Zoology 147 (2021) 125928

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.com/locate/zool

# Fluctuating asymmetry as biomarker of pesticides exposure in the Italian wall lizards (*Podarcis siculus*)

Giulia Simbula [a,*], Leonardo Vignoli [a], Miguel A. Carretero [b,c], Antigoni Kaliontzopoulou [b]

[a] Dipartimento di Scienze, Università Roma Tre, Viale G. Marconi 446, Rome, 00146, Italy
[b] CIBIO – Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, Vairão, 4485-661, Portugal
[c] Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, R. Campo Alegre, s/n, Porto, 4169 - 007, Portugal

ARTICLE INFO

*Keywords:*
Agro-environments
Developmental instability
Femoral pores
Geometric morphometric
Head morphology

ABSTRACT

The extensive use of pesticides in agricultural environments produces drastic effects on wildlife, hence the need for less invasive indicators of environmental stress to monitoring the impact of agriculture treatments on biological systems. Fluctuating asymmetry (FA), as measure of developmental instability, has recently been proposed as reliable biomarker of populations stress due to environmental disturbance. We investigate femoral pores (FP) and dorsal head shape (HS) traits in populations of the Italian wall lizard inhabiting agricultural environments to examine whether different pesticide exposures (conventional, organic and control) can cause distinctive degree of FA. High-resolution photographs of FP and HS were taken in the field with a digital camera. The number of FP were counted twice on both sides and HS was analysed using geometric morphometrics with 25 landmarks and 12 semilandmarks. Individuals under conventional management showed higher levels of FA compared to control ones, and females exhibited higher FA levels than males for the FP. However, no significant difference was found for the HS trait. Our study provided evidence that FA may have a real potential as biomarker of population stress in wall lizards, highlighting the importance in the choice of the experimental design and the traits adopted for estimating DI.

## 1. Introduction

The intentional release of chemical contaminants in the environment by humans has greatly increased along with the intensification of agricultural practices in the past few decades, causing significant detrimental effects on different levels of biological organization (Pimm et al., 1995; Hughes et al., 1997; Böhm et al., 2013). A wide range of effects on organisms may result through indirect/direct exposure to pesticides via different routes i.e. dermal exposure, inhalation, ingestion of contaminated food (Amaral et al., 2012a) resulting, for instance, in shortened lifespan, reduced number of offspring, more mutations, or weight loss, among others (Stark and Banks, 2003). Moreover, subtle behavioural changes can also be induced by chemical exposure as a loss of sexual competitiveness or a reduced ability to capture prey (Stark et al., 2004). Such effects appear to be particularly intense during early life stages, which seem to be the most vulnerable (Kleinow et al., 1999; Stark et al., 2004). Indeed, chemical contaminants may affect developmental pathways of wildlife species more directly and quickly than any naturally environmental stressor, eventually leading to a decline of individual fitness (Hoffmann and Hercus, 2000; Graham et al., 2010). In this context, the use of biomarkers as sensitive indicators and early-warning signals of declines in environmental quality and population health may play a crucial role in determining the presence of a stressor and in identifying the degree of disturbance it provokes before irreversible damage has occurred (Beasley et al., 2013).

In this context, chemical biomarkers (i.e. AChE or cytochrome P450) are usually costly, time consuming and extremely invasive techniques that often require killing the animals (Miller, 2003). Conversely, fluctuating asymmetry (FA), a morphological measure of developmental instability (DI), defined as a random deviation from perfect symmetry in bilateral organisms (Palmer and Strobeck, 2003), has been suggested as a reliable, cheap, and less invasive indicator of the effects of stress on populations (Mirč et al., 2019). As the two sides of the body of bilaterally symmetric organisms are under the influence of the same genes and environmental conditions, deviations from perfect symmetry are considered to emerge due to imprecisions and perturbations of

* Corresponding author.
*E-mail addresses:* giulia.simbula@uniroma3.it (G. Simbula), leonardo.vignoli@uniroma3.it (L. Vignoli), carretero@cibio.up.pt (M.A. Carretero), antigoni@cibio.up.pt (A. Kaliontzopoulou).

https://doi.org/10.1016/j.zool.2021.125928
Received 25 November 2020; Received in revised form 5 February 2021; Accepted 14 April 2021
Available online 19 April 2021

developmental mechanisms as a result of various environmental stressors, potentially causing overall negative effects on population health over time (Parsons, 1990; Markow, 1995; Polak, 2003). FA as a biomarker has been examined in both laboratory experiments and natural populations of various invertebrate and vertebrate species (Sarre, 1996; Blanckenhorn et al., 1998; Allenbach et al., 1999; Eeva et al., 2000; Parris and Cornelius, 2004). FA has been associated with reductions in fitness caused by urbanization (Lazić et al., 2013, 2015), exposure to pollutants (Hardersen, 2000), extreme temperatures (Mpho et al., 2002), habitat fragmentation (Crnobrnja-Isailović et al., 2005), inbreeding (Gomendio et al., 2000), outbreeding (Kurbalija et al., 2010), and hybridization (Dosselman et al., 1998).

Among reptiles, lacertid lizards are the most used model organism to estimate FA as a proxy of developmental disturbance in relation to a variety of environmental conditions as island isolation in *Podarcis siculus*, *P. bocagei*, *P. hispanicus* and *P. lilfordi* (Vervust et al., 2008; Băncilă et al., 2010; Garrido and Pérez-Mellado, 2014), agricultural pollution in *P. bocagei* (Amaral et al., 2012a); and urbanization in *P. muralis* (Lazić et al., 2013, 2015; Urošević et al., 2015). Indeed, lizards present a large set of measurable (i.e. length of limbs, femurs, head) or countable (i.e. meristic features such as scales, femoral, and anal pores) bilateral traits that can be recorded easily in the field (Laia et al., 2015). Different studies, however, obtained antithetical results across species and environmental stressors, questioning the utility of FA as a reliable biomarker (Clarke, 1995, 1998; Valkama and Kozlov, 2001; Dauwe et al., 2006; Sacchi et al., 2018). Indeed, the relationships between FA and environmental features could be feeble, not detectable due to small sample size, underestimated if only one trait is considered (Lens et al., 2002; Crnobrnja-Isailović et al., 2005), or masked by premature individual decease under high stress levels before adulthood (Polak, 2003). Despite these contradictory results, a recent meta-analysis (Beasley et al., 2013) advises the use of FA as a biomarker in conservation biology provided that (i) highly sensitive FA measurement tools are used (i.e. geometric morphometrics), (ii) the environmental stressor acting on the organism is known.

Agricultural areas have nowadays become one of the major terrestrial ecosystems on Earth that cause extensive environmental damage due to the periodic and chronic disturbances of agricultural managements (Foley et al., 2005). Lacertid lizards are among the most widespread vertebrates in Mediterranean agricultural areas (Biaggini et al., 2006; Amaral et al., 2012a,b; Bicho et al., 2013). Consequently they are subjected and exposed to different anthropogenic disturbances, as pesticide application, that have been proved to provoke serious damages on a wide range of organisms and populations (Eeva et al., 2000; Marques et al., 2005; Voets et al., 2006). Although environmental contaminants are among the major identified threats for reptiles and considered among the important causes of their decline, very little is still known about the type and severity of their effects on these non-target organisms. The main goal of this study was to examine the effect of agriculture management (i.e. exposure to chemicals) on *Podarcis siculus* (Rafinesque-Schmaltz, 1810) population health through the estimation of FA using two morphological traits (i.e. number of femoral pores and dorsal head shape). We hypothesized that (i) lizards exposed to any treatment (those from conventional and organic agricultural fields) show a higher level of FA in respect to lizards from control fields; (ii) lizards from conventional treatment show a higher degree of developmental instability than those from organic treatment (Coda et al., 2016).

## 2. Materials and Methods

### 2.1. Study species and area

We used the Italian wall lizard, *Podarcis siculus*, as model species, with populations living in three different agricultural polluted systems (see below). This and other wall lizards have already proven to be good models for field ecotoxicological studies (Amaral et al., 2012b,c,d; Bicho et al., 2013; Mingo et al., 2017) since they are widespread in agricultural habitats (Bologna et al., 2000; Maura et al., 2011), show strong site fidelity, and have small to medium home ranges. In spite of the numerous reports of *P. siculus* ubiquitous presence, very little is known about the ecology of the Italian Wall Lizard in agro-environments (Biaggini and Corti, 2017). Adult individuals of *P. siculus* were collected by hand or noosing (Simbula et al., 2019) between May and July 2019 in hazelnut orchards in central Italy (Rome and Viterbo province, Latium; mean altitude 500 m a.s.l.). Viterbo province has been extensively used for hazelnut management activities (about 17,000 hectares used in the whole area, Carbone et al., 2004) for more than 50 years and it has been regularly treated with pesticides (mainly fungicides and insecticides) in order to control pests throughout the years (Carbone et al., 2004; Fabi and Varvaro, 2010; Roversi, 2016). In such an environment, the Italian wall lizard utilizes the hazelnut roots, trunks, and low branches as the main habitat elements (e.g. for thermoregulation, foraging, as hiding place, and even as oviposition site; pers. obs.).

### 2.2. Data collection

Overall, we sampled 15 lizard populations living in hazelnut plantations with similar abiotic factors (i.e. geology, soil types and climate), but exposed to different types of treatments. Five populations were captured from conventional management areas where different pesticides have been applied regularly from May to July; five from organic fields where copper sulphate is used; and five from pesticide-free fields, with no history of chemical application for at least 10 years, used as control (Fig. 1). All the areas were separated by at least 2 km from each other, surrounded by bushes, other fields, or rural tracks. The current and historical land and pesticide usage were obtained through consultation with the landowners. In the conventional sites, a combination of fungicides and insecticides is repeatedly applied once every two/three weeks (from 3 to a maximum of 6 applications) (Table S1). After captured, lizards were sexed using secondary sexual characters as head size and femoral pores (Arnold and Ovenden, 2002), and measured for snout-vent length (SVL) with a calliper (precision 0.01 mm). We selected as traits the femoral pores (FP) and dorsal head shape (HS) because they are easy to identify and count, and they have been demonstrated to display FA in wall lizards and to be sensitive to different levels of environmental stress (Lazić et al., 2013, 2015). High-resolution photographs of FP and HS were taken in the field with a digital camera (Canon SX620 HS, automatic configuration with ISO rank from 160-500, diaphragm aperture f/9, f/11, f/13 and shutter speed of 1/60, 1/80 sec.) fixed on a tripod at a distance of 15 cm from the subject which was unable to move. Animals were marked permanently by toe-clipping (Perry et al., 2011) and immediately released in the collection site. No specimen was killed during this study.

For each individual, the number of FP were counted twice on both

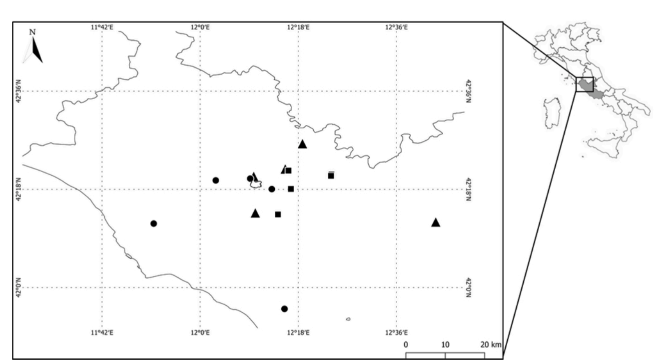


**Fig. 1.** Location of the sampling sites in central Italy, in the region of Lazio (highlighted in grey). Conventional fields are shown by squares, the organic fields by triangles, and control fields by circles.

sides of the body from digital photos by the same person (GS), allowing at least one week between counting sessions to guarantee the independence of trait counts (Fig. 2A). HS was analysed using geometric morphometrics (GM): 25 landmarks and 12 semilandmarks were digitized using TpsDig2 (Rohlf, 2005; "http://life.bio.sunysb.edu/morph) (Fig. 2B). Landmark recording was repeated twice for each animal a week apart to assess digitizing error. Specimens in which the landmarks could not be appropriately identified were not considered.

### 2.3. Statistical analysis

Preliminary analyses were performed for FP (i) to test total body size and trait size dependence, (ii) to evaluate the presence of directional asymmetry (DA) or fluctuating asymmetry (FA) by a two-way ANOVA on log-transformed (to achieve normality) trait values with side as a fixed factor, individual ID as a random factor, and their interaction as an additional term. We analysed trait size dependence by regressing absolute values on the right side of the body minus that on the left side (R-L) on SVL to test whether bigger individuals were less asymmetric due a lower measure error, and on $(R + L)/2$ to test dependence on trait size, which can yield leptokurtosis in the frequency distributions of R - L or obscure subtle antisymmetry (Palmer and Strobeck, 2003). The presence of DA is identified by a significant main effect of side, while a significant interaction of side-individual indicates the occurrence of FA (Palmer and Strobeck, 1986; Palmer, 1994). Since fluctuating asymmetry was found in all populations for FP (see Results), an individual asymmetry index ($AI_{FP}$) was estimated following Palmer and Strobeck (2003), as the absolute R-L difference between the log-transformed average of trait values across the two replicate counts: $|\ln(R\ average) - \ln(L\ average)|$.

As for the previous trait, we assessed the presence of directional and/or fluctuating asymmetry while accounting for measurement error for the head landmark configuration, applying a Procrustes ANOVA on replicate measurements following Klingenberg et al. (2002), as implemented in the *bilat.symmetry* function of R-package geomorph (Adams et al., 2020), to evaluate the same effects as described above for FP (i.e. individual, side and their interaction). This analysis was performed on each population separately. Furthermore, an individual asymmetry index for HS expressed as sums of squared differences between the original and reflected copies of all bilateral landmark coordinates was obtained following Lazić et al. (2015). This index represents the Procrustes distance between the right and left sides of the head of each individual.

Differences in the degree of absolute FA among treatments were tested for FP through a nested ANOVA, and for HS with permutational ANOVA implemented in the R-package RRPP (Collyer and Adams, 2018), considering treatment, population (nested in treatment) and sex, as well as the interaction between the main factors as the independent variables.

All tests were performed using Statistica (v8.0; Statsoft) or R, version 3.6.3 (R Core Team, 2020).

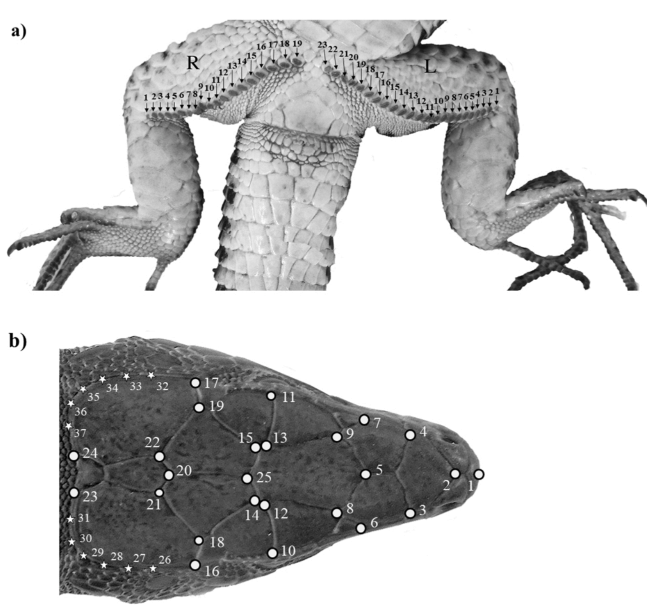


**Fig. 2.** Studied traits in *Podarcis siculus*. A) Number of femoral pores in left (L) and right (R) sides; B) landmarks (circles) and semilandmarks (stars) used to quantify dorsal head shape.

## 3. Results

A total of 293 individuals were captured and investigated, except for two specimens which were not considered for the HS analyses. The operative sex ratio (males:females) was on average 1.37 (conventional: 1.21; organic: 1.60; control: 1.31) (Table TS1). No body size or trait size dependence for the FP were revealed with the linear regression of absolute R-L values on SVL ($F_{1,307} = 0.81$, $p = 0.37$) and on $(R + L)/2$ ($F_{1,307} = 0.71$, $p = 0.40$). ANOVA comparisons taking into account individual variation and measurement error to identify asymmetry patterns in FP, indicated that the side effect was not significant, whereas the interaction term was, thus discarding DA and revealing the existence of FA in this trait in all examined populations (Table S2). The Procrustes ANOVAs performed on each population for HS (Table S3) revealed statistically significant variation due to both DA and FA (i.e. both the "side" and "side-individual interaction" terms were significant). Significant differences in the degree of absolute FA were identified for FP but not for HS (Table 1). FP presented statistically significant differences among treatments and between sexes (Table 1). Populations from conventional fields exhibited higher degrees of FA compared to populations from control fields (Post hoc Fisher's test, $p = 0.02$), whereas populations from organic fields did not differ from the remaining ($p \geq 0.61$). Females showed a higher degree of FA than males (Table 1, Fig. 3).

## 4. Discussion

Our research contributed to implement the use of FA in the study of pesticide stress. To date, just few studies examined FA patterns in lizards inhabiting agriculture polluted systems and they did not find any significant pattern. However, these works suffered of various drawbacks, for example, very low number of examined populations (i.e. absence or reduced number of control ones, Amaral et al., 2012a). By contrast, our study was robust as for the experimental design (presence of control fields) and the number of considered units (relatively high number of populations evenly distributed among treatments). As predicted, we observed a discrepancy between the considered traits as for the estimated FA among the three experimental conditions, with only femoral

**Table 1**
ANOVA on absolute asymmetry index for **(a)** femoral pores (FP) and **(b)** head dorsal scales (HS). Significant differences are highlighted in bold.

| | df | SS | F | p |
|---|---|---|---|---|
| a)*FP* | | | | |
| Intercept | 1 | 1.00 | 448.71 | <0.001 |
| **Treatment** | 2 | 0.016 | 3.65 | **0.027** |
| Population(Treatment) | 12 | 0.042 | 1.56 | 0.104 |
| **Sex** | 1 | 0.019 | 8.91 | **0.003** |
| Treatment*Sex | 2 | 0.011 | 2.55 | 0.079 |
| Error | 26 | 0.585 | | |
| b)*HS* | | | | |
| Treatment | 2 | <0.001 | 0.71 | 0.515 |
| Population(Treatment) | 12 | <0.001 | 0.59 | 0.785 |
| Sex | 1 | <0.001 | 1.00 | 0.500 |
| Treatment*Sex | 2 | <0.001 | 1.29 | 0.293 |
| Residuals | 273 | 0.018 | | |
| Total | 290 | 0.019 | | |

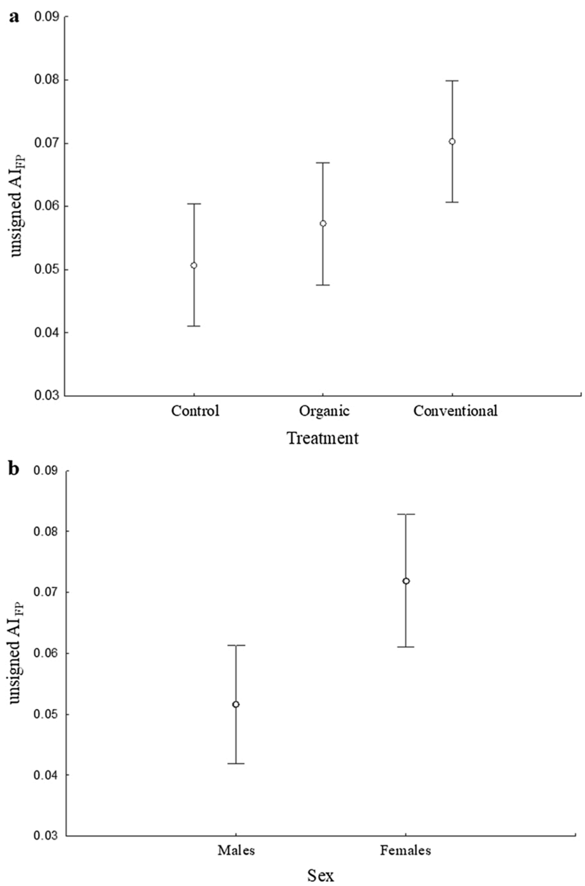


**Fig. 3.** Comparison among conventional, organic and control treatments **(A)** and between sex **(B)** of mean of absolute individual asymmetry index ($AI_{FP}$) for femoral pores. Vertical bars denote 0.95 confidence intervals.

pores (FP) highlighting distinct patterns of FA between conventional and control fields. However, contrary to our expectation, no difference was found between conventional and organic fields, indicating that any treatment affects lizards in some way.

### *4.1. Fluctuating Asymmetry in femoral pores and head shape*

When considering femoral pores (FP), since no body or trait size dependence were showed, the possible inferred differences in DI could not be either enhanced or obscured by size-dependent variability (Palmer and Strobeck, 1986). Furthermore, increased levels of FA were observed in lizard populations living in conventional fields compared to control ones. According to other studies, the number of femoral pores seems to be an appropriate measure for FA analyses in lizards subjected to environmental stressors (Lazić et al., 2013; Mirč et al., 2019). It is long recognised that toxic chemicals can accumulate in adult females inhabiting polluted areas and transferred to their eggs (Marco et al., 2004a). Moreover, in species as lizards with permeable eggshells, pollutants can also be absorbed either from the soil in which the eggs develop, or through gas and water exchange with the environment (Marco et al., 2004b). Thus, in our study, it is likely that FA may arise via maternal pesticide burden and/or via soil absorption of agrochemicals. Although it seems the most feasible hypothesis, alternative explanations should be considered as genetic stressors or temperature and water availability, which are other major sources of disturbance of development in ectotherms. Inbreeding and habitat fragmentation often have been associated to high levels of FA (Vervust et al., 2008). Although it would be impossible to assess such an effect without data on genetics, based on our observations, we can state that *P. siculus* is widespread in the study area and hazelnut plantations are relatively large patches, usually connected one to another by small unpaved roads bordered with rows of shrubs and trees, thus ensuring inter-patch connections and gene flow among the studied populations. Therefore, the observed differences in FA across the three considered managements could be more likely associated to the use of chemicals, rather than being the result of inbreeding via habitat fragmentation. In addition, high levels of FA and a decrease of fitness were observed in lizards hatched from eggs incubated at extreme temperatures (Van Damme et al., 1992; Ji et al., 2002). It has also been shown that decreased water absorption during incubation reduces hatchling survival and fitness (Marco et al., 2004a). Such hypotheses are unlikely to explain the patterns observed here, because that would imply that lizards and their eggs would have experienced different abiotic conditions among areas with different management conditions, whereas all the considered fields presented similar microclimate, soil, and vegetation characteristics.

An unexpected and interesting pattern emerged from the analysis of the variation in $AI_{FP}$ across individuals, with females exhibiting higher AI levels than males (Fig. 3b). This is remarkable and begs an explanation, as previous studies on FA failed to find a relationship with sex in lizards (Crnobrnja-Isailović et al., 2005; Lazić et al., 2013; Mirč et al., 2019). Recently, it has been found how environmental stresses (summer drought and anthropization) have stronger repercussions on females than on males in *Gallotia galloti*, evidenced as differences in reproductive cycle and investment (Megía-Palma et al., 2020). However, since FA in meristic traits depends on the conditions during the development of embryos, and not during juvenile or adult growth (Carretero, 2001), the stress of females due to mating, vitellogenesis and egg laying could be reasonably discarded as an explanation for the observed sexual difference in FA levels. On the other hand, individuals affected by developmental instability should be negatively selected for their lower body condition rather than for the femoral pore asymmetry per se, even if some studies recovered a relationship among male pore symmetry, chemical cues and mate choice by females (López and Martín, 2000). Further comparisons of FA levels in juveniles will be necessary to disentangle such an issue by testing the hypothesis of higher mortality rate in juvenile asymmetric males in respect to juvenile females that could explain the observed pattern. However, this hypothesis is not supported by data and should assume a biased sex ratio at birth towards males. Indeed, the observed male biased operative sex ratio makes such a hypothesis more unlikely since sex ratio at birth considering all other factors being equal, should be even more biased towards males to balance their hypothesized higher mortality rate for asymmetric juveniles.

Regarding head shape (HS), although FA exists in all populations, we did not find coherent variation in $AI_{HS}$ in our system, in contrast with other studies on lacertids (Băncilă et al., 2010; Lazić et al., 2015, 2016). Different explanations have been summoned in this regard. First, not all toxins may affect HS fluctuating asymmetry. For example, the applications of sodium pentachlorophenate on zebrafish, sufficiently severe to suppress cranial growth, did not produce increased fluctuating asymmetry of shape (López-Romero et al., 2012). Therefore, we could hypothesize that the pesticides applied in our system may not represent the main cause of head shape FA as heavy metals and other toxins found in urban populations (Lazić et al., 2015). Second, the level of head shape FA may vary through lizard size and age in unpredictable ways across species (Lazić et al., 2016, 2017). Moreover, head shape FA was found to be higher early after hatching and then be reduced through either actively or passively mechanisms of compensatory growth only in groups developing under stressful conditions (Lazić et al., 2016, 2017). Further integrated studies are in necessary to establish whether agricultural chemicals may have a direct effect on this trait.

Many studies used FA with multiple traits to quantify organism-wide developmental instability (DI) as a measure of individual quality or exposure to stress. If FA reflects organism-wide DI, a similar response to the same external stresses should be expected in terms of absolute FA of distinct developmentally unrelated traits (Clarke, 1998; Polak, 2003).

Although FP and HS have both an important biological significance, they are measured in very different ways. On one side, FP are limited in their variation but can be assessed with a very small margin of error, thus yielding accurate measures. By contrast, HS quantified through GM can be very efficient at identifying very fine-scale signals but, at the same time, they can be easily confounded by background noise and other biological sources of variation. Therefore, the response to a stress, measure as FA degree, should be considered as trait specific. Different explanations have been proposed: (i) there could be a weak association between a single expression of fluctuating asymmetry and developmental instability; (ii) even if all asymmetry measures are related to DI, distinct traits may have different buffering capacities at different developmental stages, varying in susceptibility to environmental stressor (due to heterogeneity in biochemical pathways in development), dissimilar ontogenetic patterns and timing of asymmetry development (Swaddle, 2003). (iii) Traits may present different functional significance resulting in divergent degree of FA, where characters with a high functional importance show lower FA (Palmer and Strobeck, 1986; Clarke, 1998). All these hypotheses suggest that the estimate of asymmetry in one trait may not reveal the same information in other independent traits. In our case, FP seem a reliable and useful indicator of FA associated to pesticides exposure.

In conclusion, our research emphasizes the importance of taking particular care in the choice of the experimental design and the traits adopted when attempting in estimating DI. Different chemicals and multiple traits cross-examination must be taken into consideration with both laboratory and field experiments to better disentangle how pesticide application may induce high levels of DI in lizards in agricultural environments. From a conservation point of view, our study provides important insights on the management of wall lizards in agro-environments. Indeed, until now, few studies proved agricultural land to be a major factor related to reptile's diversity deficit (Anadón et al., 2007; Wisler et al., 2008; Ribeiro et al., 2009). Considering the increasingly expansion of agricultural land use due to persistent higher market demand and competition (e.g. hazelnut), we would expect a demographic reduction of lizard, and in general, no-target organisms. Further studies should focus whether a balance between agriculture and conservation could be achieved through more sustainable agricultural practices maintaining semi-natural shelter habitats (Billeter et al., 2008).

## Declaration of Competing Interest

The authors report no declarations of interest.

## Acknowledgement

This research was authorized by the Ministry for Environment, Land and Sea Protection of Italy (MATTM) (Prot. 0013659/MATTM). We sincerely thank Anna Del Fabbro and Emanuele Berrilli who helped us in the field. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.zool.2021.125928.

## References

Adams, D.C., Collyer, M.L., Kaliontzopoulou, A., 2020. Geomorph: Software for geometric morphometric analyses. R package version 3.3.1. https://cran.r-project.org/package=geomorph.

Allenbach, D.M., Sullivan, K.B., Lydy, M.J., 1999. Higher fluctuating asymmetry as a measure of susceptibility to pesticides in fishes. Environ. Toxicol. Chem.: An International Journal 18 (5), 899–905. https://doi.org/10.1002/etc.5620180512.

Amaral, M.J., Carretero, M.A., Bicho, R.C., Soares, A.M.V.M., Mann, R.M., 2012a. The use of a lacertid lizard as a model for reptile ecotoxicology studies-Part 1 Field demographics and morphology. Chemosphere 87 (7), 757–764. https://doi.org/10.1016/j.chemosphere.2011.12.075.

Amaral, M.J., Bicho, R.C., Carretero, M.A., Sanchez-Hernandez, J.C., Faustino, A.M.R., Soares, A.M.V.M., Mann, R.M., 2012b. The use of a lacertid lizard as a model for reptile ecotoxicology studies: Part 2–Biomarkers of exposure and toxicity among pesticide exposed lizards. Chemosphere 87 (7), 765–774. https://doi.org/10.1016/j.chemosphere.2012.01.048.

Amaral, M.J., Sanchez-Hernandez, J.C., Bicho, R.C., Carretero, M.A., Valente, R., Faustino, A.M., Faustino, A.M.M.R., Mann, R.M., 2012c. Biomarkers of exposure and effect in a lacertid lizard (*Podarcis bocagei* Seoane) exposed to chlorpyrifos. Environ. Toxicol. Chem. 31 (10), 2345–2353. https://doi.org/10.1002/etc.1955.

Amaral, M.J., Bicho, R.C., Carretero, M.A., Sanchez-Hernandez, J.C., Faustino, A.M.R., Soares, A.M.V.M., Mann, R.M., 2012d. The usefulness of mesocosms for ecotoxicity testing with lacertid lizards. Acta Herpetol. 7 (2), 263–280.

Anadón, J.D., Giménez, A., Martínez, M., Palazón, J.A., Esteve, M.A., 2007. Assessing changes in habitat quality due to land use changes in the spur-thighed tortoise *Testudo graeca* using hierarchical predictive habitat models. Divers. Distrib. 13 (3), 324–331. https://doi.org/10.1111/j.1472-4642.2007.00343.x.

Arnold, E.N., Ovenden, D., 2002. A Field Guide to the Reptiles and Amphibians of Britain and Europe. Collins, London.

Băncilă, R., Van Gelder, I., Rotteveel, E., Loman, J., Arntzen, J.W., 2010. Fluctuating asymmetry is a function of population isolation in island lizards. J. Zool. 282, 266–275. https://doi.org/10.1111/j.1469-7998.2010.00736.x.

Beasley, D.E., Bonisoli-Alquati, A., Mousseau, T.A., 2013. The use of fluctuating asymmetry as a measure of environmentally induced developmental instability: a meta-analysis. Ecol. Indic. 30, 218–226. https://doi.org/10.1016/j.ecolind.2013.02.024.

Biaggini, M., Corti, C., 2017. Variability of breeding resource partitioning in a lacertid lizard at field scale. Anim. Biol. 67 (2), 81–92. https://doi.org/10.1163/15707563-00002523.

Biaggini, M., Corti, C., Paggetti, E., Dapporto, L., 2006. Distribution of lacertid lizards in a Tuscan agro-ecosystem (central Italy). In: Corti, C., Lo Cascio, P., Biaggini, M. (Eds.), Mainland and Insular Lacertid Lizards: a Mediterranean Perspective. Firenze University Press, Florence, pp. 13–21. https://doi.org/10.1400/73920 (2006).

Bicho, R.C., Amaral, M.J., Faustino, A.M.R., Power, D.M., Rêma, A., Carretero, M.A., Soares, A.M.V.M., Mann, R.M., 2013. Thyroid disruption in the lizard *Podarcis bocagei* exposed to a mixture of herbicides: a field study. Ecotoxicology 22, 156–165. https://doi.org/10.1007/s10646-012-1012-2 (2013).

Billeter, R., Liira, J., Bailey, D., Bugter, R., Arens, P., Augenstein, I., et al., 2008. Indicators for biodiversity in agricultural landscapes: a pan-European study. J. Appl. Ecol. 45 (1), 141–150. https://doi.org/10.1111/j.1365-2664.2007.01393.x.

Blanckenhorn, W.U., Reusch, T., Mühlhäuser, C., 1998. Fluctuating asymmetry, body size and sexual selection in the dung fly *Sepsis cynipsea*—testing the good genes assumptions and predictions. J. Evol. Biol. 11 (6), 735–753. https://doi.org/10.1046/j.1420-9101.1998.11060735.x.

Böhm, M., Collen, B., Baillie, J.E., Bowles, P., Chanson, J., et al., 2013. The conservation status of the world's reptiles. Biol. Con. 157, 372–385. https://doi.org/10.1016/j.biocon.2012.07.015.

Bologna, M.A., Capula, M., Carpaneto, G.M., 2000. Anfibi e Rettili del Lazio. Fratelli Palombi Editori, Roma.

Carbone, A., Franco, S., Pancino, B., Senni, S., 2004. Dinamiche territoriali e profili produttivi dell'Agricoltura del Lazio. Quaderni di Informazione Socioeconomica 11, 149.

Carretero, M.A., 2001. Using femoralia for testing fluctuating asymmetry in Lacertidae. Biota 2 (Suppl). Abstracts 11th OGM SEH, Zalec, Slovenia, July 13-17 2001: 15.

Clarke, G.M., 1995. Relationships between fluctuating asymmetry and fitness: how good is the evidence? Pac. Conserv. Biol. 2 (2), 146–149. https://doi.org/10.1071/PC960146.

Clarke, G.M., 1998. Developmental stability and fitness: the evidence is not quite so clear. Am. Nat. 152 (5), 762–766. https://doi.org/10.1086/286207.

Coda, J., Gomez, D., Martínez, J.J., Steinmann, A., Priotto, J., 2016. The use of fluctuating asymmetry as a measure of farming practice effects in rodents: A species-specific response. Ecol. Indic. 70, 269–275. https://doi.org/10.1016/j.ecolind.2016.06.018.

Collyer, M.L., Adams, D.C., 2018. RRPP: An r package for fitting linear models to high-dimensional data using residual randomization. Methods Ecol. Evol. 9 (7), 1772–1779. https://doi.org/10.1111/2041-210X.13029.

Crnobrnja-Isailović, J., Aleksic, I., Bejakovic, D., 2005. Fluctuating asymmetry in *Podarcis muralis* populations from Southern Montenegro: detection of environmental stress in insular populations. Amphibian-Rept. 26 (2), 149–158. https://doi.org/10.1163/1568538054253500.

Dauwe, T., Janssens, E., Eens, M., 2006. Effects of heavy metal exposure on the condition and health of adult great tits (*Parus major*). Environ. Pollut. 140 (1), 71–78. https://doi.org/10.1016/j.envpol.2005.06.024.

Dosselman, D.J., Schaalje, G.B., Sites Jr., J.W., 1998. An analysis of fluctuating asymmetry in a hybrid zone between two chromosome races of the *Sceloporusgrammicus complex* (Squamata: Phrynosomatidae) in central Mexico. Herpetologica 434–447. https://www.jstor.org/stable/3893437.

Eeva, T., Tanhuanpää, S., Råbergh, C., Airaksinen, S., Nikinmaa, M., Lehikoinen, E., 2000. Biomarkers and fluctuating asymmetry as indicators of pollution-induced stress in two hole-nesting passerines. Funct. Ecol. 14 (2), 235–243. https://doi.org/10.1046/j.1365-2435.2000.00406.x.

Fabi, A., Varvaro, L., 2010. La Moria del nocciolo nel Viterbese: procedure di indagine epidemiologica mediante tecniche GIS. Corylus Co 2, 23–30.

Foley, J.A., DeFries, R., Asner, G.P., Barford, C., Bonan, G., Carpenter, S.R., et al., 2005. Global consequences of land use. Science 309 (5734), 570–574. https://doi.org/10.1126/science.1111772.

Garrido, M., Pérez-Mellado, V., 2014. Assessing factors involved in determining fluctuating asymmetry in four insular populations of the Balearic lizard *Podarcis lilfordi*. Salamandra 50, 147–154.

Gomendio, M., Cassinello, J., Roldan, E.R.S., 2000. A comparative study of ejaculate traits in three endangered ungulates with different levels of inbreeding: fluctuating asymmetry as an indicator of reproductive and genetic stress. Proc. Royal Soc. B. Biol. Sci. 267 (1446), 875–882. https://doi.org/10.1098/rspb.2000.1084.

Graham, J.H., Raz, S., Hel-Or, H., Nevo, E., 2010. Fluctuating asymmetry: methods, theory, and applications. Symmetry 2 (2), 466–540. https://doi.org/10.3390/sym2020466.

Hardersen, S., 2000. The role of behavioural ecology of damselflies in the use of fluctuating asymmetry as a bioindicator of water pollution. Ecol. Entomol. 25 (1), 45–53. https://doi.org/10.1046/j.1365-2311.2000.00204.x.

Hoffmann, A.A., Hercus, M.J., 2000. Environmental stress as an evolutionary force. Bioscience 50 (3), 217–226. https://doi.org/10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2.

Hughes, J.B., Daily, G.C., Ehrlich, P.R., 1997. Population diversity: its extent and extinction. Science 278 (5338), 689–692. https://doi.org/10.1126/science.278.5338.689.

Ji, X., Qiu, Q.B., Diong, C.H., 2002. Influence of incubation temperature on hatching success, energy expenditure for embryonic development, and size and morphology of hatchlings in the oriental garden lizard, *Calotes versicolor* (Agamidae). J. Exp. Zool. 292 (7), 649–659. https://doi.org/10.1002/jez.10101.

Kleinow, K., Baker, J., Nichols, J., Gobas, F., Parkerton, T., Muir, D., Monteverdi, G., Mastrodone, P., 1999. Exposure, uptake, and disposition of chemicals in reproductive and developmental stages of selected oviparous vertebrates. Reproductive and developmental effects of contaminants in oviparous vertebrates, pp. 9–111.

Klingenberg, C.P., Barluenga, M., Meyer, A., 2002. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. Evolution 56 (10), 1909–1920. https://doi.org/10.1111/j.0014-3820.2002.tb00117.x.

Kurbalija, Z., Stamenkovic-Radak, M., Pertoldi, C., Andjelkovic, M., 2010. Outbreeding causes developmental instability in *Drosophila subobscura*. Evol. Ecol. 24 (4), 839–864. https://doi.org/10.1007/s10682-009-9342-0.

Laia, R.C., Pinto, M.P., Menezes, V.A., Rocha, C.F.D., 2015. Asymmetry in reptiles: What do we know so far? Springer Sci. Rev. 3 (1), 13–26. https://doi.org/10.1007/s40362-014-0028-9.

Lazić, M.M., Kaliontzopoulou, A., Carretero, M.A., Crnobrnja-Isailović, J., 2013. Lizards from urban areas are more asymmetric: using fluctuating asymmetry to evaluate environmental disturbance. PloS one 8 (12), e84190. https://doi.org/10.1371/journal.pone.0084190.

Lazić, M.M., Carretero, M.A., Crnobrnja-Isailović, J., Kaliontzopoulou, A., 2015. Effects of environmental disturbance on phenotypic variation: an integrated assessment of canalization, developmental stability, modularity, and allometry in lizard head shape. Amer. Nat. 185 (1), 44–58. https://doi.org/10.1086/679011.

Lazić, M.M., Carretero, M.A., Crnobrnja-Isailović, J., Kaliontzopoulou, A., 2016. Postnatal dynamics of developmental stability and canalization of lizard head shape under different environmental conditions. Evol. Biol. 43 (3), 368–379. https://doi.org/10.1007/s11692-016-9377-4.

Lazić, M.M., Rödder, D., Kaliontzopoulou, A., 2017. The ontogeny of developmental buffering in lizard head shape. Evol. Dev. 19 (6), 244–252. https://doi.org/10.1111/ede.12238.

Lens, L., Van Dongen, S., Matthysen, E., 2002. Fluctuating asymmetry as an early warning system in the critically endangered taita thrush. Conserv. Biol. 16, 479–487. https://doi.org/10.1046/j.1523-1739.2002.00516.x.

López, P., Martín, J., 2000. Chemoreception, symmetry and mate choice in lizards. Proc. Royal Soc. B. Biol. Sci. 267 (1450), 1265–1269. https://doi.org/10.1098/rspb.2000.1137.

López-Romero, F., Zúñiga, G., Martínez-Jerónimo, F., 2012. Asymmetric patterns in the cranial skeleton of zebrafish (*Danio rerio*) exposed to sodium pentachlorophenate at different embryonic developmental stages. Ecotoxicol. Environ. Saf. 84, 25–31. https://doi.org/10.1016/j.ecoenv.2012.06.008.

Marco, A., Hidalgo-Vila, J., Díaz-Paniagua, C., 2004a. Toxic effects of ammonium nitrate fertilizer on flexible-shelled lizard eggs. Bull. Environ. Contam. Toxicol. 73 (1), 125–131. https://doi.org/10.1007/s00128-004-0403-3.

Marco, A., López-Vicente, M., Pérez-Mellado, V., 2004b. Arsenic uptake by reptile flexible-shelled eggs from contaminated nest substrates and toxic effect on embryos. Bull. Environ. Contam. Toxicol. 72 (5), 983–990.

Markow, T.A., 1995. Evolutionary ecology and developmental instability. Annu. Rev. Entomol. 40 (1), 105–120. https://doi.org/10.1146/annurev.en.40.010195.000541.

Marques, J.F., Costa, J.L., Cabral, H.N., 2005. Variation in bilateral asymmetry of the Lusitanian toadfish along the Portuguese coast. J. App. Ichthyol. 21 (3), 205–209. https://doi.org/10.1111/j.1439-0426.2005.00627.x.

Maura, M., Vignoli, L., Bologna, M.A., Rugiero, L., Luiselli, L., 2011. Population density of syntopic, differently sized lizards in three fragmented woodlands from Mediterranean Central Italy. Community Ecol. 12 (2), 249–258. https://doi.org/10.1556/ComEc.12.2011.2.14.

Megía-Palma, R., Arregui, L., Pozo, I., Žagar, A., Serén, N., Carretero, M.A., Merino, S., 2020. Geographic patterns of stress in insular lizards reveal anthropogenic and climatic signatures. Sci. Total Environ. 749, 141655 https://doi.org/10.1016/j.scitotenv.2020.141655.

Miller, K.A., 2003. Cytochrome P450 1A as a biomarker of contaminant exposure in free-ranging marine mammals. Doctoral dissertation. University of British Columbia. https://doi.org/10.14288/1.0090772.

Mingo, V., Lötters, S., Wagner, N., 2017. The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles—a field study. Sci. Total Environ. 590, 114–124. https://doi.org/10.1016/j.scitotenv.2017.02.178.

Mirč, M., Kolarov, N.T., Stamenković, S., Vukov, T.D., 2019. Asymmetry in the common wall lizard *Podarcis muralis* under different levels of urbanization: the effect of trait and FA index selection. Arch. Biol. Sci. 71 (3), 501–508. https://doi.org/10.2298/ABS190225033M.

Mpho, M., Callaghan, A., Holloway, G.J., 2002. Temperature and genotypic effects on life history and fluctuating asymmetry in a field strain of *Culex pipiens*. Heredity 88 (4), 307–312. https://doi.org/10.1038/sj.hdy.6800045.

Palmer, A.R., 1994. Fluctuating asymmetry analyses: a primer. Developmental Instability: Its Origins and Evolutionary Implications. Springer, Dordrecht, pp. 335–364. https://doi.org/10.1007/978-94-011-0830-0_26.

Palmer, A.R., Strobeck, C., 1986. Fluctuating asymmetry: measurement, analysis, patterns. Annu. Rev. Ecol. Evol. Syst. 391–421. https://www.jstor.org/stable/2097002.

Palmer, A.R., Strobeck, C., 2003. Fluctuating asymmetry analyses revisited. In: Polak, M. (Ed.), Developmental Instability (DI): Causes and Consequences. Oxford University Press, Oxford, pp. 279–319.

Parris, M.J., Cornelius, T.O., 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology 85 (12), 3385–3395. https://doi.org/10.1890/04-0383.

Parsons, P.A., 1990. Fluctuating asymmetry: an epigenetic measure of stress. Biol. Rev. 65, 131–145. https://doi.org/10.1111/j.1469-185x.1990.tb01186.x.

Perry, G., Wallace, M.C., Perry, D., Curzer, H., Muhlberger, P., 2011. Toe clipping of amphibians and reptiles: science, ethics, and the law1. J. Herpetol. 45 (4), 547–555. https://doi.org/10.1670/11-037.1.

Pimm, S.L., Russell, G.J., Gittleman, J.L., Brooks, T.M., 1995. The future of biodiversity. Science 269 (5222), 347–350.

Polak, M., 2003. Developmental Instability: Causes and Consequences. Oxford University Press, Oxford.

R Core Team, 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Ribeiro, R., Santos, X., Sillero, N., Carretero, M.A., Llorente, G.A., 2009. Biodiversity and land uses at a regional scale: is agriculture the biggest threat for reptile assemblages? Acta Oecol. 35 (2), 327–334. https://doi.org/10.1016/j.actao.2008.12.003.

Rohlf, F.J., 2005. tpsDig, digitize landmarks and outlines, version 2.05. Department of Ecology and Evolution, State University of New York at Stony Brook.

Roversi, A., 2016. Observations on hazelnut organic farming. Bulg. J. Agric. Sci. 22 (2), 171–175.

Sacchi, R., Polo, M.S., Coladonato, A.J., Mangiacotti, M., Scali, S., Zuffi, M.A., 2018. The exposition to urban habitat is not enough to cause developmental instability in the common wall lizards (*Podarcis muralis*). Ecol. Indic. 93, 856–863. https://doi.org/10.1016/j.ecolind.2018.05.035.

Sarre, S., 1996. Habitat fragmentation promotes fluctuating asymmetry but not morphological divergence in two geckos. Res. Popul. Ecol. 38, 57–64. https://doi.org/10.1007/BF02514971.

Simbula, G., Luiselli, L., Vignoli, L., 2019. Lizards and the city: A community study of Lacertidae and Gekkonidae from an archaeological park in Rome. Zool. Anz. 283, 20–26. https://doi.org/10.1016/j.jcz.2019.08.001.

Stark, J.D., Banks, J.E., 2003. Population-level effects of pesticides and other toxicants on arthropods. Annu. Rev. Entomol. 48 (1), 505–519. https://doi.org/10.1146/annurev.ento.48.091801.112621.

Stark, J.D., Banks, J.E., Acheampong, S., 2004. Estimating susceptibility of biological control agents to pesticides: influence of life history strategies and population structure. Biol. Control. 29 (3), 392–398. https://doi.org/10.1016/j.biocontrol.2003.07.003.

StatSoft Inc, 2007. STATISTICA (data analysis software system), version 8.0.

Swaddle, J.P., 2003. Fluctuating asymmetry, animal behavior, and evolution. Adv. Study Behav. 32, 169–205. https://doi.org/10.1016/S0065-3454(03)01004-0.

Urošević, A., Ljubisavljević, K., Ivanović, A., 2015. Fluctuating asymmetry and individual variation in the skull shape of the common wall lizard (*Podarcis muralis* Laurenti, 1768) estimated by geometric morphometrics. J. Herpetol. 25, 177–186.

Valkama, J., Kozlov, M.V., 2001. Impact of climatic factors on the developmental stability of mountain birch growing in a contaminated area. J. Appl. Ecol. 38, 665–673. https://doi.org/10.1046/j.1365-2664.2001.00628.x.

Van Damme, R., Bauwens, D., Braña, F., Verheyen, R.F., 1992. Incubation temperature differentially affects hatching time, egg survival, and hatchling performance in the lizard *Podarcis muralis*. Herpetologica 220–228. https://www.jstor.org/stable/3892675.

Vervust, B., Van Dongen, S., Grbac, I., Van Damme, R., 2008. Fluctuating asymmetry, physiological performance, and stress in island populations of the Italian wall lizard (*Podarcis sicula*). J. Herpetol. 42, 369–377. https://doi.org/10.1670/07-1202.1.

Voets, J., Talloen, W., de Tender, T., van Dongen, S., Covaci, A., Blust, R., Bervoets, L., 2006. Microcontaminant accumulation, physiological condition and bilateral asymmetry in zebra mussels (*Dreissena polymorpha*) from clean and contaminated surface waters. Aquat. Toxicol. 79 (3), 213–225. https://doi.org/10.1016/j.aquatox.2006.06.001.

Wisler, C., Hofer, U., Arlettaz, R., 2008. Snakes and monocultures: habitat selection and movements of female grass snakes (*Natrix natrix L.*) in an agricultural landscape. J. Herpetol. 42 (2), 337–346. https://doi.org/10.1670/07-027.1.