easily buffer against others (Klingenberg 2003*b*). There are many mechanisms operating from the molecular to the organismal level that could potentially stabilize development within and across individuals (Klingenberg 2003*b*). For instance, higher stability could be achieved by increasing the number of gene copies or regulatory proteins, or it could be due to the robustness of molecular mechanisms of development (Klingenberg 2003*b*). Although our results suggest that both DS and canalization are somehow disrupted in urban populations, the data presented here do not allow us to decipher whether this is due to common underlying mechanisms or to parallel responses to urban conditions.

Visualization of dorsal head shape patterns reveals the posterior region of the head as an area of high variation. Indeed, both intra- and interindividual shape differences include deformations of the head areas corresponding to the parietal, occipital, interparietal, and frontoparietal scales (figs. 1, 2). This observation is in line with previous studies examining dorsal head shape variation in lacertids,
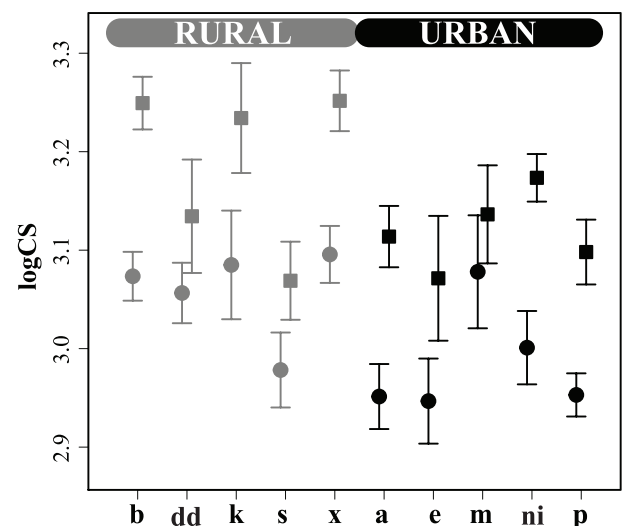


**Figure 4:** Mean logarithmic centroid size (CS) values in urban (black) and rural (gray) populations of *Podarcis muralis* from southern Serbia. Squares indicate males, and circles indicate females. Error bars show 95% confidence intervals.

**Table 4:** Results from permutational multivariate regression of symmetric head shape on centroid size (CS), with sex, population type, and all interactions as factors

|  | df | SS | P |
|---|---|---|---|
| Sex | 1 | .050 | $\mathbf{1 \times 10^{-4}}$ |
| Type | 1 | .035 | $\mathbf{1 \times 10^{-4}}$ |
| Sex × type | 1 | .001 | .588 |
| CS | 1 | .031 | $\mathbf{1 \times 10^{-4}}$ |
| Sex × CS | 1 | .002 | .405 |
| Type × CS | 1 | .007 | $\mathbf{2 \times 10^{-4}}$ |
| Sex × type × CS | 1 | .001 | .737 |
| Total | 358 | .613 | |

Note: Significant effects are highlighted in boldface type.

which indicate that shape variation associated with allometry (Bruner and Constantini 2007; Kaliontzopoulou et al. 2008; Piras et al. 2011; Urošević et al. 2013), sexual dimorphism (Bruner et al. 2005; Kaliontzopoulou et al. 2007; Ljubisavljević et al. 2010, 2011), intraspecific (Kaliontzopoulou et al. 2010; Raia et al. 2010), and interspecific (Bruner and Constantini 2007; Urošević et al. 2012, 2013) differentiation is also frequently localized in the distal area of the lizard head. Indeed, our analysis of modularity indicates that the posterior head region (open symbols in fig. 1) constitutes an independent module that, although tightly integrated to the anterior region, presents increased internal cohesiveness and particular variation patterns.

From a developmental perspective, this head region is the last to ossify in lacertids, and it continues growing until late developmental stages (Bellairs and Kamal 1981; Barahona and Barbadillo 1998). As such, the timing of developmental events may be involved in determining the concentration of high shape variation in the posterior dorsal region of the head in this lizard family. Hypothetically, the longer duration of tissue formation, activity, and restructuring in this region, compared with other head parts, may facilitate an increased plasticity and augment the differentiation between biological groups (e.g., sexes, populations, and species) and environmental disturbance factors. Interestingly, increased variation in the posterior dorsal region of the cranium is also a common pattern in other lizard families (e.g., Teiidae [Monteiro and Abe 1997], Phyllodactilidae [Zuffi et al. 2011], and *Anolis* [Sanger et al. 2013]). Furthermore, the same proximal-distal dorsal head shape modularity pattern observed here has been reported to constrain head shape variation in *Anolis* lizards (Sanger et al. 2012). Given the recurrence of this phenotypic pattern across such phylogenetically divergent squamate taxa (Pyron et al. 2013) as well as in other reptiles (Crocodylia [Monteiro et al. 1997; Piras et al. 2010]), it appears plausible that common, highly preserved developmental mechanisms are involved in determining dorsal skull phenotypic variation in this group.

*Individual Variation and Developmental Instability*

Despite the global concordance of head shape patterns across different hierarchical levels, we also find multiple signs of increased phenotypic variation due to human disturbance, which point to a disruption of developmental buffering. Indeed, examination of shape variation levels across and within individuals reveals that, compared with rural populations, urban ones exhibit significantly higher total head shape variance and an inflated degree of FA (table 2; fig. 3). Increased phenotypic variance has been documented under various stress factors (Valentine and Soulé 1973; Imasheva et al. 1998, 1999a, 1999b; Gonzalez et al. 2011a, 2011b). Populations experiencing novel or stressful environments often show higher phenotypic variation than those living under more "natural" conditions. When environmental conditions are suboptimal buffering mechanisms can be challenged, resulting in higher phenotypic variance (Willmore et al. 2007).

Traditionally, this increase in phenotypic variance has been linked to expression of cryptic genetic variation, as canalization can allow the storage of genetic variation under optimal conditions. In addition, suboptimal environments could increase the frequency of mutations and recombination rates potentially increasing phenotypic variation (Hoffmann and Parsons 1997). According to this view, enhanced expression of cryptic genetic variation or mutations can either affect genes that regulate the development of phenotypic traits or trigger the expression of specific, stress-induced genes (Debat and David 2002). VCV matrices associated with among-individual variation and congruence in the orientation of corresponding first PCs between the two population types were significantly correlated. This suggests that phenotypic variation among individuals is regulated by the same mechanisms in rural and urban populations. This result also suggests that disturbance to genes regulating head development could be responsible for increased head shape variation rather than specific, stress-induced genes. Bergman and Siegal (2003) suggested that most genes should display high phenotypic variation when compromised and that canalization is less likely to be a result of a single gene. Apart from cryptic genetic variation and mutations, increased shape variance in urban populations can be due to developmental mechanisms themselves. Activity of hormones and growth factors can be affected by various environmental perturbations (Husak et al. 2006a). Nutritional stress, for example, has been shown to affect growth of the rat skull, where individuals experiencing malnutrition in early developmental stages showed higher shape variance in the skull

base, vault, and face modules compared with the unstressed group (Gonzalez et al. 2011*b*). Similarly, intrauterine growth retardation has been shown to cause an increase in variance of rat skull shape (Gonzalez et al. 2011*a*). Whether the observed increase in total head shape variance observed in urban populations of the common wall lizard is due to the expression of cryptic genetic variation, to an increase in the frequency of new mutations, or to endocrine factors is hard to assess here. To address this question, one would need to integrate the study of phenotypic variation with molecular methods that would allow assessment of the levels of genetic variation across populations and establishing whether urbanization has a direct effect on it. Alternatively, experiments evaluating the effects of incubation conditions, growth hormones, and growth factors on head shape variance and testing whether biochemical pathways are disrupted in urban environments could establish the precise mechanisms that amplify variance levels.

Furthermore, lizards from urban areas showed significantly higher head shape FA. Increased head shape FA due to urbanization effects is strongly concordant with our previous findings obtained using multiple meristic traits (Lazić et al. 2013). VCV matrices associated with within-individual variation were significantly correlated, and the orientation of corresponding first PCs did not differ significantly between the two population types. These results suggest that the same mechanisms regulate DS in the two groups. Despite their similarity, however, the efficacy of the mechanisms underlying DS and canalization is apparently compromised in urban populations. Several environmental factors may be contributing to this. Pollution and temperature tend to be higher and humidity tends to be lower in cities than in rural areas. In organisms with soft-shelled eggs, such as *Podarcis*, toxic chemicals and heavy metals can be easily absorbed from the environment or transferred to the eggs by females (Marco et al. 2004*a*, 2004*b*). These factors are known to influence the precision of development in various taxa. In addition, it has been shown that increased incubation temperatures beyond the optimal level lead to increased FA levels in lizards (Ji et al. 2002). Furthermore, inbreeding and habitat fragmentation have also been associated with high FA in lizards (Soule et al. 1973; Sarre 1996), whereas population isolation has been suggested to increase head shape FA in other *Podarcis* species (Băncilă et al. 2010). However, in our study system lizards seem to be present through the whole city and populations are well connected, which should ensure gene flow. Moreover, a study of insular populations of *Podarcis muralis* could not establish an association between heterozygosity levels and FA (Crnobrnja-Isailović et al. 2005). In other lizard species, increased DS has been found to be positively correlated with secondary structure stability of mitochondrial origins of light-strand replication (Seligmann and Krishnan 2006). Again, only a study incorporating the use of molecular tools to assess levels of genetic variability within populations and quantify connectivity among populations could definitely assess the role played by genetic factors in shaping the observed phenotypic patterns.
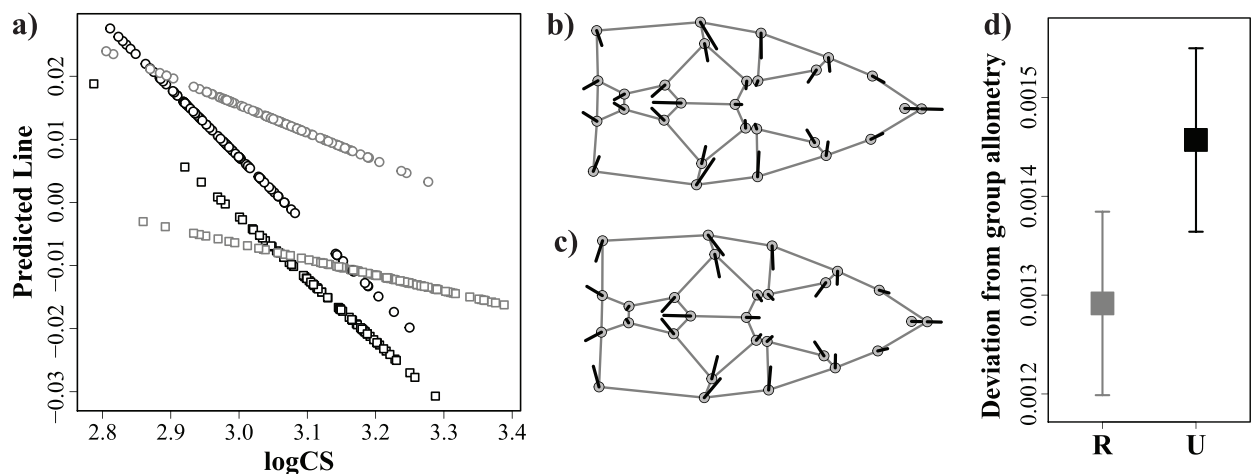


**Figure 5:** *a*, Allometric relationship between symmetric head shape and centroid size (CS) across population types (black = urban, gray = rural) and sexes (circles = females, squares = males), visualized as the first principal component of the predicted values from a multivariate regression of head shape on CS (Adams and Nistri 2010). *b*, Allometric shape change observed in rural populations. *c*, Allometric shape change observed in urban populations. *d*, Mean deviations from within-group allometric trajectory (black = urban, gray = rural). Note that shape patterns in *b* and *c* are corrected for differences in shape between the sexes and are magnified twofold compared with the actual data to enhance visualization. Error bars in *d* show 95% confidence intervals.

### Environmental Effects on Allometric Trajectories

The hypothesis that human-mediated environmental effects related to urbanization may be responsible for disturbances to developmental buffering mechanisms is further supported by our results regarding size-shape allometric trajectories. Indeed, mean adult head size was lower in urban than in rural populations (table 3; fig. 4). This limited head growth in urban sites is not a side effect of body size, as it remained the same after controlling for that effect (results not presented). In animals, body size and the relative size of different body structures are mainly determined by mechanisms that control growth rates and the duration of growth (Shingleton 2011). From this perspective, smaller adult head size can be a result of slower growth in cities as a result of malnutrition due to lower resource availability. Alternatively, decrease in adult head size could be due to smaller head size at birth or to earlier cessation of growing. Furthermore, the observed differences can also be due to trade-offs between head development and some life-history traits, so that urban lizards invest less in head size. Irrespective of the precise underlying mechanisms, however, reduced head size may have important consequences for individual fitness. In lizards, the head is involved in many important ecological (i.e., feeding, habitat use) and social (i.e., copulation, male antagonistic behavior) tasks, where head size is known to be the main determinant of biting performance (Herrel et al. 2001*a*). As such, reduced head size in urban populations may translate into reduced biting capacity, which could in turn disrupt mate acquisition and sexual selection mechanisms and/or limit the range of potential preys, increasing intraspecific competition for resources (Herrel et al. 2001*b*; Verwaijen et al. 2002; Huyghe et al. 2005; Husak et al. 2006*b*).

Potential signs of disturbance to development are also detected through the comparison of size-shape allometric slopes between population types, which revealed a divergence of allometric trajectories (table 4; fig. 5*a*–5*c*). As we explored static allometries, we cannot at present determine whether the observed differences between types emerge early during head ontogeny or in the adult stage. Nevertheless, this result suggests that different mechanisms could be regulating size-shape relationships under different environmental conditions. Alternatively, the mechanisms controlling head shape allometry may be disturbed in urban populations. This second explanation appears more feasible, given the high cohesiveness of shape patterns across hierarchical levels reported here. Further support for this hypothesis comes from the higher deviation from within-group allometric trajectories, observed in lizards from urban populations (table 5; fig. 5*d*). These deviations represent size-independent variation in shape, meaning

**Table 5:** Results from permutational ANOVA on deviations from within-group allometric trajectories of symmetric head shape on centroid size, with sex, population type, and their interaction as factors

|              | df  | SS                   | *F* ratio | *P*  |
|--------------|-----|----------------------|-----------|------|
| Sex          | 1   | $3.81 \times 10^{-7}$ | .615      | .44  |
| Type         | 1   | $2.48 \times 10^{-6}$ | 4.006     | **.044** |
| Sex × type   | 1   | $1.59 \times 10^{-6}$ | 2.579     | .108 |
| Residuals    | 355 | $2.19 \times 10^{-4}$ |           |      |
| Total        | 358 | $2.24 \times 10^{-4}$ |           |      |

Note: Significant effects are highlighted in boldface type.

that size and its consequences explain less shape variation in urban lizards. Since size and shape generally change jointly, allometry has been traditionally considered a strong integrating factor and a powerful evolutionary constraint (Klingenberg 2010). However, in recent years it has been shown that closely related species can show different allometric trajectories (Adams and Nistri 2010; Urošević et al. 2013), providing evidence that allometry itself can evolve. Furthermore, experiments on *Drosophila* show that allometry can be strongly dependent on environmental factors, such as nutrition and temperature (Shingleton et al. 2009). Similarly, growth hormone deficiency is known to affect skull shape allometry in mice (Gonzalez et al. 2013). These findings suggest that differences in allometry can be due to many environmental factors affecting complex developmental pathways directly or indirectly.

Alternatively, the smaller head size observed in urban populations could be a result of strong directional selection, which could consequently reduce developmental robustness (Clarke 1997). For instance, differences in habitat use or predation pressure may modify selective pressures between population types, yielding the head size patterns observed here (e.g., Kaliontzopoulou et al. 2010). If such a selective modification is relatively recent, the mechanisms involved in developmental buffering and DS may have not had time to adjust to such an evolutionary change. Such a hypothesis would explain highest FA in urban populations as well as the alterations observed in allometric trajectories (Leamy and Atchley 1984). Unfortunately, most studies investigating the effect of specific environmental factors on allometry alteration come from experiments on well-established model organisms, such as insects or small mammals, with no studies on lizards. Investigating the role played by specific factors, such as contaminants, nutrition, humidity, and temperature, on head shape allometry in lizards would require a more experimental approach.

Taken together, the results obtained in this study using different indexes to evaluate phenotypic variation are highly consistent across multiple hierarchical levels and

provide evidence of a disruption of developmental buffering in urban populations of the common wall lizard. Studies investigating the relationship between disturbance and phenotypic variation are usually restricted to one of these indexes, which limits their potential to detect disturbance or to sustain that the observed phenotypic patterns are indeed a result of interference with developmental mechanisms. Our results support the view that integration of multiple indexes may increase the chance of detecting disturbance and, most importantly, broaden our understanding of how the developmental mechanisms involved in the regulation of phenotypic variation react to disturbance. The evidence obtained here indicates that urbanization factors have deep effects on lizard head shape development, a trait that is highly correlated with various fitness components, suggesting that urban populations could be suffering fitness reduction. Future studies incorporating information on genetic variability and its effect on phenotypic patterns as well as experimental manipulation to understand exactly how environmental factors related to urbanization interfere with developmental pathways would shed more light on the precise mechanisms involved in the patterns observed here.

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*Podarcis muralis* individuals from the city of Niš, Serbia. Photograph by Jelka Crnobrnja-Isailović.