

Divergent scute asymmetry among pure and crossed individuals of *Testudo hermanni* (Gmelin, 1789)

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Abstract

Divergent scute asymmetry among pure and crossed individuals of Testudo hermanni (Gmelin, 1789). Fluctuating asymmetry (FA) refers to subtle differences between left and right sides in bilaterally symmetrical organisms or their parts. Both genetic and environmental changes can increase FA, reflecting deterioration in developmental homeostasis of adult morphology due to a loss of developmental stability. In this study, we used geometric morphometric techniques to examine plastral scute asymmetries in a sample of 31 pure and crossed *Testudo* species (*T. hermanni hermanni* n = 23 and crosses with *T. hermanni boettgeri* n = 8) only females by means of 19 anatomical landmarks. Procrustes ANOVA indicated that FA in crossed individuals was significantly higher than that in pure individuals. Crossed individuals also showed a greater degree of phenotypic plasticity than *T. hermanni hermanni*. We conclude that crosses among *T. hermanni hermanni* and *T. hermanni boettgeri* can increase homozygosity and are responsible for greater developmental instabilities. Nonetheless, more information on crossed phenotypes could be of great interest to raise pure Hermann's tortoises for reintroduction programmes.

Key words: Carapace, Geometric morphometrics, Hybridization, Plastron, Testudines

Resumen

Asimetría divergente en las placas de Testudo hermanni (Gmelin, 1789) puras e híbridadas. La asimetría fluctuante (AF) se define como la presencia de diferencias sutiles entre los lados derecho e izquierdo de los organismos bilateralmente simétricos o sus partes. Tanto los cambios genéticos como ambientales pueden incrementar la AF, que viene a representar el deterioro en la homeostasis del desarrollo de la morfología adulta debido a una menor estabilidad del desarrollo. En este estudio examinamos, mediante técnicas de morfometría geométrica, asimetrías en las placas del plastrón en una muestra de 31 individuos puros y cruces de *Testudo* (*T. hermanni hermanni*, n = 23 y sus cruces con *T. hermanni boettgeri*, n = 8), únicamente hembras utilizando para ello un conjunto de 19 referencias anatómicas. El ANOVA de Procrustes indicó una AF significativamente mayor en los cruces, que además exhibieron un mayor grado de plasticidad fenotípica. Los autores concluimos que el cruce entre esas subespecies podría producir un incremento en la homocigosidad que

seria responsable de un aumento de la inestabilidad del desarrollo. Por otra parte, una mayor información sobre los fenotipos de los cruces resultaría de gran interés para la cría de la tortuga mediterránea de cara a los programas de reintroducción.

Palabras clave: Caparazón, Morfometría geométrica, Hibridación, Plastrón, Testudines

Resum

Asimetria divergent a les plaques de Testudo hermanni (Gmelin, 1789) pures i híbrides. L'asimetria fluctuant (AF) es defineix com la presència de diferències subtils entre els costats dret i esquerre dels organismes bilateralment simètrics o les seves parts. Tant els canvis genètics com els ambientals poden incrementar l'AF, que representa la deterioració en l'homeòstasi del desenvolupament de la morfologia adulta a causa d'una disminució en l'estabilitat del desenvolupament. En aquest estudi examinem, mitjançant tècniques de morfometria geomètrica, asimetries en les plaques del plastró en una mostra de 31 individus purs i encreuats de *Testudo* (*T. hermanni hermanni*, n = 23 i els seus encreuaments amb *T. hermanni boettgeri*, n = 8), només femelles. Per a això utilitzem un conjunt de 19 referències anatòmiques. L'ANOVA de Procrustes va indicar una AF significativament més gran en els encreuaments, que a més van mostrar un grau més elevat de plasticitat fenotípica. Els autors concloem que l'encreuament entre aquestes subespècies podria produir un increment en l'homozigositat que seria responsable d'un augment de la inestabilitat del desenvolupament. D'altra banda, disposar de més informació sobre els fenotips dels encreuaments seria de gran interès per a la cría de la tortuga mediterrània amb vista als programes de reintroducció.

Paraules clau: Closca, Morfometria geomètrica, Hibridació, Plastró, Testudines

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Introduction

Developmental instability arises from genetic or environmental stressors that disturb the normal developmental pathways of continuous characters, producing developmental noise. This index results from the difference between left and right in ideal bilaterally symmetrical organisms or parts of organisms, which we expect to be zero, and it provides a measure of how well an individual can buffer its development against internal and external environmental stress during ontogeny (Klingenberg and McIntyre, 1998). Developmental instability in phenotypic traits is commonly measured as fluctuating asymmetry (FA) (Van Valen, 1962). Many publications consider FA is a good estimator of developmental stability, that is, the ability of an organism to buffer minor developmental accidents (Alibert et al., 1994). Both genomic and environmental changes can increase FA, representing a possible deterioration in developmental homeostasis that is apparent in adult morphology (de Coster et al., 2013; Ducos and Tabugo, 2014). Hybridization is one of the biological factors underlying the changes in levels of FA (De Heredia et al., 2018). Genetic hypotheses have been proposed to explain the changes of FA in hybrids, as any change in genetic pool can result in a change of developmental stability (Carter et al., 2009; Albarrán–Lara et al., 2010; Demontis et al., 2010).

Hermann's tortoises are small to medium–sized tortoises from southern Europe (Berry and Shine, 1980; Wyneken et al., 2008). Two subspecies are currently recognized (Soler

et al., 2012; Rhodin et al., 2017): the western Hermann's tortoise (*T. h. hermanni*) and the eastern Hermann's tortoise (*T. h. boettgeri*). The eastern subspecies, *T. h. boettgeri*, is larger, reaching up to 28 cm in length, while *T. h. hermanni* is rarely larger than 18 cm. Crosses between the two subspecies have been described (Soler et al., 2012).

Geometric morphometric (GM) approaches help to understand the geometry of the symmetries through the analysis of form, also providing graphic visualizations of the statistical findings that can support the explanation. In this study, we examined, for the first time, morphological differences in ventral plastron symmetry between pure and crossed *Testudo* species using GM techniques, in captive animals managed under similar conditions, thus rejecting a priori environment as the cause of possible differences.

Material and methods

Sample

Specimens in the study were adult female turtles without any detectable abnormality such as injuries or unusual additional scutes or plates. Pure animals in the study were naturally deceased specimens, while crossed individuals had been collected as private individuals and euthanized in accordance with procedures established by conservation and welfare laws concerning prevention of genetic pollution in wild populations of the Hermann's tortoise. Corpses were obtained from CRARC (the Amphibian and Reptile Rescue Centre of Catalonia in Masquefa, Catalonia, Spain). To avoid possible bias due to sexual dimorphism (Wyneken et al., 2008; Bulté and Blouin–Demers, 2010), we considered only one females in this sample, giving a final data set of 31 animals (23 *T. hermanni hermanni* and eight individuals crossed with *T. hermanni boettgeri*).

Each turtle was levelled in accordance with a horizontal plan. Image capture was performed with a Nikon® D70 digital camera (image resolution of 2,240 x 1,488 pixels) equipped with a Nikon AF Nikkor® 28–200 mm telephoto lens. The camera was placed so that the focal axis of the camera was parallel to the horizontal plane and centred on the plastral (ventral) aspect. A scale was put over each specimen.

Landmark selection

Pictures were transported to TPSUtil (Rohlf, 2015) to convert the file. TPSDig2 was used for the digitation process (Rohlf, 2015). Nineteen discrete anatomical landmark points (seven of them mid–sagittal), located on intersections of different scutes, were taken from the samples (fig. 1). Digitalization was bi–replicated to establish the measurement error (Fruciano, 2016). To apply the usual GM methods, it is necessary to project shape space, which is curved, non–Euclidean, onto a linear, Euclidean space (Klingenberg and McIntyre, 1998). To do so we used the linear tangent space as a location of the consensus configuration on the curved shape space (Klingenberg and McIntyre, 1998).

Digitation and shape analysis

A Generalized Procrustes analysis (GPA) approach eliminates the scale and the translational and rotational differences of the coordinate data of the landmarks between subjects (Webster and Sheets, 2010). The coordinate data for each specimen are usually scaled by its centroid size (CS, the square root of the sum of squared distance between each landmark and the plastron centroid) (Bookstein, 1991). The CS and GPA–scaled coordinates represent surrogates of size and shape, respectively (Webster and Sheets, 2010) (fig. 2).

To detect the components of variances and deviations, we used a Procrustes ANOVA. In this analysis, the individual effect denoted the individual variations of shape and size of

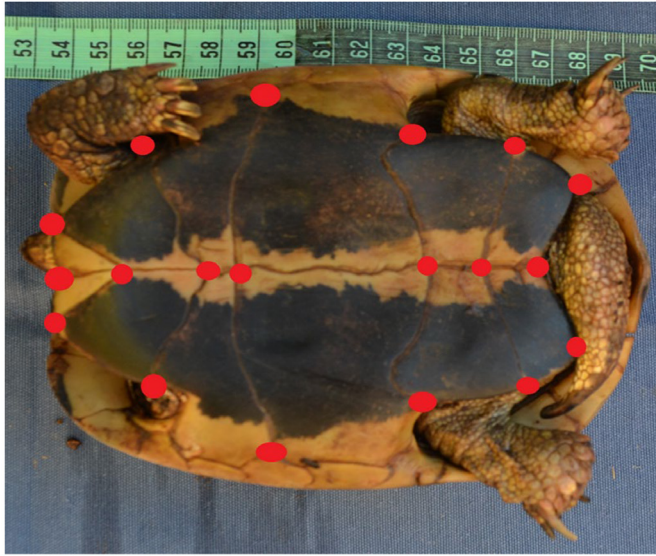


Fig. 1. Set of 19 discrete anatomical landmarks used for this study on plastrons, all situated on intersection of scutes. Seven were mid-sagittal. The individual in this photo is a pure *Testudo hermanni hermanni*.

Fig. 1. Conjunto de 19 referencias anatómicas diferenciadas utilizadas para este estudio en los plastrones, todas ellas situadas en la intersección de las placas. Siete de ellas fueron medio sagitales. Este individuo es un Testudo hermanni hermanni puro.

each turtle. The individuals mean square is a measure of total phenotypic variation and it is random. The main effect of sides indicates the variation between sides and is considered as the measure of DA, and the individuals x sides is the mixed effect, this indicating FA in the data (Klingenberg and McIntyre, 1998). Lastly, measurement error represents the variation due to measurement error in taking landmarks of the same individual in separate sessions (Klingenberg and McIntyre, 1998). There are more degrees of freedom in Procrustes ANOVA than in conventional ANOVA because the squared deviations are summed over all the landmark coordinates (instead of a single sum of squares in conventional ANOVA). Therefore, the number of degrees of freedom is that for ordinary ANOVA multiplied by the shape dimension, which is, for our two-dimensional coordinate data, twice the number of landmarks minus four (the number of coordinates minus two dimensions for translation and one each for scaling and rotation) (Klingenberg and McIntyre, 1998).

Allometry and size correction

The effect of allometry was verified using the multivariate regression of shape (Procrustes coordinates) on size (\log_{10} -transformed CS) which was treated here as a proxy for general size. A canonical variate analysis (CVA) was used to assess asymmetric shape differences associated with each group. CVA is a discriminant analysis designed to maximize variation between groups and to minimize variation within groups to obtain the best possible segregation among groups. Since allometric effect of size or shape was significant, for CVA

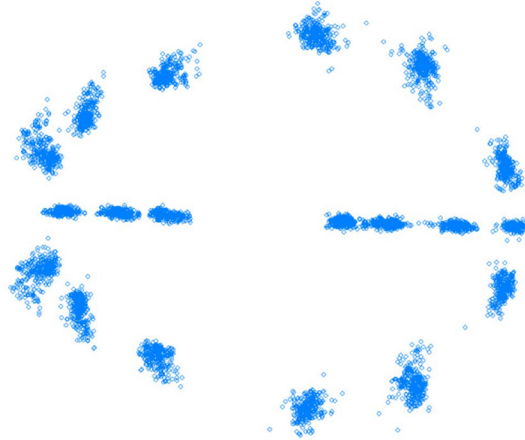


Fig. 2. Procrustes superimposition for the 19 anatomical landmarks used in this study.

Fig. 2. Superimposició Procrustes para 19 referencias anatómicas utilizadas en este estudio.

we used the residual component of the regression of shape on CS, making it possible to compare shapes with minimum interference from differing size. The statistical significance of pairwise differences in mean shapes was assessed by a permutation test using Mahalanobis distance, which measures distances between points for multiple variables, even when they are correlated.

All analyses were carried out using the PAST v. 2.17c (Hammer et al., 2001) and MorphoJ v. 1.06c (Klingenberg, 2011) packages. For all tests, statistical significance was demarcated at the 5% level.

Results

The process of extracting landmark coordinates is associated with some degree of error. Among others, digitalization error can result from non-coplanarity of landmarks (Webster and Sheets, 2010). Analyses of the data set using TpsSmall v. 1.33 (Rohlf, 2015) indicated an excellent correlation between the tangent and the shape space (uncentered correlation between the tangent space onto Procrustes distance = 0.9990), so the linear tangent space touching the curved shape space was acceptable for further statistical analysis.

The Procrustes ANOVA also showed that measurement error was negligible as the mean square (MS) for individual variation for each group exceeded the measurement error (table 1). The significant 'side*individual' effect confirmed the presence of an FA level of 9.22% for crossed individuals compared to 4.48% for *T. h. hermanni* (table 1). Crossed specimens also showed a higher degree of phenotypic plasticity than parenteral species (75.6 versus 69.3%).

The multivariate regression of the Procrustes coordinates on \log_{10} -transformed CS showed that allometry was statistically significant ($p < 0.001$, permutation test with 10,000 random permutations). \log_{10} -transformed CS accounted for 8.69% of the total shape variation, so the allometric relationship (the scaling) between plastral shape and size was clear. The

Table 1. Measurement error Procrustes ANOVA for shape of plastral scute symmetry for female *T. hermanni hermanni* ($n = 23$) and their crosses ($n = 8$), with a significant effect of 'side*individual' effect (FA) for both groups. Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances (dimensionless).

Tabla 1. Error de medición ANOVA Procrustes para la forma de simetría de las placas plastrales en la hembra de *T. hermanni hermanni* ($n = 23$) y sus cruces ($n = 8$), con un significativo efecto de "lado*individual" (FA) para ambos grupos. Las sumas de cuadrados (SS) y las medias de cuadrados (MS) son en unidades de distancias Procrustes (sin dimensiones).

	SS	MS	df	F	P
<i>T. h. hermanni</i>					
Individuals	0.15338392	0.0004101174	374	15.44	<.0001
DA	0.00225439	0.0001326110	17	4.99	<.0001
FA	0.00993115	0.0000265539	374	1.20	0.019
Error	0.01731491	0.0000221418	782		
Crosses					
Individuals	0.01757016	0.0001476484	119	8.20	<.0001
DA	0.00045249	0.0000266173	17	1.48	0.114
FA	0.00214309	0.0000180092	119	6.24	<.0001
Error	0.00078505	0.0000028862	272		

first canonical discriminant axis derived from morphometric data of the residuals of the regression explained 100% of asymmetry variation between groups, and discrimination in asymmetry between the two groups was significant ($p < 0.001$, 10,000 permutation rounds).

Discussion

In this study, we measured the levels of fluctuating asymmetry in *T. hermanni hermanni* and *T. hermanni hermanni* crossed with *T. hermanni boettgeri*. The results of the Procrustes ANOVA indicated random differences (fluctuating asymmetry) between the shape of left and the right scute patterns of the plastrons. The level of fluctuating asymmetry was significantly higher in crossed individuals than in pure individuals, suggesting that crossing in this case increases fluctuating asymmetry. A plausible explanation for higher levels of fluctuating asymmetry detected among crossed specimens of *T. hermanni* would be the differences in genetic composition of the crossed population, because as management was similar for all individuals, external factors would therefore be responsible for similar developmental instability. The problem is that although it may be simple to measure FA, it is difficult to determine the underlying cause that increases bilateral asymmetry (Rego et al., 2006).

In the literature there are well-documented situations in which inter-species hybridisation increases bilateral asymmetry, both in vegetal species (Albarrán–Lara et al., 2010; Cuevas–Reyes et al., 2018; De Heredia et al., 2018) and in animals (Alibert et al., 1994;

Vishalakshi and Singh, 2009; Demontis et al., 2010; Ducos and Tabugo, 2014; Lajus et al., 2015). This has usually been interpreted as a direct consequence of the fact that the genome of such hybrids is a combination of two different parental genomes and, hence, the mechanisms that stabilize the interferences due to development noise are disrupted (Rego et al., 2006). The same occurs in our study where *Testudo hermanni hermanni* and *T. hermanni boettgeri* do not coexist in natural populations due to the barriers of isolation between these distinct subspecies (Rego et al., 2006). This increase in asymmetry can be the result of a misbalance between the stabilizing effect due to increased heterozygosity and the disruptive effect caused by breakdown of genomic co-adaptation (Alibert et al., 1994; Albarrán–Lara et al., 2010) and thus they have a more unstable development (understood as a mechanism which offsets random interference). Or it could simply occur that some of the genes involved in the symmetrical development of crossed individuals do not work properly (Rego et al., 2006). The degree of phenotypic plasticity was also higher among crossed individuals, as has been described in hybrids for other species (Carreira et al., 2008). In any case, fluctuating asymmetry, as a biomarker of stress and developmental instability, could thus be used as an indicator of populational quality and adaptation of crossed *Testudo hermanni* specimens.

As far as we know, our study is the first to compare the level of asymmetries among pure and crossed genotypes of *Testudo* subspecies. On the basis of our findings, changes in morphological asymmetry among crossed individuals could be used as a measure of selection on hybrid genomes. Although measures of levels of fluctuating asymmetry can be viewed as an indirect method to determine how genetic composition acts under crossing events, the exact genetic basis and its effects on symmetry are still unknown, and future studies are needed.

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