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Different Carapace Asymmetry Among Sexes in Vulnerable Tortoise: *Chelonoidis carbonarius* Spix 1824 (Testudines: Testudinidae)

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ABSTRACT

Developmental stability (DS) is characterized by an individual's ability to resist random environmental and/or genetic perturbations during their development. Fluctuating Asymmetry (FA) is the most common means of assessing developmental stability (DS) in bilateral traits. To date, little attention has been paid to sexual differences in FA among tortoises. The aim was to assess the levels of carapace scutation symmetries in a sample of 45 wild adult red-footed tortoises, *Chelonoidis carbonarius* (13 males and 32 females), from the Arauca plains (Colombia) using geometric morphometric techniques. The landmark configuration, based on the dorsal scute sutures of the carapace, consisted of 7 symmetric pairs with three landmarks along the axial plane, and was tested. Procrustes ANOVA reflected sex-related FA ($p<0.05$), with males tending to exhibit a more pronounced asymmetry. Although we cannot identify the potential sources responsible for the detected developmental instability, our results suggest a high degree of stress and highlight that human intervention in Arauca is affecting wildlife. Similar studies in the future, correlated with an estimate of human impact, could provide irrefutable proof of causality between FA and environmental stressors. The results of this study may represent an estimate of the physical status of the *Chelonoidis carbonarius* (Spix 1824) population currently being studied.

1. Introduction

Bilaterally symmetrical organisms can exhibit different types of asymmetry, including Fluctuating Asymmetry (FA), Directional Asymmetry (DA), and Antisymmetry (AS) (Graham *et al*. 1993; Graham *et al*. 2010). FA is defined as the deviation of the symmetry of an individual from its ideal symmetry. It is considered a negative indicator of the individual's ability to resist random and small developmental accidents (Palmer 1996). These developmental accidents are commonly the result of genetic or environmental stress (Auffray *et al*. 1999; Carter *et al*. 2009). Inbreeding has been linked to reduced health in populations and, consequently, to body symmetry. DA occurs whenever there is a greater development of a character on one side than the other (Kharlamova *et al*. 2010; Lotto and Béguelin 2014). DA typically has a genetic basis (Leśniak 2018). Finally, AS is a bimodal asymmetry that is random concerning side, where the lack of symmetry is distinguished by a departure from a Gaussian distribution of side differences (Mancini *et al*. 2005). For the quantification of these asymmetries, size and shape must be assessed separately (Sforza *et al*. 1998). Geometric morphometric approaches have been used for this purpose.

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Chelonoidis carbonarius was described as Testudo carbonaria by Spix in 1824 (Echeverry-Alcendra 2019). It is a tortoise species from South America, distributed in open areas (Barros *et al*. 2012; Rhodin *et al*. 2021) from northern Paraguay and Argentina through the cis-Andean region to Panama (Gallego-García *et al*. 2015; Cacciali *et al*. 2016; Rhodin *et al*. 2021). They are medium-sized tortoises that can reach over 40 cm (Barros *et al*. 2012), characterized by dark-colored, loaf-shaped carapaces with a lighter patch in the middle of each scute. However, they exhibit considerable variation in morphological characteristics (Barros *et al*. 2012; Gallego-García *et al*. 2015). No size or sex dimorphism has been described (Barros *et al*. 2012), but morphological population differences are recognized within the species between areas, primarily in plastron scutes, carapace width, and head length (Barros *et al*. 2012). *C. carbonarius* is frequently kept as a pet, and over-collection, as well as habitat destruction, have caused it to be close to extinction (Gallego-García *et al*. 2015), appearing as "Vulnerable" in the 2021 TFTSG Draft Red List (Rhodin *et al*. 2021).

The anthropogenic disturbance in the habitat, resulting from the continuous human exploitation of the Arauca plains, contributes to environmental deterioration, subjecting *C. carbonarius* to ecological stress and thereby promoting developmental instability in the species. Therefore, we hypothesize that human intervention in the environment where these turtles live may be related to the presence of possible asymmetries. Hybridization and consanguinity are excluded, as we have worked with an open wild population. To date, not much attention has been paid to asymmetric studies or sexual differences in FA in *C. carbonarius*. In the present study, the scutation asymmetries between sexes in the shell of the wild red-footed tortoise (*C. carbonarius*) from the Arauca plains were analyzed and compared using geometric morphometric techniques. Our data aims to compare possible differences in sexual homeostasis among these animals.

2. Materials and Methods

2.1. Sampling Area

The present study was carried out in the department of Arauca (Colombia) located on the Orinoco Basin (the "Llanos Orientales") in the extreme East, bordering Venezuela, situated between the Arauca and Casanare rivers, and covering a huge area of 23.818 km^2 (latitude: 07°05′25″ N, longitude: 70°45′42″ W, altitude: 128 m) (Figure 1). Seventy-five per cent of the land is flat. The soil is covered by native grass. The temperature varies from 28°C to 35°C (Arauca 2024). This area experiences minimal tourist impact, with waste from multinational crop companies posing its primary threat.

2.2. Data Collection

Data were obtained from 45 wild adult individuals (13 males, 34.8-53.7 cm total carapace length; and 32 females, 20.2-47.2 cm total carapace length) of *C. carbonarius*, during the dry season (February to March 2019) (Salamanca-Carreño *et al*. 2024). This tortoise is

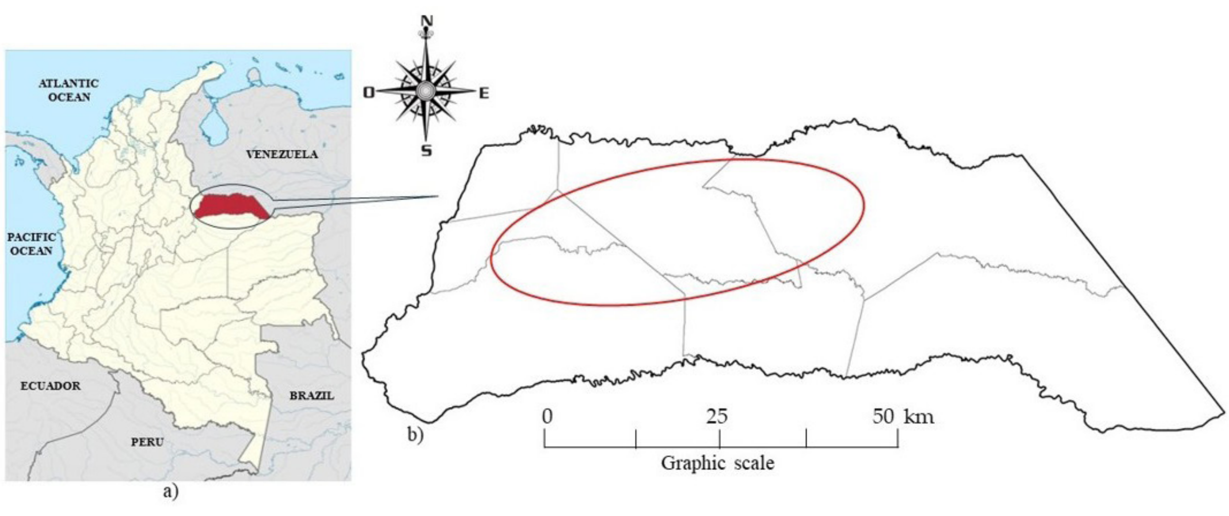

Figure 1. a) Map of Colombia. b) Arauca Department is located in the "Llanos Orientales" of Eastern Colombia. Red circle: sampling location. Source: authors

diurnal and typically inhabits tropical dry forests. It is an omnivorous animal.

In different areas, digital pictures were obtained in the field, with the camera held approximately 40-50 cm above each tortoise, focused on the center of their central dorsal aspect. We only used the carapacial scores because the carapacial photographs are less susceptible to tilting during the photographing process (the animal can rest flat and inside its shell). A single digital picture of each animal was obtained. To prevent distortion and optical aberrations, no zoom was used, and the tortoises were maintained in a neutral position, using props to minimize parallax errors. All the field-collected specimens displayed no injuries from predators or additional scutes. Photographs were taken with a Nikon P530 camera featuring a 42× optical zoom. Identification of sex was based on the presence or absence of a pronounced plastron concavity. An average straight carapace length of 44.9 cm is reported, although records of up to 51 cm are also available (Echeverry-Alcendra 2019). A ruler was inserted previously on each image. After their measurement and photography, the animals were released at the same site where they were captured. In no case were animals sacrificed, or traumatic manipulation or samples obtained that could cause pain.

2.3. Geometric Morphometrics

A landmark configuration based on the dorsal scute sutures of the carapace, comprising seven symmetric pairs with three landmarks along the axial plane, was tested (Figure 2).

Three types of landmarks can be recognized. Our landmarks were of type I, e.g., localized at the intersection of sutures and therefore easy to identify repeatedly. The captured images were transformed using TpsUtil v. 1.40 software (Rohlf 2015a), and landmarks were recorded using TpsDig v. 2.26 software (Rohlf 2010). Scale was eliminated by setting the centroid size (the square root of the sum of squared distances between each landmark and the carapace centroid) the same in all specimens (Bookstein 1992). Because measurement error may either hide or bias fundamental differences between the sides (Palmer 1994), the first author repeated the measurements for all traits in all samples twice to minimize inconsistencies or errors in plotting landmark points. The set of original configurations and mirrored copies (including the replicated measurements) was then superimposed (scaled, translated, and rotated) following the Procrustes method of generalized least squares superimposition.

Shape spaces are curved, non-Euclidean spaces. To apply the usual methods of statistics, it is necessary to project shape space onto a linear Euclidean space, so that the distribution of points in the tangent space can be used as a good approximation of their distribution in shape space (Klingenberg and McIntyre 1998a). As the process of extracting tangent landmark coordinates from curved objects can introduce errors due to non-coplanarity

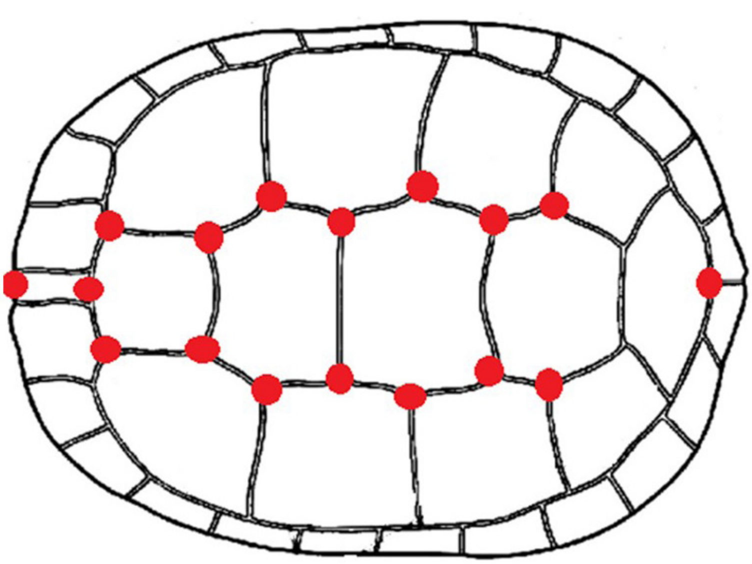

Figure 2. Pairs of landmarks (14) situated on sutures of carapace scutes (right: anal region). As the carapace is a symmetric structure with object symmetry, characterized by an axis of symmetry that passes through the landmark configuration, the symmetry axis can partially align with the three remaining landmarks on the mid-sagittal line

(Klingenberg and McIntyre 1998a; Webster and Sheets 2010), we tested whether the observed shape variation was sufficiently slight. This analysis was conducted using TpsSmall v. 1.33 software (Rohlf 2015b).

The asymmetrical component was quantified as the difference between the original and the ideally symmetrical configuration, based on the displacement of unpaired landmarks along the mid-sagittal axis and the average displacement of paired landmarks moving in any direction. For the quantification of asymmetry, a displacement of unpaired landmarks is allowed in the direction perpendicular to the mid-sagittal axis while paired landmarks move freely. The analysis done was specifically for object symmetry, with the symmetry axis passing through the structure.

2.4. Statistical Analysis

A multivariate regression of the asymmetric component against centroid size was also performed to detect allometry for each sex. There was no change in asymmetry according to size, shape asymmetric variables, and centroid size, which were found to be largely uncorrelated in the sample ($p = 0.0661$; 10,000 randomization rounds).

To examine the amount of asymmetric variation, we used a Procrustes two-way mixed model ANOVA (Klingenberg *et al*. 1998b; Klingenberg & Monteiro 2005) for each sex and shape separately. The "individuals" effect refers to the variation in size and shape among individuals. The main effect is "sides," which indicates the variation between sides. The "individual x side interaction" is a mixed effect, representing the failure of individuals to be the same between sides (left and right). Lastly, measurement error, estimated from the total variation of the entire landmark configuration, was included.

The "individuals" effect refers to the variation among individual samples and can be interpreted as variation in symmetry. The effect of "individual x side interaction" is the measure of FA, and the effect of "side" is the measure of DA (Klingenberg 2002). To verify the data for AS, we visually examined scatter plots of vectors representing left-right differences for each landmark after superimposition using the Procrustes algorithm. This vector plot depicts the displacement of all landmarks between the target form and those on the reference form. Analytical procedures that incorporate geometric morphometrics in testing for FA are available from numerous seminal publications (Klingenberg *et al*. 1998b; Klingenberg 2002; Klingenberg & Monteiro 2005). Finally, a Canonical Variate Analysis (CVA) was performed to compare the asymmetric components between sexes. Mahalanobis distances were used for this test.

All analyses were performed using MorphoJ v. 1.07a (Klingenberg 2011), with permutation tests conducted across 10,000 permutation runs. For all analyses, $\alpha=0.05$.

3. Results

Although the carapace is markedly convex, when we compared the 2D Procrustes distances to the tangent space distances (i.e., Euclidean distances in tangent space), the relation was very close to linear for all the data, e.g., it appeared an excellent correlation between the tangent and the shape space (correlation between the tangent space onto Procrustes distance=0.999034). This result proved the acceptability of the dataset for further statistical analysis in the Euclidean space.

Initially, intra-observer error, which is associated with the placement of landmarks, was evaluated. The results showed that the variation among samples ($MS = 0.0003500863$) was higher than the variation between double distributions of landmarks ($MS = 0.0000001508$); hence, high intra-observer agreement was ascertained.

The absence of clustering of vectors (as the equivalent of bimodal distributions of left-right differences) provided no support for the presence of AS (Figure 3). Procrustes ANOVA shows the mean squares for the mixed-model ANOVA and its corresponding effects (Table 1). The significant interaction of "individual x side" confirmed the presence of FA for both sexes, but the side factor (indicating DA) was not detected to be statistically significant. Canonical Variate Analysis (CVA) revealed statistically significant differences between sexes; that is, a significant factor analysis (FA) was found for both sexes ($p<0.0001$; 10,000 permutation rounds).

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 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).

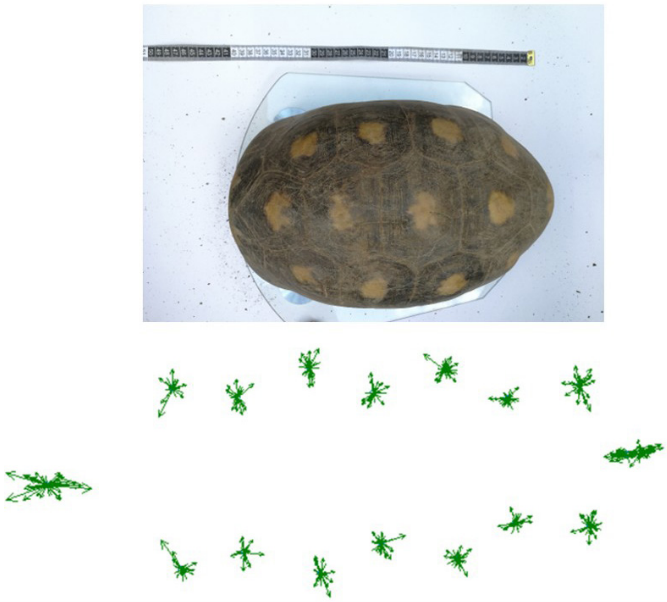

Figure 3. Clustering of vectors for 45 individuals (13 males and 32 females) of *Chelonoidis carbonarius*. The vector plot depicts the displacement of all landmarks between the target form and those on the reference form. The absence of clustering of these vectors does not suggest antisymmetry

Table 1. ANOVA Procrustes analysis of dorsal shape of 45 red-footed tortoise *Chelonoidis carbonarius* carapaces (13 males and 32 females)

A/A/Males (n=13)

Effect	SS	MS	df	F	P
Individual	0.060659	0.000337	180	5.97	<.0001
Side	0.001229	8.19×10^{-05}	15	1.45	0.1277
Individual* Side	0.010155	5.64×10^{-05}	180	835.44	<.0001
Error 1	2.63×10^{-05}	6.75×10^{-08}	390		

A/Females (n=32)

Effect	SS	MS	df	F	P
Individual	0.163378	0.000351	465	4.81	<.0001
Side	0.001166	7.77×10^{-05}	15	1.06	0.3875
Individual* Side	0.033961	7.30×10^{-05}	465	395.53	<.0001
Error 1	0.000177	1.85×10^{-07}	960		

4. Discussion

Developmental instability (DI) refers to an organism's inability to buffer the perturbation of a developmental process under given genetic and/or environmental conditions (Jung *et al*. 2016). Many studies have shown that DI can be caused by genetic and environmental stresses, such as nutritional deficiencies, genetic diseases, and parasitosis (Jung *et al*. 2016). Fluctuating asymmetry (FA, random departures from perfect symmetry) has been established as an indicator of developmental stress, implying a measurable degree of instability (DI) (Graham *et al*. 2010; Jung *et al*. 2016). The use of geometric morphometrics, combined with multivariate statistical techniques, yielded a low measurement error, providing clear information on the carapacial asymmetry of adult males and females of *C. carbonarius*. Our results reflected a significant FA for both sexes.

To date, available studies of asymmetries among testudines are scarce and sometimes yield contradictory conclusions. For instance, Dillard (2017) found no shell symmetric differences between the two populations of Pseudemys species (*P. concinna* and *P. rubriventris*). Still, Davis and Grosse (2008) found FA in *Trachemys scripta*, as did Băncilă *et al*. (2012) in Testudo graeca ibera, while Buică and Cogălniceanu (2013) detected low levels of FA in this same species. In a study on *T. hermanni* hermanni and *T. hermanni* boettgeri, fluctuating asymmetry was observed between the shape of the left and right shield patterns of the plastrons (Parés-Casanova *et al*. 2020). Similarly, Goessling *et al*. (2017) detected fluctuating asymmetry in *Gopherus polyphemus*. The lack of a relationship between asymmetry and body size, another conclusion of our study, has been observed in studies on other species (Băncilă *et al*. 2012; Buică and Cogălniceanu 2013; Goessling *et al*. 2017).

Considering that FA is the product of cumulated effects from stresses over the development history, detected levels of FA demonstrate an inability to buffer developmental noise and achieve an equilibrium. In any case, as animals were sampled from different areas in Arauca and no consanguinity—a possible developmental perturbation —was expected, our results suggest a high degree of stress and highlight that human intervention in Arauca is affecting wildlife. No FA studies have been reported in *C. carbonarius*; therefore, it cannot be compared.

In conclusion, wild *Chelonoidis carbonarius* from the Arauca plains in eastern Colombia exhibit fluctuating asymmetry on their carapaces, with sex differences. These results may suggest a high degree of stress and highlight that human intervention in Arauca is affecting the sexes of *C. carbonarius* differently. The results of this study may represent an estimate of the physical status of the *C. carbonarius* Spix 1824 population currently studied. Globally, results highlight the need for increased conservation and management of red-footed tortoises in populations experiencing reduced fitness. Similar studies in the future, correlated with an estimate of human impact, could provide proof of causality between developmental instabilities and environmental stressors, in Araucanian and other wild populations.

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Institutional Review Board Statement

This study conforms to the appropriate guidelines for the welfare of reptiles. No ethical approval was necessary, as field research consisted of gentle manipulation of animals for measuring, weighing, and photographing, which were immediately released at the same capture point. Neither sample collection nor pain manipulation was done. The procedures of the National Environmental License Authority of Colombia, as outlined in Resolution No. 01877 (October 22, 2018), were followed.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the second author on reasonable request.

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