



Original Articles

The exposition to urban habitat is not enough to cause developmental instability in the common wall lizards (*Podarcis muralis*)

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ABSTRACT

Developing trustworthy biomarkers is of primary importance in monitoring impacts of environmental stressors on biological systems. Recently, fluctuating asymmetry in morphological traits of the common wall lizard (*Podarcis muralis*) has been proposed as useful tools for assessing environmental disturbance in urban habitats. However, the experimental design used in previous studies lacked both site replication (only one urban area was sampled) and independent assessment of the physiological conditions of lizards. Hence, we replicated the analysis involving three large cities in Northern Italy and measured lizards' health conditions as assessed by blood sampling analyses. We found a weak statistical association between fluctuating asymmetry and urban habitat. No significant differences occurred for white blood cell concentration and heterophils-to-lymphocytes ratio, whereas parasite load was significantly lower in urban than natural populations. Finally, somewhat of the variability in fluctuating asymmetry correlated with one or more latent variables not directly observed, but still not related to the habitat. Contrary to previous studies, we concluded that fluctuating asymmetry cannot be regarded as a reliable measure of urban environmental stresses. Despite this, fluctuating asymmetry in the morphology of common wall lizards has a real potential for working as biomarkers, but we need to clarify first the developmental mechanisms, the true effectors of the stress, their interactions and their effects on individual fitness in order to solve the inconsistencies occurring among different studies.

1. Introduction

Urban environments make animal populations experience a variety of new conditions, such as different predatory pressure, increased temperatures, and unusual food sources (Gering and Blair, 1999; Ditchkoff et al., 2006; Murphy et al., 2016) just to name a few. Such new conditions may stress individuals leading to homeostasis disruption and, ultimately, to individual fitness decline. However, not all species respond negatively to human-related habitat alterations, and some species may actually take advantage of the new characteristics (Martín and López, 1999a,b, 2015; Diego-Rasilla, 2003). In this scenario, conservation biologists are trying to figure out sensitive biomarkers to reliably measure exposition to various stressors, and early detect any decline in population health and consistence before it is too late (Forbes et al., 2006).

Among all possible biomarkers proposed till now (including molecular, cellular, and histological biomarkers, McCarthy and Shugart,

1990) morphological traits are particularly promising as they are cheap, low time-consuming, and not invasive. Beasley et al. (2013) suggested the use of fluctuating asymmetry (FA) as a morphological biomarker, basing on the idea that FA is a proxy for environmentally induced developmental instability. FA is defined as small, random deviations of bilateral traits from perfect symmetry due to subtle variations in the developmental environment (Palmer and Strobeck, 2003). Thus, environmental stressors interfering with the ability of individuals in maintaining precise development (resulting in negative effects on the population over time) could be detected by significantly increased FA levels. However, the initial attractiveness of FA as a potential biomarker has been weakened due to inconsistent results across species. In their meta-analysis, Beasley et al. (2013) concluded that the use of FA as a biomarker is legitimate provided that the measurement tool used to assess FA is highly sensitive (e.g., geometric morphometrics compared to linear and/or meristic measures), individuals are not able to buffer a developmental pathways against the stressor (e.g., anthropogenic

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compared to natural stressors), and the environmental stressor acting on the organism is known (e.g., the negative effects on fitness-related traits are verified). Last but not the least, stress related to FA is detected more easily in laboratory compared to field populations.

Lizards offer a good model for measuring exposition to environmental stressors since they are widespread and well adapted to urban habitats, they live at high density, move across very small home ranges (hundreds of square meters) and are easy to catch and keep in the laboratory. Accordingly, a lot of studies have used lizards for biomarker essays during the last two decades (e.g., *Gallotia galloti*, Fossi et al., 1995; Sanchez-Hernandez et al., 1997; *Diplodactylus conspicillatus* and *Rhynchoedura ornata* Read (1998); *Podarcis carbonelli*, Mann et al., 2007; *Agama agama*, Oyekunle et al., 2012; *Podarcis siculus*, Marsili et al., 2009; Verderame et al., 2016).

FA has been widely detected in lizards, and correlations with a variety of environmental conditions have been found, including population, habitat and fragmentation, temperature, pollution, and insularity (Sarre and Dearn, 1991; Sarre, 1996; Tull and Brussard, 1944; Vervust et al., 2008; Băncilă et al., 2010; Garrido and Pérez-Mellado, 2014; Urošević et al., 2015; Lazić et al., 2013, 2015). However, results are once again contradictory, and there are studies that failed to detect relationships between FA and environmental features (e.g., Crnobrnja-Isailović et al., 2005; Warner and Shine, 2006; Amaral et al., 2012). By contrast, only a few studies have investigated the effects of FA on fitness-related traits, once again with opposite results: active effects have been found on exertion (Vervust et al., 2008), escape speed (López and Martín, 2002), femoral pore secretions (Martín and López, 2000; López et al., 2002; López et al., 2006), incubation temperature (Ji et al., 2002), but not on bite force (Vervust et al., 2008), incubation temperature (Longson et al., 2007), female mating preference (Lailvaux and Irschick, 2006), testosterone plasma level (Veiga and Salvador, 1997), and hybridization (Dosselman et al., 1998).

Despite these non-univocal results, most studies have analyzed the correlation between the magnitude of FA and some environmental features without an independent validation of the potential relation linking stressors to FA (reviewed in Leung et al., 2003). That is, some environmental features have been assumed to be stressful for lizards *a priori*, without any assessment of their actual power to stress individuals settled in those habitats. A typical example of this approach concerns urban habitat: it is often considered suboptimal (i.e., more stressful) than natural ones based on the assumed negative effects of human alterations. If this reasonably applies for larger species, such mammals or birds, it becomes questionable for such small species as *Podarcis* lizards, which have been evolved in rocky and dry habitats, resulting pre-adapted to human-made environments. Hypothetically, lizards in human environments might experience lower predation risk as well as higher food availability, thus achieving higher densities than in natural habitats. Paradoxically, we could detect higher FA in human-made environments because of increased competition due to increased density rather than to human-related stressors. Without a direct measure of individuals' healthy condition, these two opposite states cannot be fully disentangled.

Recently, Lazić et al. (2013, 2015) compared the FA levels of the common wall lizard (*Podarcis muralis*) across urban and rural populations around the city of Niš (Serbia). Data collected consistently suggested that urban lizards showed higher levels of FA in four morphological traits, and this was interpreted as an evidence that anthropogenic disturbance causes an increase in FA, and hence FA can be a reliable indicator of exposition to environmental stressors following human activities. However, authors did not replicate the experiment in other cities and did not make an independent assessment of the physiological conditions of the sampled lizards. Indeed, they assumed *a priori* common wall lizards living in urban habitat being in suboptimal habitat conditions. Consequently, the experimental design did not allow to disentangle the effects of the urban environment in itself, apart from the specific features of the City of Niš. Even more, the

conclusion that fluctuating asymmetry of common wall lizards is a reliable measure of urban environmental stresses needs further experimental support.

In the present study, we analyzed the magnitude of FA in four morphological traits of the common wall lizards across urban and rural populations and we compared the degree of FA observed in urban and rural habitats with lizards' healthy conditions as assessed by blood sampling analyses. We replicated the comparison in three separate urban areas of Northern Italy in order to generalize results as representative of the effects of urbanization on the developmental instability of the species.

2. Methods

The Common wall lizard is a small lizard (snout-vent length, SVL, 45–75 mm) widespread in southern and central Europe, which mates multiply and produces two clutches per year on average (Sacchi et al., 2012) during its life (max lifetime 5 years, Barbault and Mou, 1988). Breeding season starts from late February and ends in July (Sacchi et al., 2012), and body temperature during activity is near 33 °C, being slightly higher (33–36 °C) in warmer regions (e.g., Central Italy) and lower (32 °C) in mountain areas (Tosini and Avery, 1994).

2.1. Study area and design

The study was carried out during April–July in three cities of the Liguria region in Northern-Italy at least 25 km apart from one another: Genoa (44°25'N, 8°54'E), Recco (44°21'N, 9°08'E), and Pietra Ligure (44°8'N, 8°16'E) respectively (Fig. 1). For each site we sampled three populations in as many different habitats, one in a fully urbanized area in the city center (urban habitat), one in a partially urbanized area with gardens and urban parks (sub-urban habitat), and one in a natural environment in the surrounding of the city (natural habitat). Adult common wall lizards (snout-vent length, SVL > 54 mm, Sacchi et al., 2012) of both sexes were collected by noosing, between 0900 and 1400 h, and not less than 10 individuals for each sex were caught. Overall, we collected 211 lizards (on average 23 for population, range 21–25), including 107 males and 104 females. After capture, lizards were transported to the laboratory of the University of Pavia where they were weighted and measured for snout-vent-length (SVL). For each individual we took high resolution digital images of ventral scales (VS), ocelli on both flanks (OC), femoral pores (in males only, FP), and head dorsal scales (HS) using a Nikon D50 camera at a 1.2-million-pixel resolution, equipped with a Nikkor 60 mm AF-S Micro lens, and fixed on stand at a distance of 18 cm.

2.2. Fluctuating asymmetry quantification

For each individual, one of us (MP) recorded the number of VS, OC, and FP twice from the digital images, with one week between the first and the second counting and randomizing the order of examined specimens, to ensure the independence of trait counts. We then calculated for each trait the difference between the value on the right side of the body minus that on the left side (R-L). Hence, for each individual we calculated three overall asymmetry indexes (AI_{VS} , AI_{OC} , AI_{FP} respectively) following Lazić et al. (2015) as the unsigned R-L difference between the log-transformed average of trait values across the two replicate counts as it follows:

$$AI = |\ln(R_{average}) - \ln(L_{average})|$$

Digital images of HS were analysed using geometric morphometrics. One of us (MP) digitized 36 landmarks (Fig. 2), twice for each lizard, using TpsDig2 (Rohlf, 2005; <http://life.bio.sunysb.edu/morph/>). As for previous traits, we allowed at least one week between subsequent digitalizations of the same image, and the order was randomized. Subsequently, we first reflected all landmark configurations to obtain their

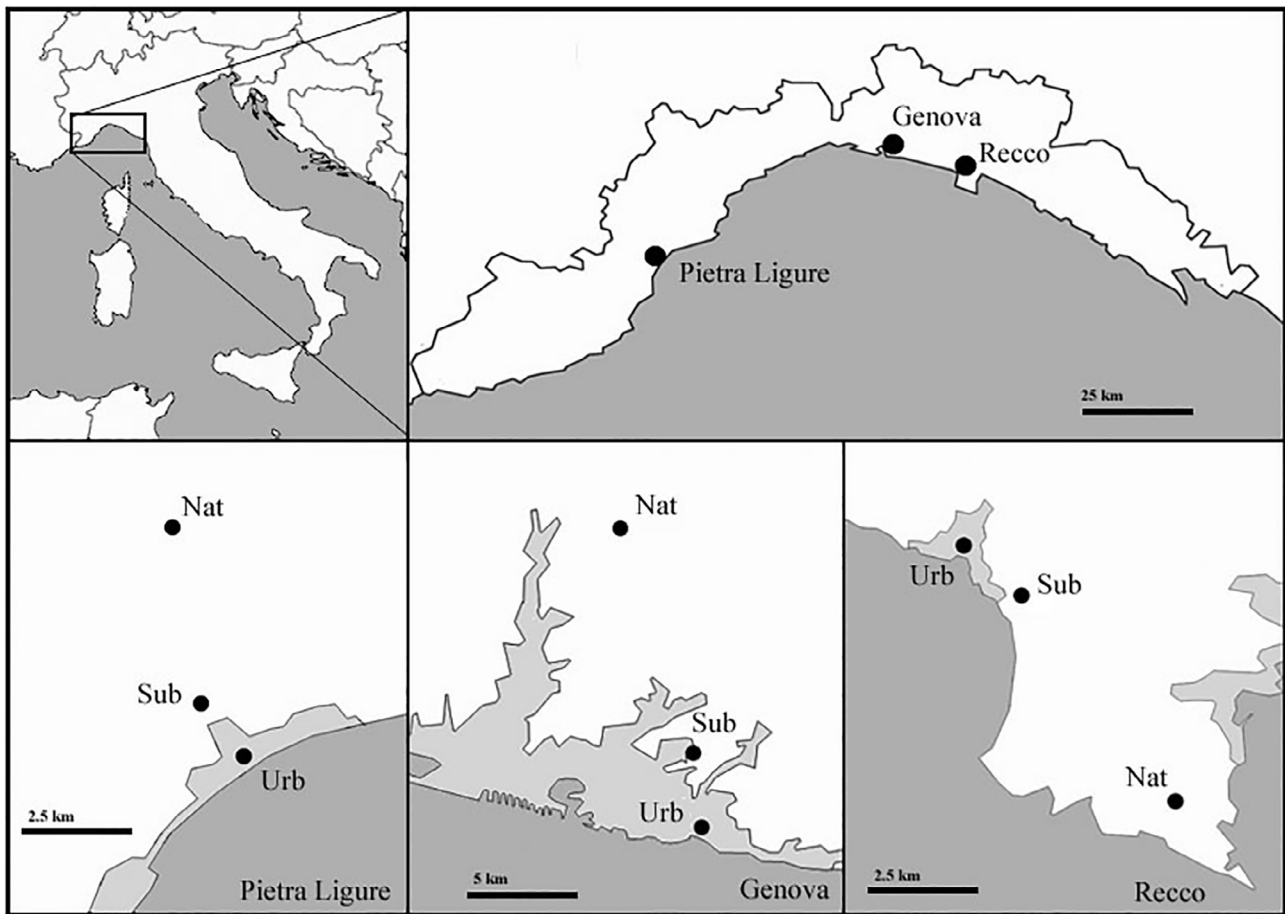


Fig. 1. Map of the three cities used to compare fluctuating asymmetry among urban (Urb), sub-urban (Sub) and natural (Nat) populations of the Common wall lizard.

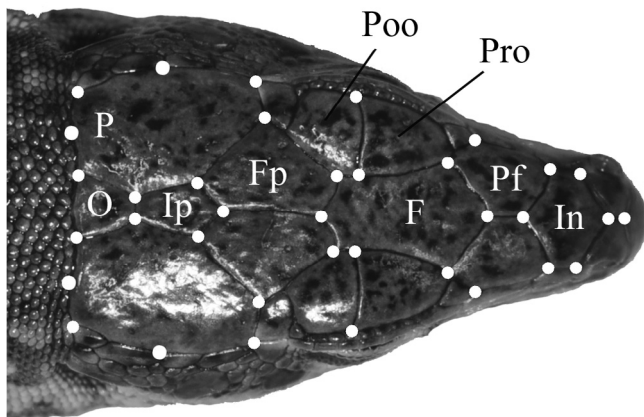


Fig. 2. Landmarks used to quantify fluctuating asymmetry in head shape. O = occipital scale, P = parietal scales, Ip = inter-parietal scale, Fp = frontoparietal scales, F = frontal scale, Pf = prefrontal scales, In = internasal scale, Pro = preocular scales, Poo = postocular scales.

mirror images (Klingenberg et al., 2002). Then, we scaled all the configurations to unit centroid size and superimposed by a generalized procrustes analyses (GPA). Superimposition of the original landmark configurations and their mirror images allows the separation of symmetric and asymmetric variation components (Mardia et al., 2000; Klingenberg et al., 2002). Then, we derived a FA index for head shape (AI_{HS}) following the procedure proposed by Klingenberg and McIntyre (1998) for structures with object symmetry. In summary, we considered only paired landmarks and we computed the difference of landmark

coordinates between original and mirrored configurations for each individual. These differences were used to calculate AI_{HS} as the squared root of the sum of squared differences from all landmark coordinates. This index represents the Procrustes distance between the right and left sides of the head of each individual.

2.3. Blood sampling and leukocyte counts

Blood samples (3–5 μ l) were collected from the postorbital plexus (MacLean et al., 1973) from 51 males and 47 females (at least 5 males and 5 females of each population). Blood slides were prepared by placing a drop of blood directly onto a glass slide and smearing it with a second slide to produce a blood layer one cell thick. Smears were stained by the May–Grunwald/Giemsa method. Leukocytes (classified as heterophils, eosinophils, neutrophils, basophils, lymphocytes, and monocytes) and red blood cells were counted by randomly scanning smears at $60\times$ magnification, and at least 50 microscope fields were considered (Sacchi et al., 2011). By this procedure, we counted on average 150–200 leukocytes each smear, and white blood cell concentration (WBC) was expressed as the number of leukocytes every 10,000 red blood cells. The heterophil:lymphocyte ratio (HL) was calculated as a sensitive measure of immunosuppression and long-term stress; an increase in this ratio may reflect a reduction in the responsiveness of the immune system due to an increase of corticosterone plasma level following a stressful experience (Davis et al., 2008). Finally, we recorded blood parasite load (Haemogregarines, PA) defined as the number of parasites found every 10,000 red blood cells.

2.4. Statistical analyses

We performed a preliminary analysis to i) assess trait size or body size dependence in FA indices of all morphological traits and ii) check for the occurrence of fluctuating and directional asymmetry in each trait. Due to the different structure of data, preliminary analyses were performed separately for the three meristic traits (i.e., number of ventral scales, ocelli and femoral pores) and head shape. The R-L values did not deviate from normality in any meristic trait (Kolmogorov-Smirnov test, $P > 0.05$). We checked for trait size dependence using a linear regression of unsigned R-L values on SVL (to test for dependence on total body size) and on $(R + L)/2$ (to test for dependence on trait size), and we did not find any significant relationship (see Table S1 for statistics). Then, we used a two-way ANOVA where the log transformed trait was the dependent variable with side, individual and their interaction as grouping factors. In this models, a significant main effect of side accounts for directional asymmetry, whereas a significant side \times individual interaction points out the occurrence of FA (Klingenberg et al., 2002). The analysis supported the occurrence of FA in all traits, and showed the presence of directional asymmetry in VS and OC (see Table S3 for statistics).

As for previous traits, we test for directional and fluctuating asymmetries in head scales' landmark configurations by performing a Procrustes ANOVA following Klingenberg et al. (2002) on the full data set. The analysis revealed statistically significant individual variation as well as variation due to directional and FA (see Table S4 for statistics). Then we checked size dependence effects through a multivariate regression of shape coordinates on centroid size. We used the first 9 principal components of the variance-covariance matrix (VCV) of the asymmetric component as shape variables, snout to vent length (SVL, log transformed) as size proxy and both site and sex as grouping factors. We used SVL in spite of centroid size since it is a better predictor of age. The model included all the main effects and the size \times population and size \times sex two-way interactions. The null hypothesis of parallel slopes among sites was rejected ($F_{195,197} = 1.181$, $P = 0.37$).

As additional preliminary test, we examined the correlation between traits (signed and unsigned R-L) to test respectively whether i) traits belonging to the same body part show similar asymmetry patterns, and ii) asymmetry in a single trait can be used as an indicator of individual quality. Significant, though weak, correlations were found between VS and OC, but not with FP, so we analyzed each trait separately (see Table S2 for statistics).

Following Lazić et al. (2013), we first looked for differences among habitats compared to the total variance of FA in the four morphological traits we considered (i.e., VC, OC, FP, and HS). Since HS is a multivariate trait, we calculated the shape variance for each habitat as the trace of the within-habitat-VCV matrix. We compared variances across habitats through a resampling procedure ($n = 999$).

We analysed the relationship between habitat and FA in each morphological trait (as estimated by AI_{VS} , AI_{OC} , AI_{FP} , and AI_{HS}) using linear mixed models. The AIs were the dependent variable with habitat, sex, and body as main effects and habitat \times sex (but not for femoral pores) and habitat \times svl as interaction effects. The site (as a three levels factor) was the random intercept to account for unexplained variation at site level (σ_{site}^2) after we controlled for the explanatory variables. The initial model was simplified using backward elimination of the not significant terms (Zuur et al., 2009), and we checked residuals of the initial model for normality and homoscedasticity (Zuur et al., 2009). We used Satterthwaite approximation to estimate the degrees of freedom of denominators, and we performed four independent analyses, one for each trait. Finally, pseudo- R^2 accounting for the variance explained by the model (fixed and random effect combined) and for the fixed effects alone were computed according to Nakagawa and Schielzeth (2013).

We compared total variances of blood variables (i.e., WBC, HL, and PA) across habitats through a resampling procedure with 999

Table 1

Comparison of the total variances of FA across habitats (U = urban, S = suburban, N = natural) in four morphological traits of the Common wall lizards. P-values are computed through resampling procedure ($n = 999$).

Trait	V_u/V_s	V_u/V_n	V_s/V_n
Ventral scales (VS)	0.84 ($P = 0.49$)	0.94 ($P = 0.95$)	1.12 ($P = 0.10$)
Ocelli (OC)	1.23 ($P = 0.12$)	0.86 ($P = 0.033$)	0.71 ($P = 0.22$)
Femoral pores (FP)	1.04 ($P = 0.12$)	0.99 ($P = 0.35$)	0.86 ($P = 0.78$)
Head shape (HS)	1.16 ($P = 0.042$)	1.34 ($P < 0.001$)	1.15 ($P = 0.048$)

permutations, as we did for morphological traits. Hence, we used linear mixed models to investigate the relationships between habitat and hematological variables. Model structure was the same used for morphological traits (PA was log-transformed to achieve normality), and we used backward elimination to remove not significant terms.

We used linear-mixed model once again to investigate relationships between FA and hematology. In this case, the AI indexes were the dependent variable with hematological variables as predictors, and the site as a random effect. As in previous analyses, we simplified models by removing not significant effects.

Analyses were performed using the package lme4 (Bates et al., 2015) and MuMIn (Barton, 2016) in R ver. 3.2.4 (R core Team, 2016), and otherwise stated, data reported are means \pm standard errors.

3. Results

3.1. Fluctuating asymmetry and habitat

The comparison of variances of the four morphological traits across habitats did not supply consistent results (Table 1). Indeed, the highest variances were observed in urban habitat for HS, in natural habitat for both OC and FP, and in sub-urban habitat for VS. However, in most cases, the ratio between variances did not significantly deviate from one. Urban populations clearly exhibited significant higher total HS variance compared to both suburban and natural habitats, while sub-urban populations were more variable than natural ones, but the opposite occurred for OC, where natural populations exhibited significant higher variance compared to urban habitat.

The final models revealed no significant association of both AI_{VS} and AI_{OC} with habitat ($P > 0.45$ at removal, Fig. 3). We detected a negative relationship between AI_{OC} and SVL ($\beta_{\text{natural}} = -0.008 \pm 0.004$, $F_{1,208.6} = 4.141$, $P = 0.043$), suggesting that smaller lizards were more asymmetric. All other predictors did not enter any of the two final models. The random effect of site (σ_{site}^2) was significant in both models (VS: $LR-X^2 = 6.48$, $df = 1$, $P = 0.011$; OC: $LR-X^2 = 3.83$, $df = 1$, $P = 0.049$), and it accounted respectively for 6.7% and 4.0% of unexplained variance in trait asymmetry, after controlling for fixed effects. This last result suggested that an (albeit small) amount of the variability in FA of VS and OC depended on some unknown features of sites not related to habitat, sex, and body size.

The habitat was the only predictor with a (even marginally) significant effect ($F_{2,97.4} = 2.962$, $P = 0.056$) entering the final mixed model of AI_{FP} . For this trait the asymmetry was significantly lower in sub-urban compared to both urban and natural habitats (Fig. 3), even the difference was statistically significant for the last one only ($\beta_{\text{urban}} = -0.005 \pm 0.007$, $t_{97.2} = 0.599$, $P = 0.55$; $\beta_{\text{natural}} = -0.017 \pm 0.007$, $t_{97.4} = 2.347$, $P = 0.021$). The final model accounted for 8.0% of the total variance in the FP FA, while the habitat alone (the fixed effect) accounted for 5.4% of the whole variance. Consistently, the site (the random effect) did not significantly affect AI_{FP} .

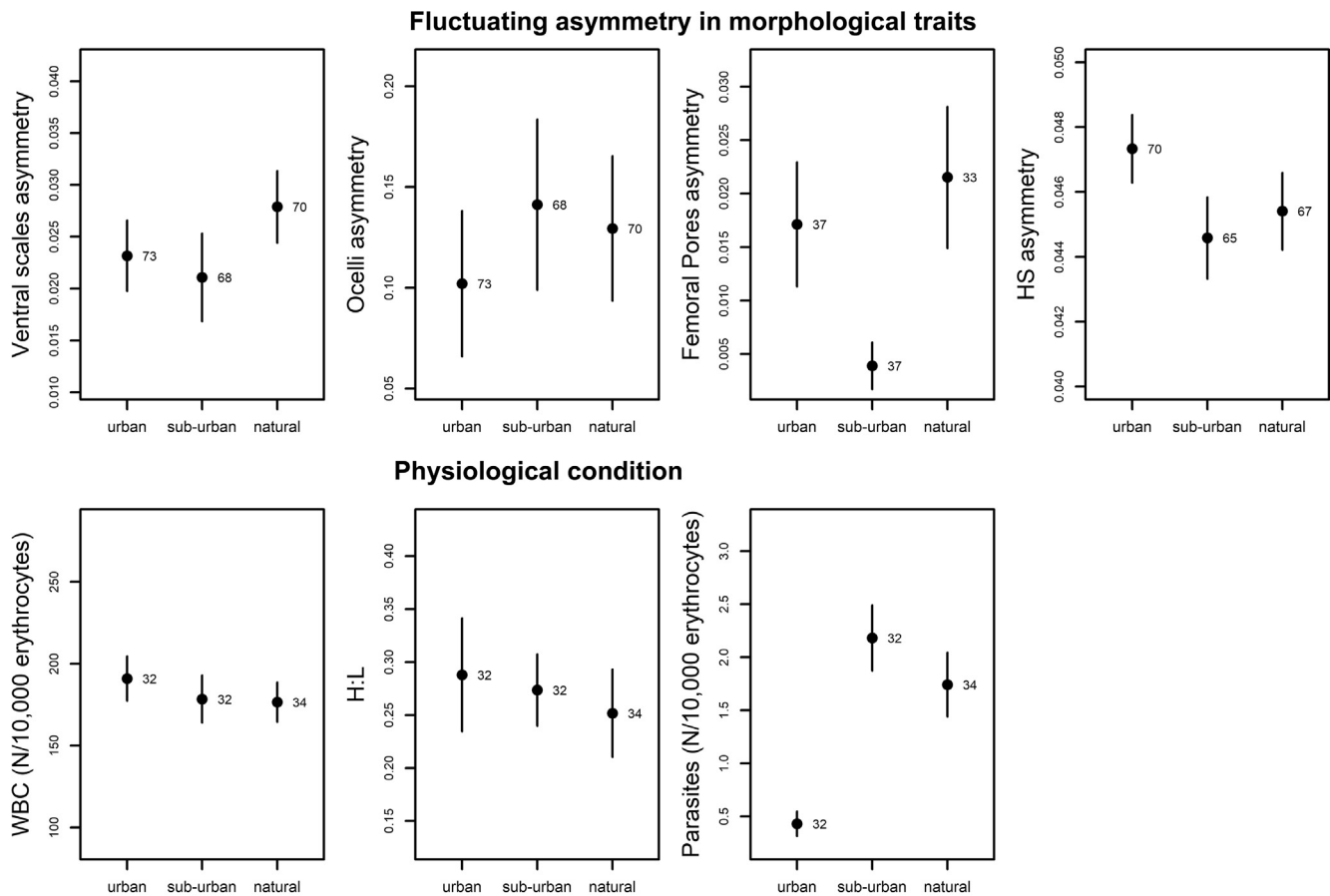


Fig. 3. Comparisons among urban, sub-urban and natural population for (upper panels) the intensity of fluctuating asymmetry in four morphological traits, and (lower panels) physiological condition assessed by total with blood cells (WBC), heterophils-to-lymphocytes ratio (H:L) and parasite load (Haemogregarins).

As to AI_{HS} , we did not find any significant effect of the habitat ($P > 0.44$ at removal), but we found a highly significant effect of sex \times SVL interaction ($F_{1,196} = 27.21, P < 0.001$), suggesting that asymmetry in males increased with increasing body size ($\beta_{\sigma} = 0.039 \pm 0.012, t_{198.1} = 3.281, P = 0.0012$), while the opposite occurred in females ($\beta_{\varnothing} = -0.044 \pm 0.010, t_{198.1} = 4.449, P < 0.001$). Finally, the random effect in the final model was not significant, suggesting that variation in HS asymmetry was not affected by site.

3.2. Hematology and habitat

The comparison of the variances of the three hematological variables across habitat did not supply any significant results (Table 2), notwithstanding the lowest variances of WBC and HL, and the highest variance of PA was observed in natural habitat.

We found no significant variation across habitats for both WBC and

Table 2

Comparison of the total variances of FA across habitats (U = urban, S = suburban, N = natural) for hematological variables of the Common wall lizards. P-values are computed through resampling procedure (n = 999).

Trait	V_u/V_s	V_u/V_n	V_s/V_n
White blood cells (WBC)	0.88 (P = 0.60)	1.18 (P = 0.55)	1.35 (P = 0.44)
heterophil:lymphocyte ratio (HL)	2.51 (P = 0.32)	1.67 (P = 0.36)	0.67 (P = 0.55)
Parasite load (PA)	0.14 (P = 0.24)	0.14 (P = 0.15)	0.99 (P = 0.39)

HL, as the corresponding final models did not include any of the predictors ($P > 0.16$ at removal). The random effect of site was not significant for HL, but it was for WBC ($LR-X^2 = 47.08, df = 1, P = < 0.001$). The among-site variation (σ_{site}^2) accounted 53.8% of unexplained variance in WBC, suggesting that a relevant amount of variation in WBC depend on local conditions not related to habitat.

At the opposite, PA significantly depended on habitat ($F_{2,93.1} = 14.22, P < 0.001$), being lower in urban compared to both sub-urban and natural habitats ($\beta_{suburban} = -1.80 \pm 0.35, t_{97.2} = 5.082, P < 0.001$; $\beta_{natural} = -1.40 \pm 0.35, t_{97.4} = 3.979, P < 0.001$, Fig. 3). Sex and SVL did not enter the final model ($P > 0.17$ at removal), neither the random effect was significant.

3.3. Fluctuating asymmetry and hematology

Linear mixed models did not reveal any association between FA and hematological variables for AI_{VS} and AI_{FP} , as none of the predictors entered the final models ($P > 0.10$ at removal). Only PA had a significant effect on AI_{OC} ($F_{1,94.0} = 2.453, P = 0.016$), and asymmetry increased with increasing parasite load ($\beta = 0.04 \pm 0.02$, Fig. 4a). Finally, the simplified model for AI_{HS} included only HL as a significant term ($F_{2,94.0} = 5.077, P = 0.027$), showing that asymmetry decreased with increasing values of the ratio ($\beta \times 10^{-3} = 9.8 \pm 4.4$, Fig. 4b).

4. Discussion

In the present study we replicated the analysis by Lazić et al. (2013, 2015) in order to compare once again the intensity of FA in morphological traits between urban and natural populations of three large cities in Northern Italy. In their studies carried out in the City of Niš

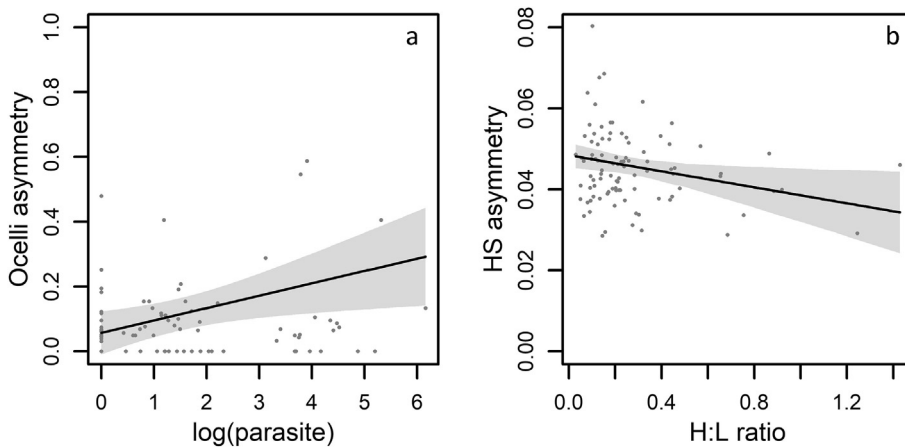


Fig. 4. Relationships between fluctuating asymmetry ([R-L]) and lizards' physiological condition: (a) Number of ocelli vs parasite load (Haemogregarins/10.000 erythrocytes), and (b) head shape vs heterophils-to-lymphocytes ratio.

(Serbia) Lazić et al. (2013, 2015) reached the conclusion that the environmental stress caused by urban environment causes increased FA. Despite the data clearly supported the authors' hypothesis, it cannot however be generalized, and bolster the use of FA as a reliable biomarker in this species. Indeed, the experimental design lacked both site replication (only one urban area was sampled) and independent assessment of the physiological conditions of the sampled lizards. Hence, the analyses did not have the power of disentangling the effects of the urban environment in itself, apart from the specific features of the city of Niš, and assumed *a priori* that common wall lizards living in urban habitat were in suboptimal habitat conditions compared to natural ones. Generalization is well apart to be a trivial issue, since the development of cheaper, trustworthy and easy to use biomarkers (such as morphological biomarkers) is of primary importance in monitoring the impacts of environmental stressors on biological systems (Beasley et al., 2013).

Our results confirmed the occurrence of FA in all the analyzed traits, and were totally consistent with the patterns observed in Lazić's studies, notably for head shape. Indeed, the patterns of shape variation that we obtained by our geometric morphometric analyses reproduced exactly those observed in Niš (compare Fig. S1 with Fig. 2 in Lazić et al., 2015). Therefore, we are confident that our indices of FA captured the same processes as in the study in the city of Niš, but with three independent replicates. Despite this, we found weak, if not at all, statistical association between FA and urban habitat. The asymmetries in ventral scales, ocelli and head shape were similar among urban, sub-urban, and natural habitats on the one side, whereas the sub-urban populations were less asymmetric than the others concerning the number of femoral pores (but no difference occurred between urban and natural populations) on the other one. Thus, our results suggested no significant effects of urban habitat on FA, counter to what observed by Lazić et al. (2013, 2015). We suspect that the higher levels of FA observed in urban compared to natural populations in the city of Niš probably reflected some unmeasured features specific to that city rather than a general effect of the urbanization in itself. In support to this issue, we found a significant random effect at site level (σ_{site}^2) in two out of four indices of FA, suggesting that part of the variability in FA in those two morphological traits correlates with one or more latent variables not directly observed in the study but still not related to the habitat.

The hematological assessment confirmed that lizards from urban populations were not in a worse physiological condition than natural ones, since no significant differences among habitats occurred for WBC and HL indexes, whereas PA resulted significantly lower in urban compared to both suburban and natural populations. So, the assumption that urban habitat consistently stresses out individuals, leading to homeostasis disruption and, ultimately, to individual fitness decline is far from being realistic (at least for this species). Further, urban habitat might offer more profitable conditions than natural ones, and several

studies on different lizards, including common wall lizard, actually support this issue. For example, lizards inhabiting human-dominated habitats may develop a greater confidence to human presence and gain benefits in terms of time and costs saved for other activities such as foraging or mating (Martín and López, 1999a,b, 2015; Diego-Rasilla, 2003; Pellitteri-Rosa et al., 2017). On the other hand, there is experimental evidence that animals inhabiting rural areas, with limited human presence, consider humans as a real predatory threat and consequently show much more caution (Berger et al., 2007; Mainini et al., 1993; Nowak et al., 2014; Recarte et al., 1998). Consequently, lizards in rural habitats spend more time hidden, and have significantly lower body mass than individuals in urban environments (Martín and López, 1999a,b). If we add that the presence of more effective predators such as birds, snakes or mammals is restricted, anthropic environments might rather represent more beneficial habitats for common wall lizards, as well as for many other lizards, compared to rural counterparts. Thus, it is not so surprising if we failed to detect any cue of physiological depletion in urban populations. Accordingly to morphological measures, we found a strong significant random effect on WBC variability, suggesting that more than 50% of WBC variance depend on some latent features associated to sites but not directly related with habitat. Therefore, the physiological condition of lizards differed among populations once again depending on one or more latent variables not directly measured in this study but still not related with the urban habitat.

All what has just been said leads us to argue that it is somewhat simplistic to say that urban environments stress lizards as well as FA is a reliable measure of this stress. The framework is much more complex, since i) there is probably not a single stressor which can be linked to the urban environments, and ii) a single stressor does not replicate over different urban environments consistently. For example, concentrations of heavy metals may be higher in urban areas, but it is not a distinctive trait of that habitat (contaminations may also occur in natural sites), as well as heavy metal composition changes site by site and mixtures of heavy metals may cause new – unpredictable – effects than single components. Thus urban habitat cannot be regarded as a proxy for the high levels of FA by simply arguing that in urban area contamination by heavy metal is higher. Furthermore, heavy metals do not disrupt homeostasis all in the same way or regardless of their concentrations. Therefore, there is no *a priori* reason to think that the heavy metal-induced disruption of homeostasis found in a city must be exactly replicated in other cities. That is, FA is not expected to work consistently over different cities as biomarker for heavy metal contamination. This reasoning can be equally translated to other potential stressors such as air pollutants, temperature and water availability or loss of genetic variation due to inbreeding (Lazić et al., 2013, 2015). None of those traits can be regarded as distinctive of the urban habitat, thus weakening the possibility of using FA to evaluate environmental disturbance.

A second source of concern is that the extent of FA does not

necessarily reflect the current level of stress, since it arises as a result of a developmental process including embryogenesis and post-hatching growth, which in the case of lizards may span over more than one year (sexual maturity in common wall lizard is achieved on average in the mid of second season, Barbault and Mou, 1988). Thus, even admitting that urban habitat may stress lizards, we cannot be confident that the occurrence of high levels of FA are actually recording the occurrence of a concomitant stressor. Indeed, without repeated measures over long period of time (e.g., one year) or in more than one site, we cannot disentangle the effect of the urbanization apart from the effects of punctuated events of stress, such as drought during spring, which have nothing to do with urban areas. Our data actually suggest that such a kind of problem cannot be completely excluded, as we failed to find a clear relationship between the extent of FA in the four morphological traits and the physiological condition of lizards. Consistently with the hypothesis that stressed individuals are more asymmetric, we found that parasite load increased the extent of FA in the number of ocelli, but contrary to that hypothesis, we found a negative relationship between FA in head shape and HL ratio. No relationship with FA in any trait was found for WBC. Therefore, the experimental support for the hypothesis that FA reflects a current status of stress in our case is weak.

There are two main points that might downplay the potential importance of FA in lizards' morphological traits as a valuable biomarker, which could explain the inconsistencies between our and Lazić's studies. First, FA may not be sensitive enough as a biomarker (Leung et al., 2003). The predictive power of FA to reliably signal an environmental stress has still not been assessed for lizards, but in birds it may be rather limited and several latent variables (either biotic and abiotic) are likely to produce the most variation of FA. In their review, Lens and Eggermont (2008) concluded that just 9% of the variation of FA over different birds' morphological traits was explained by environmental stress. Helle et al. (2011) found that the habitat fragmentation explained no more than 1.4% of the variance in FA of feather length in Eurasian tree-creeper (*Certhia familiaris*) males, and this percentage increased up to 4.9% when both sex and age were included in the explaining model. FA in lizard's morphology may suffer a similar lack of power as in birds, and consequently work as a biomarker only in the few cases in which the noise-to-signal ratio is particularly high. A second, and more important, reason is that the association of FA with individual fitness is currently still unclear in this and in other species of lizards, as well. Whenever the underlying mechanisms of developmental homeostasis are unknown, it is impossible to predict when, and to what extent, FA may be a reliable indicator of the underlying developmental instability (Lens and Eggermont, 2008), which is a key to detect the relationships of FA with the other variables of interest (Lens and Eggermont, 2008; Beasley et al., 2013). Moreover, FA may work as a sensitive biomarker unless environmental stressors have a negative effect on fitness-related traits (Beasley et al., 2013). Therefore, without having first clarified the developmental mechanisms, the true effector of the stress, their interactions and their effects on individual fitness, the comparison of FA on morphological characters between urban and natural habitats will go on without a solution.

5. Conclusion

This study highlights the importance of the experimental design when dealing with the evaluation of FA as a potential indicator of environmental disturbance in urban habitats. Site replication, a clear assessment of physiological condition of model species as well as an understanding (and quantitative measure) of the true effector of the stress should be explicitly included in the experimental design in order to evaluate if and when FA is a proxy for environmental disturbance in urban habitat.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecolind.2018.05.035>.

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