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Urban Life Affects Differentiation and Phenotypic Variation but not Asymmetry in a Fully Terrestrial Salamander

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Abstract

The environmental transformations associated with cities are expected to affect organisms at the demographic, phenotypic, and evolutionary level, often negatively. The prompt detection of stressed populations before their viability is compromised is essential to understand species' responses to novel conditions and to integrate urbanization with biodiversity preservation. The presumably stressful conditions of urban environments are expected to affect organisms' developmental pathways, resulting in a reduction of the efficacy of developmental stability and canalization processes, which can be observed as increased Fluctuating Asymmetry (FA) and Phenotypic Variance (PV), respectively. Here, we investigated whether patterns of phenotypic variation of urban populations of a fully terrestrial salamander, *Salamandra salamandra bernardezi*, are affected by urban settings compared to surrounding native forest populations. We sampled populations within and around the city of Oviedo (northern Spain) and used geometric morphometrics to compare morphological differentiation, head shape deviance from the allometric slope, PV, and FA. We also compared morphological patterns with neutral genetic and structure patterns. We observed increased levels of differentiation among urban populations and in PV within certain of them, yet no differences in allometric deviance and FA were detected between habitats, and no morphological measures were found to be correlated with genetic traits. Our results do not support a clear negative impact of urban conditions over salamander populations, but rather suggest that other ecological and evolutionary local processes influence morphological variation in this urban system.

Keywords Amphibians · Canalization · Fluctuating Asymmetry · Geometric Morphometrics · Stressors

Introduction

Cities are highly transformed environments characterized by a set of conditions with important, but not yet fully explored, consequences for biodiversity (Grimm et al., 2008; Seto et al., 2012). Urbanization leads to increased levels of habitat fragmentation, pollution (i.e., air, water, soil, acoustic, and light pollution), and changes in biotic (species community richness and structure) and abiotic (chemical and physical parameters) conditions, which results in novel environments (Cadenasso et al., 2007; Grimm et al., 2008; Oke, 1973). While some species are unable to persist in cities, others have managed to develop stable and well adapted urban populations (e.g., the brown rats and feral pigeons) (McKinney, 2008). Populations inhabiting urban areas usually exhibit phenotypic, physiological, or ecological modifications that result from novel selective pressures associated to these environments, providing interesting study systems for exploring contemporary evolution in

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anthropic environments (Diamond & Martin, 2021; Johnson & Munshi-South, 2017; Santangelo et al., 2022). Indeed, the modification of eco-evolutionary dynamics in cities results in particular patterns of variability within urban areas, such as an increase of intraspecific phenotypic variation (Thompson et al., 2022). Understanding how organisms vary in response to urbanization, as well as how they are affected by the novel selective pressures, is crucial to predict the responses of biodiversity to human-dominated areas and foster conservation strategies that may conciliate urban development with biodiversity preservation (Collins et al., 2021; McDonnell & Hahs, 2015).

Many of the biotic and abiotic factors related to urbanization can alter organismal life histories, and with them the amount of energy invested in reproduction and development in urban-dwelling populations (Diamond & Martin, 2021; Kolonin et al., 2022; Sepp et al., 2018; Snell-Rood et al., 2015). When those factors negatively affect individual homeostasis and fitness, ultimately compromising population viability, they are considered as stressors (Parsons, 2005). Among the many outputs of stressful conditions, development is one of the first levels to be disturbed. It is a highly regulated process that includes stability mechanisms to buffer deviations from the predetermined developmental pathway caused by genetic or environmental disturbances (i.e., canalization and developmental stability) (Debat & David, 2001; Waddington, 1942; Willmore et al., 2007). But when conditions (e.g., environmental) exceed certain thresholds, the efficacy of such regulatory mechanisms is reduced, producing suboptimal phenotypes, which may ultimately cause a reduction in fitness (Clarke, 1995; Møller, 1997). Deviance from the predetermined phenotype can have direct effects on fitness, affecting for example individual's survival (Martín & López, 2001; Tocts et al., 2016) or mating success (Møller & Thornhill, 1998). At the same time, levels of variability around an optimal phenotype can be considered an indicator of the efficacy of regulatory mechanisms underlying development, as phenotypic variation resulting from disturbed development usually arises before any other trait that significantly compromises population survival is affected (e.g., reproductive output). Thus, it can be used as a biomarker to precociously detect stressed populations ("early warning system" Clarke, 1995).

The impact of stressors over developmental accuracy may arise at two different organismal scales: at the population level, as an outcome of the disturbance of developmental canalization processes, which results in an increased phenotypic variance (PV) across individuals; and at the individual level, resulting from the disturbance of developmental stability within individuals, usually measured as an increase in fluctuating asymmetry (FA) or the random deviation from bilateral symmetry (Beasley et al., 2013; Debat & David, 2001; Palmer & Strobeck, 1986; Willmore et al., 2007). The assessment of developmental disturbance using the aforementioned phenotypic variation measures (i.e., PV and FA) is a widely accessible, inexpensive, and non-invasive methodology, which allows increasing sample sizes and the spectrum of study systems. Considering the number of potential stressors that converge in urban environments, the assessment of developmental precision using phenotypic traits can help to evaluate the health of urban populations and constitute a preliminary approach to investigate the consequences of urban environments on a wide range of urban-dwelling organisms (e.g., plants: Shadrina et al., 2020; invertebrates: Weller & Ganzhorn, 2004; birds: Vangestel & Lens, 2011; amphibians: Zhelev et al., 2019; reptiles: Lazić et al., 2015; fish: Allenbach, 2011; and mammals: Puckett et al., 2020).

The potential to persist in urban environments and the magnitude and direction of the effects of urbanization are highly dependent upon species' specific attributes and life-history traits (Becker et al., 2007; Evans et al., 2011; Pyron, 2018). For instance, generalist species with high mobility (e.g., birds and flying insects) and short generation times are more prone to successfully persist, and even adapt (Salmón et al., 2021), in highly modified habitats than specialists with reduced dispersal abilities, long generation times, and with traits that increase their sensitivity to certain stressors, such as ectothermic physiology or ground dwelling life (McDonnell & Hahs, 2015; Møller, 2009; Vergnes et al., 2014). Despite the fact that the development of ectotherms is more sensitive to environmental factors than that of endotherms (Eyck et al., 2019; Noble et al., 2018) studies on urban ecology among vertebrates are biased towards birds and mammals, while reptiles and amphibians remain largely understudied (Collins et al., 2021). Specifically, amphibian species are expected to be highly affected by urbanization because of their relatively reduced mobility, high sensitivity to environmental cues (i.e., pollutants, through their permeable skin), and the common biphasic life-cycle that usually includes egg-lying and an aquatic larval phase (Hamer & McDonnell, 2008; Pyron, 2018). Nonetheless, amphibians present a wide variety of reproductive modes, ranging from fully aquatic life-cycles and external development, either direct or biphasic, to fully terrestrial species in which development is completed within the parents' body (and all combinations in between) (Nunes-de-Almeida et al., 2021). Thus, species with external development, both aquatic or terrestrial, have been reported to be especially sensitive to hydroperiod, habitat availability and connectivity, environmental-pollutants, and the presence of predators (Becker et al., 2007; Hamer & McDonnell, 2008; Suazo-Ortuño et al., 2008; Trimble & Van Aarde, 2014). Conversely, those reproductive strategies in which development occurs internally, including metamorphosis when it exists, are expected to be affected differently by novel environmental factors and may present different conservation requirements.

Here, we evaluated developmental disturbance in morphological variation (i.e., head shape) from urban populations of a fully terrestrial salamander with internal development (pueriparity *sensu* Greven, 2003) comparing phenotypic variation measures between urban and forest populations of the pueriparous *Salamandra salamandra bernardezi* (Alarcón-Ríos et al., 2020; Buckley et al., 2007; Mulder et al., 2022) in the city of Oviedo (northern Spain) and surrounding areas (Fig. 1a). This system is particularly well-suited for urban research in fully terrestrial amphibians due to the following reasons: (i) patterns of neutral genetic diversity and structure of urban and surrounding populations from Oviedo have already been studied and linked to historical and demographic processes (Álvarez et al., 2015; Lourenço et al., 2017). This allows for morphological comparisons between and within habitats under a well-known evolutionary history. (ii) A recent study found a higher proportion of morphological deformities within urban populations compared to forest ones (Velo-Antón et al., 2021) indicating the presence of factors that influence the morphology of some urban populations. (iii) The study of morphological variation within this species has been previously optimized using geometric morphometrics to study dorsal head shape (Alarcón-Ríos et al., 2017), which provides high-resolution tools for studying the developmental effects of urban environments in this organism. The head is a complex and functionally relevant structure (Hanken & Hall, 1993) and, thus, a likely target of selection. It is also highly variable within *S. salamandra* (Alarcón-Ríos et al., 2020a; Bas & Gasser, 1994), even at local scales (Alarcón-Ríos et al., 2017). Indeed, some of the aforementioned deformities in Oviedo populations appeared in the head (Velo-Antón et al., 2021), supporting head shape as a suitable structure to assess morphological variation in response to urban stressors. Finally, (iv) while most studies investigating amphibians´ phenotypic responses to anthropic alterations have focused on species with aquatic stages or terrestrial eggs (e.g., Rubbo & Kiesecker, 2005; Parris, 2006; Wilk et al., 2020; but see Iglesias-Carrasco et al., 2017), to our knowledge, the phenotypic changes of amphibians with a fully internal embryonic development have never been examined, making this a particularly interesting system for future comparisons with other reproductive strategies.

We aim to answer four fundamental questions: (1) are urban populations of *S. s. bernardezi* morphologically differentiated from neighbouring forest populations? Due to the high functionality of the trait under study (head morphology), the ecological disparities between habitats, and the historical isolation of urban populations from surrounding ones (see Lourenço et al., 2017) we expect some degree of differentiation between urban and forest salamanders. (2) Do urban populations exhibit higher levels of developmental disturbance than forest populations? Based on the high sensitivity of amphibians to environmental disturbance (Hamer & McDonnell, 2008; Pyron, 2018), and the observed higher incidence of deformities within urban populations (Velo-Antón et al., 2021), we hypothesize a lower performance of the mechanisms involved in developmental stability and morphological canalization in urban populations, resulting in higher levels of fluctuating asymmetry (FA) and phenotypic variance (PV). (3) Are levels of developmental instability (i.e., FA) and phenotypic variance (i.e., PV) associated with genetic variation (heterozygosity and relatedness) and effective population size (*Ne*) in these populations? Although Oviedo populations present levels of genetic variation comparable to forest ones, they generally show higher levels of relatedness and smaller *Ne*, which together with their higher isolation (Lourenço et al., 2017) might be reflected in increased FA and PV levels (e.g., Garrido & Pérez-Mellado, 2014; Eterovick et al., 2016). Finally, (4) are patterns of phenotypic and genetic differentiation among urban populations generally concordant? Considering that genetic differentiation among Oviedo city populations result from drift processes and bottleneck events (Lourenço et al., 2017), a concordance between both patterns would point to similar processes of random accumulation of variance acting on morphology, while a deviance would point to local sources of variation within the urban environment.

Materials and methods

Sampling and Study site

We sampled adult individuals during rainy nights between October-November 2020 in 10 urban populations from Oviedo, and nine neighbouring native forest populations (Table 1). Due to species-specific activity patterns, whereby males are more active during the sampling period, and the difficulty of accessing certain urban populations, which are located within historic buildings, enclosed convents, and private estates, together with their small size, obtaining either no females or a sufficiently large number of them to yield consistent results was not feasible in many of them. Thus, to avoid unbalanced sampling between sexes and populations that may add noise or provide unreliable results, only adult males were studied. Urban sampling points were considered as different populations as they are genetically isolated from each other (Lourenço et al., 2017). All populations from native forest (hereafter 'forest') were located

Table 1 Summary table of all studied populations from urban and forest habitats, sample sizes, shape variation (mean FA index and PV values considering size effects by population) and genetic traits (*Ho*, R and *Ne*). Genetic and demographic data was obtained from Lourenço et al., 2017

Population	Code	Habitat	N	mean FA index	PV	Ho	R	Ne
Facultad Biología	FB	Urban	26	1.870	1.10×10^{-3}	0.678	0.094	46
Plaza de Toros	PT	Urban	20	1.862	9.25×10^{-4}	0.68	0.099	126
Club de Tenis	TEN	Urban	18	1.901	1.08×10^{-3}	0.737	0.125	30
San Pedro de los Arcos	ARC	Urban	19	1.864	1.24×10^{-3}	0.658	0.136	70
Jardines Seminario	SEM	Urban	18	1.876	8.50×10^{-4}	0.641	0.129	348
Calle Muérdago-Otero	OTE	Urban	21	1.871	9.21×10^{-4}	0.721	0.137	21
Campus del Milán	MIL	Urban	17	1.866	9.65×10^{-4}	0.658	0.265	15
Patio Catedral	CAT	Urban	33	1.862	1.07×10^{-3}	0.663	0.116	22
Patio Peregrinos	PER	Urban	8	1.868	4.79×10^{-4}	-	-	-
Monasterio	MON	Urban	8	1.863	1.05×10^{-3}	0.619	0.288	18
Total Urban			188					
San Miguel de Lillo	LIL	Forest	18	1.867	9.41×10^{-4}	-	-	-
Villamar	VMA	Forest	14	1.843	7.56×10^{-4}	-	-	-
Bendones	BEN	Forest	19	1.850	1.4×10^{-3}	-	-	-
Pozoval	POZ	Forest	32	1.862	8.30×10^{-4}	-	-	-
Latores	LAT	Forest	17	1.879	7.66×10^{-4}	-	-	-
Brañes	BRA	Forest	26	1.851	8.52×10^{-4}	-	-	-
Soto del Rey	REY	Forest	22	1.866	9.12×10^{-4}	-	-	-
Laviada	LAV	Forest	15	1.860	6.87×10^{-4}	-	-	-
La Folguera	FOL	Forest	26	1.863	8.49×10^{-4}	-	-	-
Total Forest			189					

within forested patches of variable sizes (Fig. 1a) primarily composed of oaks (*Quercus robur*), chestnuts (*Castanea sativa*), and birches (*Betula* spp.), with some riparian species, and a well-developed understory. Forest sampling points were located more than two kilometres apart from each other, the maximum distance of dispersion in this species (Hendrix et al., 2010). All forest populations were within a radius of nine kilometres from the city to keep geographic environmental variation between habitat groups to a minimum (Fig. 1). We examined 377 individuals (188 from urban and 189 from forest sites) (Table 1).

After collection, animals were anesthetized (benzocaine; Ethyl 4-aminobenzoate; Sigma-Aldrich, Darmstadt, Germany. Product number: E1501. Ref.: 12,909) to facilitate animal handling and data collection. We obtained high-resolution pictures of the dorsal view of the head following the methodology for image acquisition described in Alarcón-Ríos et al. (2017), and released all animals, after recovery from anaesthesia, at the place of capture within the following 24 h. Salamanders were captured and processed under the collection permits provided by the regional government of Asturias, Spain (Nº Expte: AUTO/2020/671). All applicable national and institutional guidelines for the care and use of animals were followed.

Shape data Acquisition: Geometric Morphometrics

For the study and comparison of head morphological variation and asymmetry between urban and forest populations we applied landmark-based geometric morphometrics (GM), adapting the methodology described in Alarcón-Ríos et al. (2017), which has been shown to accurately identify shape differences at a very fine biological scale (population) while maintaining a low, non-systematic measurement error. We digitized 13 fixed landmarks and 32 sliding semilandmarks using tpsDig2 v 2.21 (Rohlf, 2015), which capture main morphological features of the dorsal view of salamanders' head: snout, upper jaw, eyes and parotid glands (Fig. 1d). One of us (L.A-R) digitized all individuals twice to take measurement error into account. In the total sample size ($N=377$), we did not consider any individual in which image quality or the existence of any morphological condition, such as missing eyes (Velo-Antón et al., 2021), prevented the accurate digitization of all landmarks.

To explore patterns of head morphology between (urban vs. forest sites) and within habitats, and examine developmental stability we first performed a Procrustes ANOVA using the *bilat.symmetry* function from the R-package *geomorph* (Adams et al., 2021; Baken et al., 2021). This function implements a Procrustes ANOVA with *individual*, *side*, and their interaction as main factors to test for the presence of Directional Asymmetry (DA, corresponding to the *side*

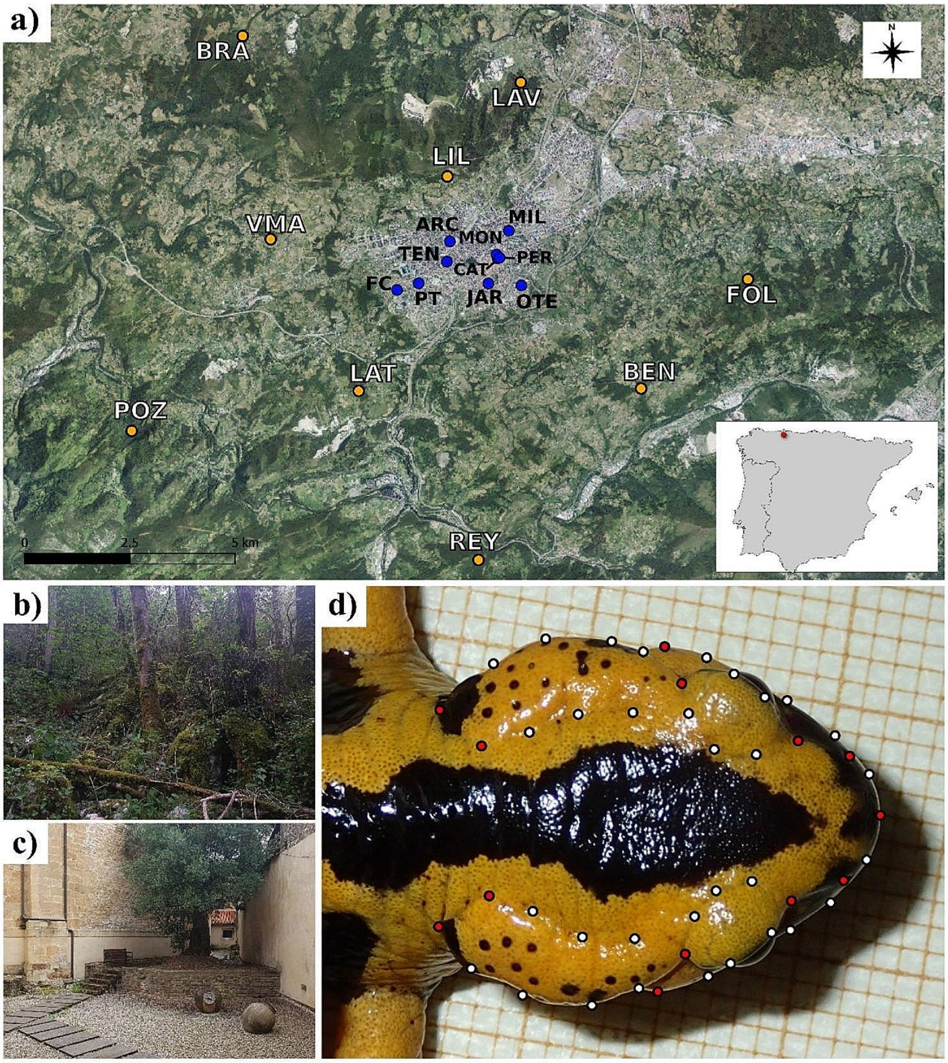

Fig. 1 Map displaying the distribution of studied populations in urban (blue) and forest (orange) habitats. The inset shows the location of Oviedo city in the Iberian Peninsula (Spain and Portugal) (**a**). Picture of Pozoval (POZ) as an example of a forest population (**b**). Picture of Peregrinos (PER) as an example of an urban population (**c**). Landmarks (red circles) and semilandmarks (white circles) recorded on the dorsal view of salamanders' head for geometric morphometrics analysis (**d**)

effect) and/or Fluctuating Asymmetry (FA, corresponding to the *individual* × *side* interaction) while accounting for measurement error (following Klingenberg et al., 2002). We performed these tests in all populations separately and on the complete data set to identify coherent shape components across the entire sample.

After testing for asymmetry patterns, we aligned the average of head shape coordinates from both replicates of each individual using a generalized least-squares Procrustes superimposition (GPA; Rohlf & Slice, 1990; Rohlf, 1999) to standardize in size, location and orientation. Then, we repeated the procedures of *bilat.symmetry* function to obtain averaged coordinates of mirrored individual configurations, thus isolating only the symmetric shape component for downstream analyses of shape variation across populations; while we used the total (averaged across individual replicates and superimposed) shape variation to examine some components of developmental (in)stability (see further on for details on statistical procedures).

We implemented all GM procedures and statistical analyses using packages *geomorph* v. 4.0 (Adams et al., 2021; Baken et al., 2021) and *RRPP* v. 1.0 (Collyer & Adams, 2018, 2019) in the R language for statistical programming R (R Development Core Team, 2022). We assessed statistical significance based on 10,000 random permutations, using Residual Randomization (Collyer et al., 2015).

Morphological Differentiation between and within Habitats

We first explored global patterns of morphological variation between and within habitats. For this purpose, we compared the symmetric component of head shape between habitats using distance-based Procrustes ANOVAs and including sampling population as a factor nested within habitat. As a measure of head size we used the logarithm of Centroid Size (logCS), calculated as the square root of the summed squared distances of each landmark from the centroid of the landmark configuration (Dryden & Mardia, 2016), which is uncorrelated with shape in the absence of allometry. Then, we analysed the allometric relationship between head shape and size by repeating the Procrustes ANOVA including head size as a covariate and its interaction with habitat and population (nested within habitat). This allowed us to investigate the covariation between head shape and size, test for common allometric slopes between habitats and among populations within habitats and evaluate habitat and population differentiation in shape while accounting for size effects on shape.

To further explore levels of differentiation among populations within each habitat, and determine which habitat presented higher levels of differentiation among the populations within it we used the function *morphol.disparity* in *geomorph* package (Adams et al., 2021; Baken et al., 2021). We compared Procrustes variances across all populations within each habitat, calculated from the residuals of a model that considered population size-corrected shape.

To visually explore patterns of differentiation in morphospace of studied populations we produced a two first principal components plot that included least-squares means for each analysed population after accounting for allometric shape variation and 95% nonparametric confidence ellipses for each group as a measure of the precision of group mean estimation.

Morphological Signs of Developmental Disturbance

To investigate whether the urban environment affected salamander head development we compared three different components of shape variation between habitats: total head shape variance (PV), deviations from the shape-size allometric slope as a measure of canalization, and fluctuating asymmetry (FA) as a measure of developmental instability (Lazić et al., 2015).

Head Shape Variance and Deviations from Allometric SlopeHead Shape Variance and Deviations from Allometric Slope

We first tested whether urban populations exhibited higher levels of head shape variance (PV) than forest ones as a proxy of the levels of developmental canalization. Using the symmetric component of head shape variation (see above), we estimated: (1) overall PV within each habitat considering habitat mean; (2) PV within each population, considering population mean in the model, using the function *morphol.disparity* as implemented in the package *geomorph* (Adams et al., 2021; Baken et al., 2021). In both analyses we accounted for size effects on shape including CS as a covariate in the model. Populations PV from different habitats were then compared using a permutational ANOVA.

As a second measure of developmental canalization, we calculated the deviation from the size-shape allometric relationship following Lazic et al. (2015), also considering only the symmetric part of shape variation. As both habitats, and populations within them, presented the same allometric slopes (see Results), we used a common allometric line for all individuals. We then tested for differences in these deviations from the allometric line through a permutational ANOVA with habitat and population nested within habitat as factors.

Fluctuating Asymmetry PatternsFluctuating Asymmetry Patterns

To evaluate differences in the level of developmental instability between habitats and among populations we first extracted an individual unsigned asymmetry index (FA index) for head shape. This index was calculated as the square root of sum of squared differences between original and reflected copies of bilateral landmark coordinates following Lazić et al. (2015). Then, we tested whether levels of FA differed between habitats and across populations within each habitat through a permutational ANOVA with habitat and population, nested within habitat, as factors. Then, to evaluate whether levels of FA varied with size, and if there were differences in allometric relationship between FA and size in each habitat and among populations we repeated the permutational ANOVA including size as a covariate.

Association between head Morphology and Genetic Background in Urban Populations

We evaluated the correlation between patterns of morphological variation in urban populations (population PV and mean FA of each population) and population mean heterozygosity (*Ho*), relatedness (*R*) and effective population size (*Ne*) using permutation tests with 1,000 resampling cycles without replacement. Genetic and demographic data were obtained from a previous study in which sampling was conducted four years earlier (Lourenço et al., 2017), and which includes all urban populations analyzed in this study except for population PER (Table 1).

To evaluate the role of drift as driver of morphological differentiation among urban populations we examined the correlation between the matrix of pairwise genetic distances (F_{ST}) (extracted from Lourenço et al., 2017) and the matrix of pairwise morphological distances (Euclidean distances of population mean shapes after correcting for size effects) using the Mantel test as implemented in the R package *ecodist* (Goslee & Urban, 2007).

Results

Morphological Differentiation between and within Habitats

Symmetric shape comparisons between habitats did not show significant differences between individuals from urban and forest populations (Table 2a). Conversely, individuals from both habitats differed in head size (Table 2b), and an allometric relationship between shape and size existed (Table 2c). However, when size effects were considered, urban and forest populations still did not differ in shape (Table 2c).

Procrustes ANOVAs also showed a significant variation across populations within each habitat, irrespective of size effects on shape (Table 2c). Furthermore, the degree of differentiation among populations was greater among urban than among forest populations when considering size effects on shape (differentiation among urban populations:10.04×10^{-4}; differentiation among forest populations: 8.91×10^{-4}; $P=0.045$) (Fig. 2).

Morphological Signs of Developmental Disturbance

Head Shape Variance and Deviation from Allometric Slope

Analysis comparing levels of overall PV between habitats resulted in significantly higher levels of morphological disparity among urban individuals (Urban PV: 12.03×10^{-4}; Forest PV: 10.26×10^{-4}; $P=0.007$) (Fig. 2). However, when comparing habitats considering population PV (Table 1), we found no significant differences between them (Table 3a).

Significant differences in the degree of deviation from the allometric slope existed among populations within each habitat but not between habitats (Table 3b).

Fluctuating Asymmetry Patterns

Procrustes ANOVAs performed for head shape revealed the existence of DA and FA (i.e., significant *side* and *individual* × *side* terms respectively), both when populations are considered separately (Table S1) or when they are all analysed together (Table 4). However, our results did not find differences in FA levels between habitats or among populations. Moreover, FA did not vary with head size, and the lack of significant differences between habitats and among populations persisted when accounting for head size (Table 3c).

Associations between Morphology and Genetic Background in Urban Populations

We find a marginally significant positive relationship between *Ho* and FA ($r=0.653$; $P=0.046$). However, this relationship was no longer significant after removing an extreme outlier value (TEN) ($r=0.179$; $P=0.315$). We therefore did not have strong evidence that either of heterozygosity (*Ho*), relatedness (*R*), or effective population size (*Ne*) were correlated with levels of developmental instability (mean FA index) (R: r = -0.223, $P=0.699$; *Ne*: $r=0.069$, $P=0.265$) or levels of PV (phenotypic disparity within each population) (*Ho*: r = -0.009, $P=0.495$; R: r = -0.043, $P=0.519$; *Ne*: r = -0.528, $P=0.949$) among urban populations.

Table 2 Results of the permutational ANOVAs used to test differences in head shape (a) and size (b) between habitats and among populations within habitats, shape-size covariation, head shape differentiation between habitats and among populations after accounting for allometric variation and common allometric slopes (c). Significant values are in bold

	df	SS	F	Z	P
a) Head Shape					
Habitat	1	0.008	2.063	1.573	0.061
Population	17	0.066	3.832	10.656	**< 0.001**
Residuals	358	0.364			
b) Head Size					
Habitat	1	1.074	18.515	2.830	**0.001**
Population	17	0.986	4.765	5.717	**< 0.001**
Residuals	358	4.358			
c) Head shape including size as covariate					
Size	1	0.010	9.828	5.086	**< 0.001**
Habitat	1	0.008	2.222	1.698	0.046
Population	17	0.063	3.743	10.193	**< 0.001**
Size × Habitat	1	0.001	0.730	-0.379	0.644
Size × Population	17	0.020	1.164	1.206	0.117
Residuals	339	0.337			

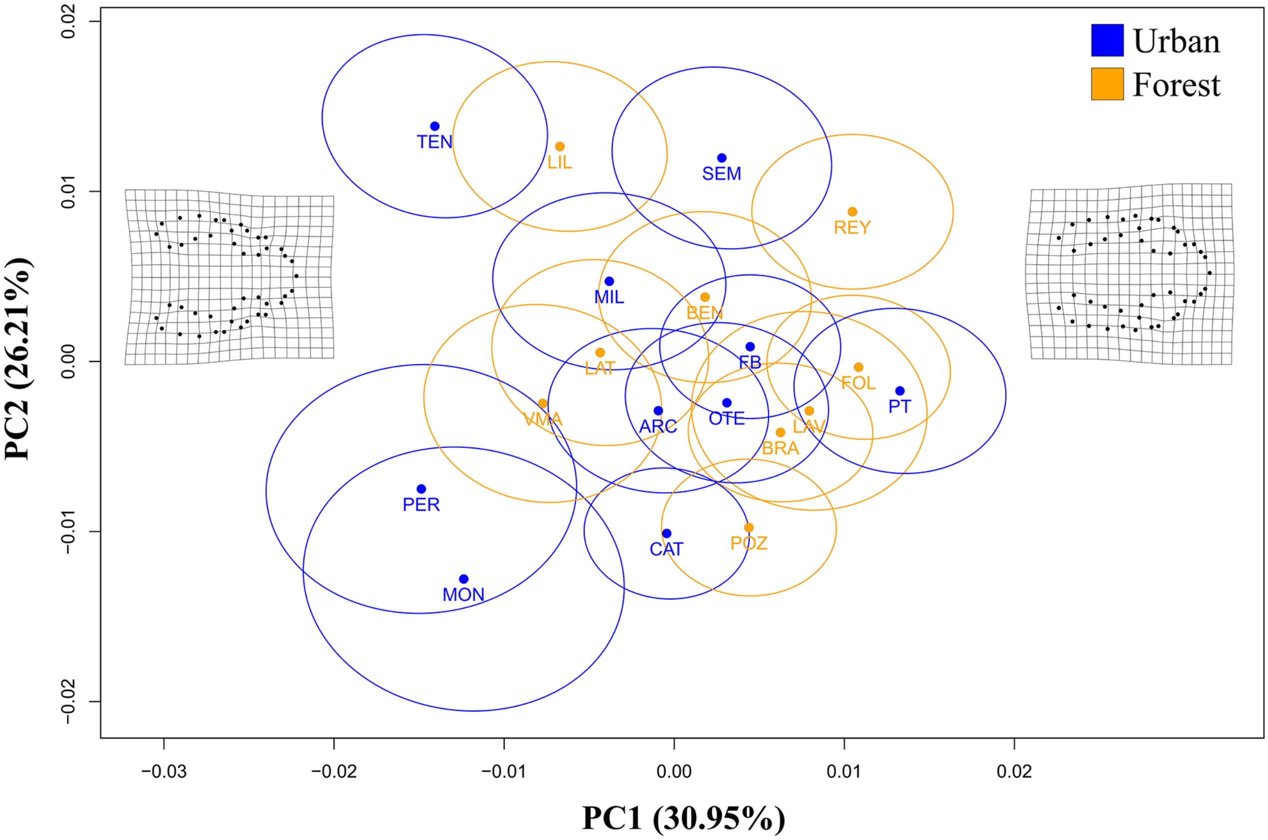

Fig. 2 Means and 95% confidence ellipses of the first and second principal components of head shape variation across individuals in each studied population from both habitats. Deformation grids depict shape change at the extremes of the first axis in comparison to the overall mean. Shape change has been magnified by a factor of three to facilitate visualization

Table 3 Results of the permutational ANOVAs used to test for differences between habitats and across populations within each habitat in phenotypic variance (PV) (a), the deviation from the allometric slope (b) and in fluctuating asymmetry (FA) index (c), without and while considering variation in size. Significant values are in bold

			Df	SS	F	Z	P
a)	**Phenotypic Variance**	Habitat	1	2.96×10^{-8}	0.689	0.231	0.420
		Residuals	17	7.29×10^{-7}			
b)	**Deviation from allometric slope**	Habitat	1	0.007	1.868	1.374	0.088
		Population	17	0.063	3.671	10.202	**< 0.001**
		Residuals	358	0.359			
c)	**Fluctuating Asymmetry Index**	**i) No considering size**					
		Habitat	1	0.009	3.815	1.447	0.070
		Population	17	0.039	0.724	-0.765	0.774
		Residuals	358	1.124			
		ii) Including size					
		Size	1	0.002	0.606	0.206	0.438
		Habitat	1	0.007	2.982	1.233	0.110
		Population	17	0.039	0.709	-0.818	0.789
		Size x Habitat	1	0.001	0.254	-0.270	0.607
		Size x Population	17	0.038	0.698	-0.844	0.802
		Residuals	339	1.085			

Table 4 Results of the Procrustes ANOVA used to test for the presence of Directional (DA, *side* effect), and Fluctuating Asymmetry (FA, *individual* × *size* effect) on the entire sample. Significant values are in bold

	df	SS	F	Z	P
Individual	376	1.753	4.238	-2.516	0.994
Side	1	0.160	145.271	7.969	**< 0.001**
Individual × Side	376	0.414	6.522	58.042	**< 0.001**
Individual × Side × Replicate	754	0.127			

Similarly, we find no significant correlation between genetic (F_{ST}) and phenotypic distances among populations (Mantel $r = 0.339$; $P = 0.271$).

Discussion

Cities represent an extreme transformation of the original habitats that are expected to affect organisms inhabiting them at different levels (e.g., phenotypic, demographic, evolutionary). Stress-related changes in phenotypic and life-history traits are rarely detected until population viability is severely compromised, but the indirect evaluation of the efficacy of developmental processes can allow the timely detection of urban populations under stress (Clarke, 1995). Urban populations' developmental pathways are expected to be affected due to the exposition to urban stressors resulting in an increased developmental instability (i.e., FA) and a higher frequency of phenodeviants (i.e., PV). Our results in the fully terrestrial salamander *S. s. bernardezi* partially support these predictions, as we found inconsistent results between different measurements of developmental disturbance: we observed a higher PV in almost all urban populations, but no differences in PV, allometric deviance and FA levels between urban and forest habitats, which prevents us from confirming a clear negative impact of urban conditions over salamander populations, as quantified through the study of head-shape developmental processes. On the other hand, we found higher morphological differentiation among urban populations than among forest ones, suggesting the existence of some mechanism driving phenotypic divergence within Oviedo city.

Increased Urban Phenotypic Differentiation but Inconsistent Patterns in PV

Increased phenotypic variability is a general trend in anthropic areas worldwide, and it can arise from developmental, ecological and evolutionary processes (Alberti et al., 2017; Thompson et al., 2022). On the one hand, high phenotypic differentiation among populations within a city might result from eco-evolutionary processes associated to the urban environment, such as isolation time, environmental heterogeneity, and the modification of selective pressures (Thompson et al., 2022). The city of Oviedo was founded during the late 8th century and urban salamander populations became progressively isolated following the expansion and urbanization of the city, suffering bottlenecks and drift processes that shaped the levels and structure of neutral genetic diversity (Lourenço et al., 2017). Drift and founder effects associated to reduced population size and high levels of isolation are expected to decrease intrapopulation genetic and phenotypic variation and increase interpopulation differentiation (Johnson & Munshi-South, 2017; Thompson et al., 2022). Accordingly, urban salamander populations present higher interpopulation differentiation than forest ones in head shape. Specifically, PER and MON populations, which have the lowest sample size ($N = 8$) and occur in small courtyards within the Cathedral (0.023 and 0.101 Ha respectively) (Fig. 1c), are two of the most differentiated populations along the first axis of variance (Fig. 2). Salamanders from the Cathedral (CAT, PER and MON) are considered to be isolated since the construction of Oviedo's first wall 12 centuries ago (Lourenço et al., 2017). Remarkably, other urban populations exhibiting considerable differentiation such as TEN and SEM (Fig. 2), are those in which isolation occurred longer ago, not considering the Cathedral ones (Lourenço et al., 2017). Thus, it could be plausible that time since isolation, and thus drift, may explain to some extent phenotypic differentiation among urban populations.

At the same time, cities are known to present high spatial and temporal heterogeneity (Alberti et al., 2020), and thus, observed pattern of differentiation might also result from local adaptive processes operating differently across the city. The mosaic of habitats within the city, which ranges from city parks to stony courtyards (Fig. 1c, Lourenço et al., 2017), may lead to ecological differences across sites (e.g., differences in type and availability of preys, shelter, or predators), which together with the restriction of gene flow among urban populations might favour local adaptation processes with the subsequent differentiation among populations (Kozak et al., 2005; Littleford-Colquhoun et al., 2017; Marques et al., 2022). In the present study, the lack of correlation between phenotypic and neutral genetic differentiation patterns, suggests that local adaptive processes within the city may be contributing to head shape differentiation, in a 'city-archipelago' fashion (Littleford-Colquhoun et al., 2017). However, in addition to local adaptation, we cannot completely rule out drift processes as an underlying driver of morphological differentiation among urban populations (Clegg et al., 2002). Additionally, cities might modify natural and sexual selection pressures (i.e., relaxed selection) (Lahti et al., 2009; Rodewald & Arcese, 2017; Santangelo et al., 2022). For instance, the high morphological differentiation observed among urban salamander populations might result from differences in selection pressures over morphological specialization across populations, allowing for higher, and potentially adaptively successful diversification in urban morphologies (e.g., Falvey et al., 2020). However, further research on the consequences of head shape changes in relationship with habitat structure and heterogeneity would be necessary to evaluate more accurately the factors underlying functional differentiation between urban sites.

On the other hand, all those eco-evolutionary processes that concur within cities can also help to understand observed levels of variability (i.e., PV) in head shape within urban populations. Despite a reduction in intrapopulation phenotypic diversity is expected in small and isolated urban populations (Johnson & Munshi-South, 2017; Thompson et al., 2022), we observed that they generally present higher PV values (but not significantly different from forest ones), with the exception of PER, which is the less variable studied population (Table 1). This trend could be suggesting a reduction in the efficacy of the mechanisms buffering developmental canalization processes, understood as an organismal property that promotes the production of consistent phenotypes from a common genetic basis (Waddington, 1942; Willmore et al., 2007). The disruption of canalization-related mechanisms can have a genetic basis (e.g., expression of cryptic genetic variation, recessive alleles, or mutations), or may result from external factors that modify the accurate functioning of developmental buffering (see Takahashi, 2019 and references therein). Accordingly, phenotypic variability has been observed to increase in inbred populations (Réaale & Roff, 2003), or under stressful environmental conditions such as nutritional or thermal stress in some organisms (Gonzalez et al., 2014; Hoffmann & Hercus, 2000; Imasheva et al., 1999). Although with the data at hand we cannot determine the exact mechanisms underlying the observed generalized increase in morphological variability of urban populations, we did not find any correlation between genetic diversity or inbreeding measures and phenotypic variance. Indeed, Oviedo urban populations do not show significantly lower levels of genetic diversity than populations outside the city (Lourenço et al., 2017), and thus, similar levels of phenotypic variation could be expected. Therefore, it seems more plausible that, in this system, the performance of developmental canalization mechanisms could be impaired in some populations due to environmental factors associated to specific urban areas, which would in turn result in the higher phenotypic diversity observed in some populations from the city.

No Differences in Fluctuating Asymmetry or Allometric Deviance

Contrary to our predictions, we did not find evidence of a disruption of developmental processes in FA or allometric deviance. First, uniformity of allometric slopes across populations and habitats points to similar mechanisms controlling head shape variation with respect to size. Additionally, the lack of significant differences in the deviance from the allometric slope between habitats reveals that allometric mechanisms are not altered in urban environments. Secondly, urban populations do not exhibit higher FA than forest ones. Here, we should emphasize that developmental stability is trait-specific (Lazić et al., 2013; Karvonen et al., 2003), and it depends upon the trait functionality, which determines the strength of the control mechanisms underlying development (Leamy & Klingenberg, 2005; Palmer & Strobeck, 1986). In this sense, the head is a highly functional and relevant structure that is expected to be strongly buffered developmentally. As such, disturbances in developmental stability may occur in a very fine scale, if at all, and not be easy to identify in this structure. In addition, the degree of asymmetry can vary along ontogeny. Our sampling design only includes adult males, so the results can be biased by two different mechanisms: selection against asymmetric individuals and lower fitness (Tocts et al., 2016), although the frequency of head deformities in adult salamanders within urban populations (Velo-Antón et al., 2021) suggest no drastic effects of asymmetry on survival; and the existence of buffering developmental mechanisms that compensate asymmetry throughout development (Lazić et al., 2016), although trends and underlying mechanisms seem to be highly variable across species (Lazić et al., 2017). Thus, investigating FA levels at earlier stages of development might uncover different patterns and the existence of stressors in urban environments.

Furthermore, our study did not observe a clear relationship between levels of genetic diversity, *Ne*, relatedness, and levels of head shape asymmetry or phenotypic variance across urban populations. Similarly, those relationships did not appear between genetic diversity and deformity frequencies neither in a previous study (Velo-Antón et al., 2021). Other studies exploring the relationship between genetic diversity and developmental stability in other organisms have provided highly variable results (Eterovick et al., 2016; Garrido & Pérez-Mellado, 2014; Gilligan et al., 2000; Graham et al., 2010; Pertoldi et al., 2006; Vøllestad et al., 1999). However, it is important to note that environmental and genetic factors may affect fitness in a combined way, and high genetic diversity might constitute a protection against other environmental stressors (Joubert & Bijlsma, 2010; Kristensen et al., 2006). Thus, the lack of differences in FA between urban and forest populations may result from the existence of moderate to high levels of genetic diversity in Oviedo (Lourenço et al., 2017), which are very similar, or even higher, to other distant populations occurring in larger forest areas throughout several *S. salamandra* subspecies (Antunes et al., 2018, 2021; Lourenço et al., 2019; Velo-Antón et al., 2012). Indeed, a high *Ne*/*N* ratio was found in a urban salamander population in Oviedo (Álvarez et al., 2015), suggesting putative mechanisms of genetic compensation (e.g., high levels of multiple paternity; Alarcón-Ríos et al., 2020) to prevent inbreeding depression, which could also buffer against developmental disturbances.

Finally, specific life-history traits of the study system should be considered when interpreting the impacts of urban environments. For *S. salamandra*, in particular, the pueriparous reproductive mode of the examined populations reduces their direct exposition to stressors (e.g., pollutants) during embryogenesis, since the developing embryos remain protected within the mother's body until birth after metamorphosis (Buckley et al., 2007). Given that this is the most sensitive stage of development (Møller, 1996; Pineda et al., 2012), this might explain the absence of clear morphological signs of developmental disturbance within the urban habitat reported here. Pueriparity is a key trait for the persistence of viable salamander populations across Oviedo, in patches where water bodies for reproduction are not available (Álvarez et al., 2015; Lourenço et al., 2017). Furthermore, genetic Álvarez et al., 2015; Lourenço et al., 2017) and morphological (this study) results suggest that despite inhabiting a highly transformed environment, these pueriparous salamanders have managed to persist and maintain stable urban populations. However, deciphering the role that this evolutionarily derived reproductive mode may play in the capacity of amphibian populations to cope with environmental stressors requires comparative studies including larviparous forms.

Conclusions and implications for urban evolutionary studies

The lack of unambiguous evidence to confirm stressed urban salamander populations, via developmental disturbance measures (i.e., developmental stability and canalization processes), together with the reasonably larger levels of genetic diversity observed across Oviedo urban populations (Lourenço et al., 2017), suggest that the viability of Oviedo urban salamanders is not apparently compromised. However, the increased levels of phenotypic variation observed in most urban populations point to several potential scenarios that would need further investigation. On the other hand, although processes such as drift cannot be completely ruled out, remarkable differences in head morphology among urban populations and the lack of correlation of such differentiation with neutral genetic patterns indicate the possibility of distinct eco-evolutionary processes influencing phenotypic disparity within cities.

As a concluding remark, in their recent review Thompson and collaborators (2022) drew attention to two key aspects of urban evolutionary studies. First, the importance of considering phenotypic disparity, more than means, when assessing phenotypic consequences of urbanization. Second, the relevance of sampling design (i.e., population definition), and the implications of making groups by contrasting habitats not considering heterogeneity and structure within each of them. Our results are in line with both ideas, as important patterns of differentiation between habitats only arise when considering morphological variation, but not when comparing morphological means. Indeed, the higher morphological differentiation that exists among urban populations highlights the need of considering multiple populations to cover the heterogeneity that could exists within each habitat to accurately understand the patterns that arise and pinpoint the possible underlying mechanisms.

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Data Availability Datasets used in the present study can be found in Figshare https://doi.org/10.6084/m9.figshare.19771855.v2

Declarations

Competing Interests The authors declare no competing interests.

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