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Using digital images in the study of fluctuating asymmetry in the spur-thighed tortoise *Testudo graeca*

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Abstract: Fluctuating asymmetry has been proposed as a useful noninvasive tool for detecting the influence of the environment on body development. In the present study, we used digital images to estimate the fluctuating asymmetry of the plastron in an isolated *Testudo graeca* population in Dobrogea, Romania. We extracted data from the photos regarding the area, height, and width of 3 plastron scutes. A total of 155 adult tortoises analysed showed reduced variation in plastron asymmetry and no significant relationship was detected between fluctuating asymmetry and sex or body size. We suggest that fluctuating asymmetry may be an individual trait, with no predictive value at the population level.

Key words: Fluctuating asymmetry, *Testudo graeca*, fitness, bioindicator, plastron

1. Introduction

Reptiles are declining worldwide, with nearly 1 in 5 reptilian species threatened with extinction, and another 1 in 5 species classed as Data Deficient (Böhm et al., 2013). Gibbons et al. (2000) identified 6 significant threats to reptile populations: habitat loss and degradation, introduced invasive species, pollution, disease, unsustainable use, and global climate change. Among reptiles, tortoises are among the world's most endangered vertebrates, with more than 300 species threatened with extinction (Turtle Conservation Coalition, 2011). A recent assessment showed that tortoises of the family Testudinidae are significantly over-threatened (Böhm et al., 2013).

There is an urgent need to develop rapid, nondestructive methods for assessing the status of wild populations of tortoises. Among the different measures proposed, developmental stability, i.e. the ability of an individual to withstand random perturbations during its development (Møller, 1994), is considered a good indicator of environmental and genetic stress. A common means of assessing developmental stability is through analysis of fluctuating asymmetry (FA) in bilateral traits, facilitated in tortoises by their large, solid carapace, which allows for measurement of body geometry (Adams et al., 2004).

It is considered that the organism can overcome environmental stress and maintain a similarity between the 2 sides of the body. Any deviation from normal body development can be underlined by FA analysis. This mode of analysis involves the demonstration of deviation from bilateral symmetry as a measure of human impact, which exceeds the body's ability to maintain a normal development in stressful conditions (Valen, 1962).

The spur-thighed tortoise *Testudo graeca* is under increasing stress due to habitat reduction and fragmentation. At the European level, it is considered vulnerable (Cox and Temple, 2009), while at the European Union level, it is a species of community interest that requires the designation of special areas of conservation (European Commission, 1992).

Human impact affects all tortoise populations directly or indirectly (Türkozan et al., 2008). The easily visible effects of human impact on *T. graeca* are shown on the carapace and plastron as blows or destruction of the scutes (Tuegel and Weise, 2006).

Evaluating FA in *T. graeca* can be achieved simply only for the plastron by using landmarks placed at boundaries between scutes for statistical analysis of the obtained shape (Băncilă et al., 2012). In this study, we used an alternative method for FA study, derived from the method proposed by Davis and Grosse (2008). This method does not require landmarks; instead, each plastron scute has its edge drawn using graphic software. The resulting shape is measured using computer software capable of measurements on digital images. This approach reduces the bias introduced by landmark placement and offers greater flexibility, but it is more time-consuming.

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The aim of this study was to assess the FA in an isolated population of *T. graeca* and test whether FA is influenced by sex, body size, and plastron abnormalities.

2. Materials and methods

2.1. Site description

The isolated population of *T. graeca* occurs within the Histria Archaeological Complex (HAC), Dobrogea Province, Romania (44°32′56″N, 28°45′56″E, altitude 0–2 m a.s.l.). This area is located in the Danube Delta Biosphere Reserve and surrounded by the water bodies of the Sinoe Lagoon and sandbanks. The HAC population inhabits an area of 32 ha. Past archaeological activities have created a complex of microhabitats consisting of slopes and pits. Although it has a reduced area, the touristic and archaeological impact remains. Protection is increased by a fence delimiting the perimeter of the HAC.

2.2. Data collection and analysis

This study is based on the data obtained between 2010 and 2012 from 155 adult tortoises, 76 males and 79 females. All tortoises were sexed, photographed, and measured. Moreover, all captured tortoises were marked with a small notch in one of marginal scutes and an identification number was written using a permanent marker, so that tortoises could be identified using solely the images (Stubbs et al., 1984). The digital images were obtained with a Nikon P100 10-MP with reduced optical aberrations, and the tortoises were maintained without inclination to any side. We considered that any optical aberrations would have negative effects on the data quality. A standard was inserted on each image for image measurement calibration.

The method used in this study required highlighting the shapes of selected scutes in each photographed plastron. This was done using the Photoshop application (Adobe Photoshop CS5 Extended). The plastron image was imported into Photoshop, followed by superimposing a graphic layer on which the edge of each analysed scute was drawn using a line of 2 pixels in width and the drawing tool of this software application. The drawing was carried out through the space between the outermost rings of each scute. For FA analyses we selected only humeral, femoral, and anal scutes, avoiding the scutes that have lateral extensions and morphological variations.

The extraction of measurements started with the calibration of the measurement tool of Photoshop Extended by setting the scale to the standard inserted into each image. The process of obtaining the scutes' measurements involved the selection of each scute shape using the "magic wand" tool of Photoshop followed by the selection of the "record measurements" option in the Analyses menu.

Using the method proposed by Davis and Grosse (2008), we measured each scute area. We also measured the height and width of each scute (Figure 1). Using this method, it is easier to obtain the area, height, or width of each scute because these values are obtained automatically. This eliminates the task of manually determining the highest or widest point of the scute for measurements. The obtained results were retained in Photoshop and exported in text format. This format is easily imported into Microsoft Office Excel to create a database with measurements.

An average value was calculated for each parameter of the selected scutes from the same side of the plastron. The selected measurements provide 3 different perspectives on the possible asymmetry of body growth. The difference between the average values for the left and right sides of the plastron was analysed for indication of deviation from bilateral symmetry in relation to straight carapace length (SCL), curved carapace length (CCL), and sex. Positive values for any of the 3 parameters will indicate higher values for the left side of plastron (LSP), while negative values will indicate higher values for the right side of plastron (RSP). The obtained data were analysed using the SigmaPlot 11 application (Systat Software, Inc., 2011). The SCL measurements were obtained along the median suture of the plastron, from the anterior edge of the carapace to the exterior margin of the supracaudal scute. The CCL was obtained by measuring the curvature of the carapace at its median line from the anterior edge of the carapace to the tip of the supracaudal scute (Stubbs et al., 1984).

3. Results

The population structure showed that the highest number of individuals has a SCL of 19–21 cm (Figure 2). The studied parameters did not differ significantly between sexes (Mann–Whitney test, $P > 0.1$). The average deviation from bilateral symmetry is reduced for all the measured parameters in both females and males (Figure 3).

The analysed parameters for females and males in respect to SCL (Figure 4) and CCL (Figure 5) showed similarities without significant correlation to SCL or CCL (Spearman rank correlation $R < 0.1$, $P > 0.3$). For area and width, the variation from bilateral symmetry is balanced for the left and right sides of the plastron. The average difference in the height shows higher values for the right side of the plastron for both sexes. Each studied parameter presents a distinct pattern of variation in deviation from bilateral symmetry with an increase in asymmetry with body size. Females tend to be more asymmetric without a clear trend over the classes of body dimensions. The asymmetry trend in males shows a reduction with increasing body dimension (i.e. SCL).

Extreme variation in asymmetry was observed in some individuals with abnormal scute development. We revealed that the height and width is preserved but the area of the scutes differs greatly for the 2 sides of the plastron

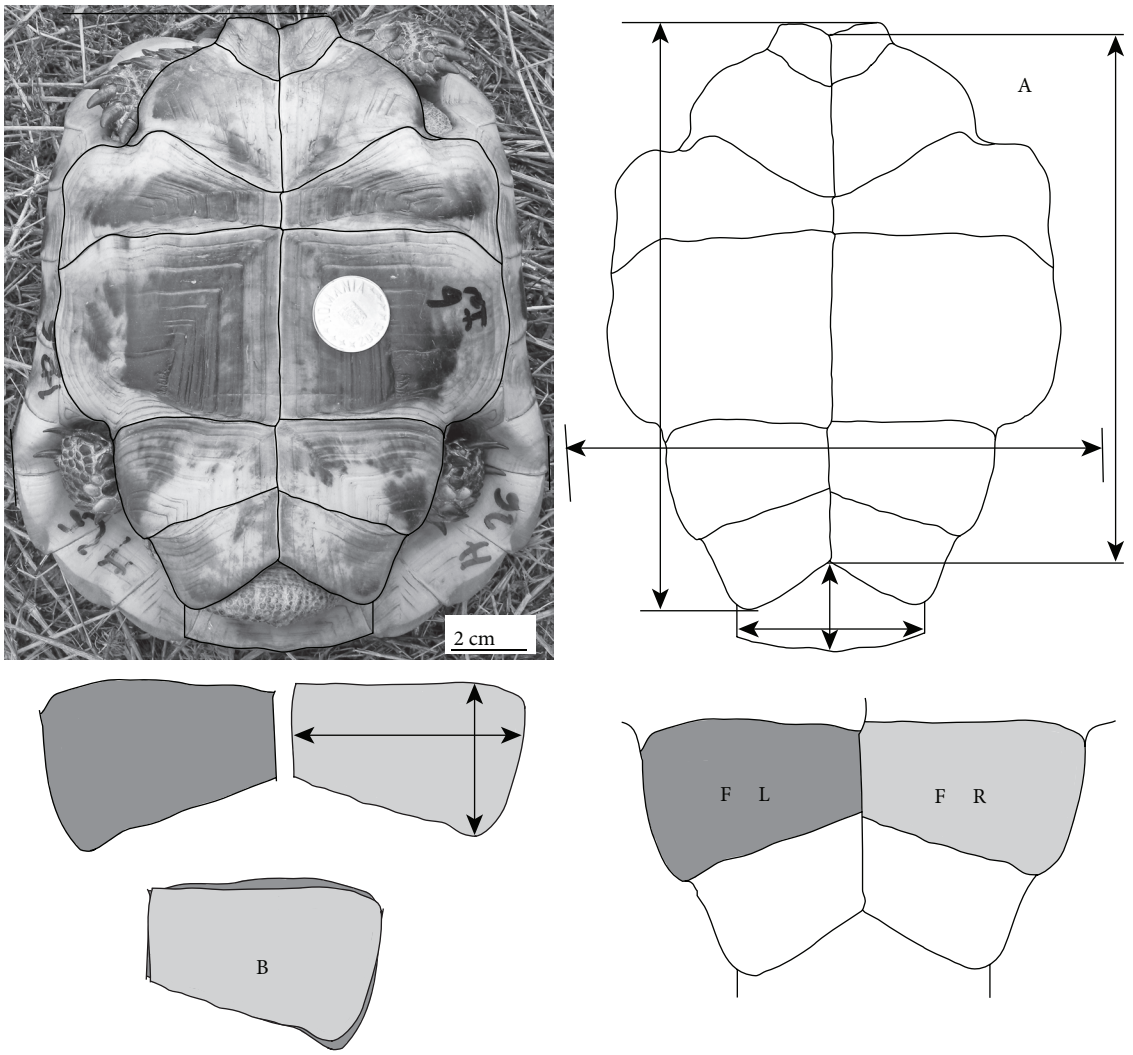

Figure 1. Outline of plastron (A) and demonstration of the difference (B) between the surface of the left (FL) and right (FR) femoral scutes (photo: Gabriel Buică).

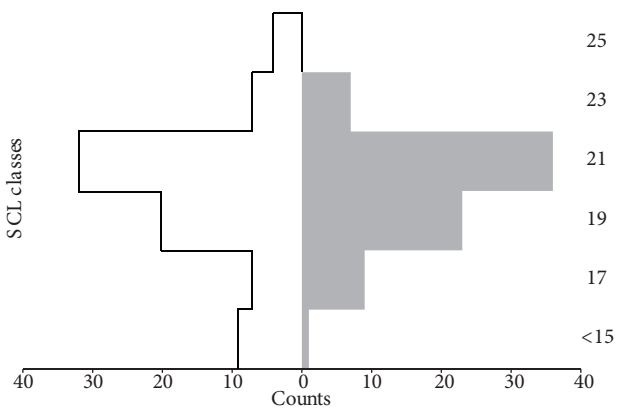

Figure 2. Adult tortoises' population structure based on SCL classes in cm (females in white, n = 79; males in grey, n = 76).

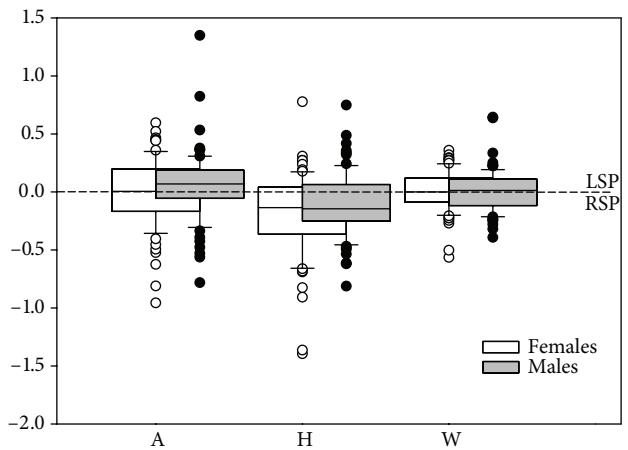

Figure 3. The deviation from bilateral symmetry in females and males for area (A) in mm^2 and height (H) and width (W) in mm (LSP: left side of plastron, RSP: right side of plastron).

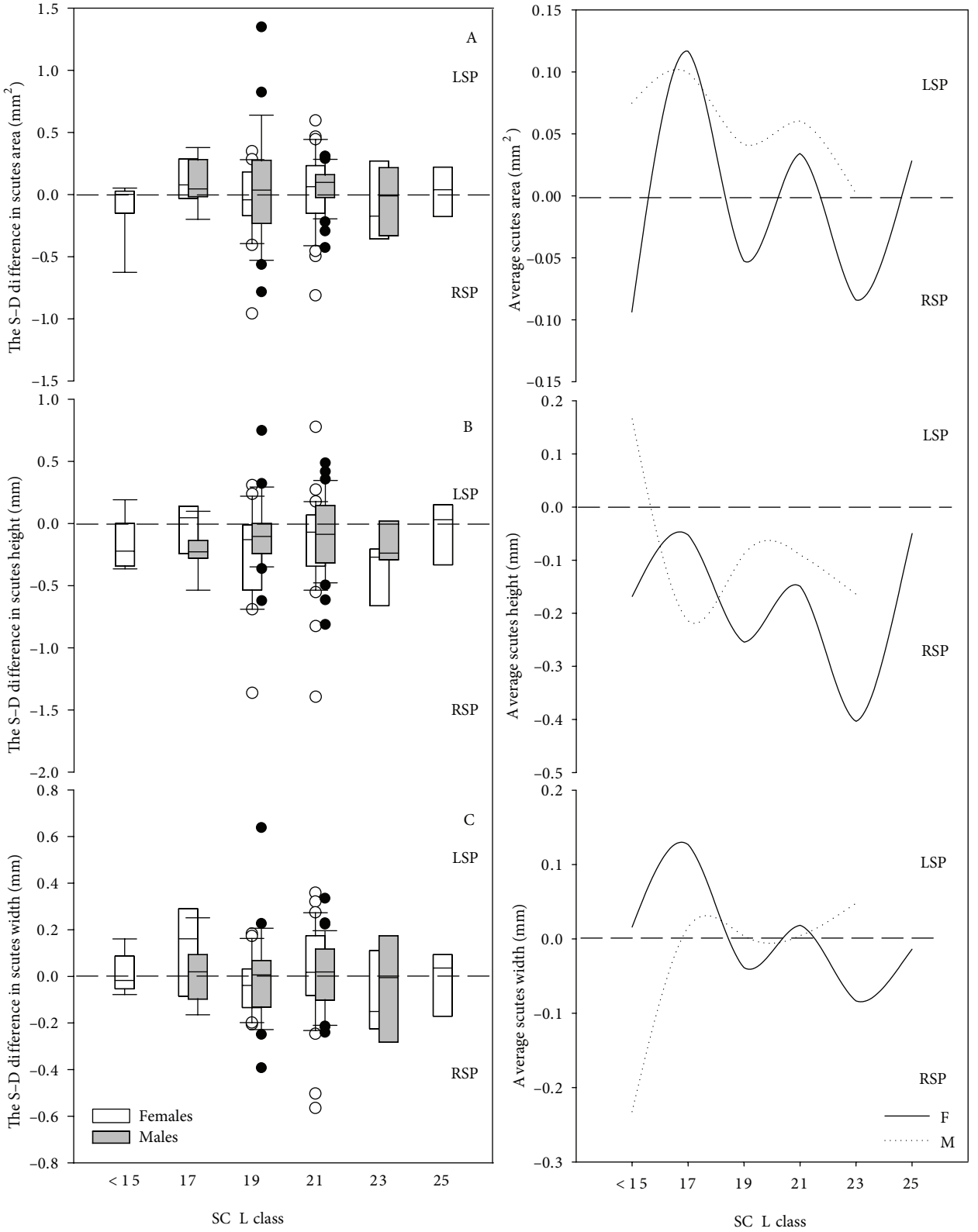

Figure 4. The distribution of the differences between the average values for area (A), height (B), and width (C) for the left (LSP) and right (RSP) sides of the plastron relative to straight carapace length (SCL) and the corresponding average value in each SCL class (females n = 79, male n = 76).

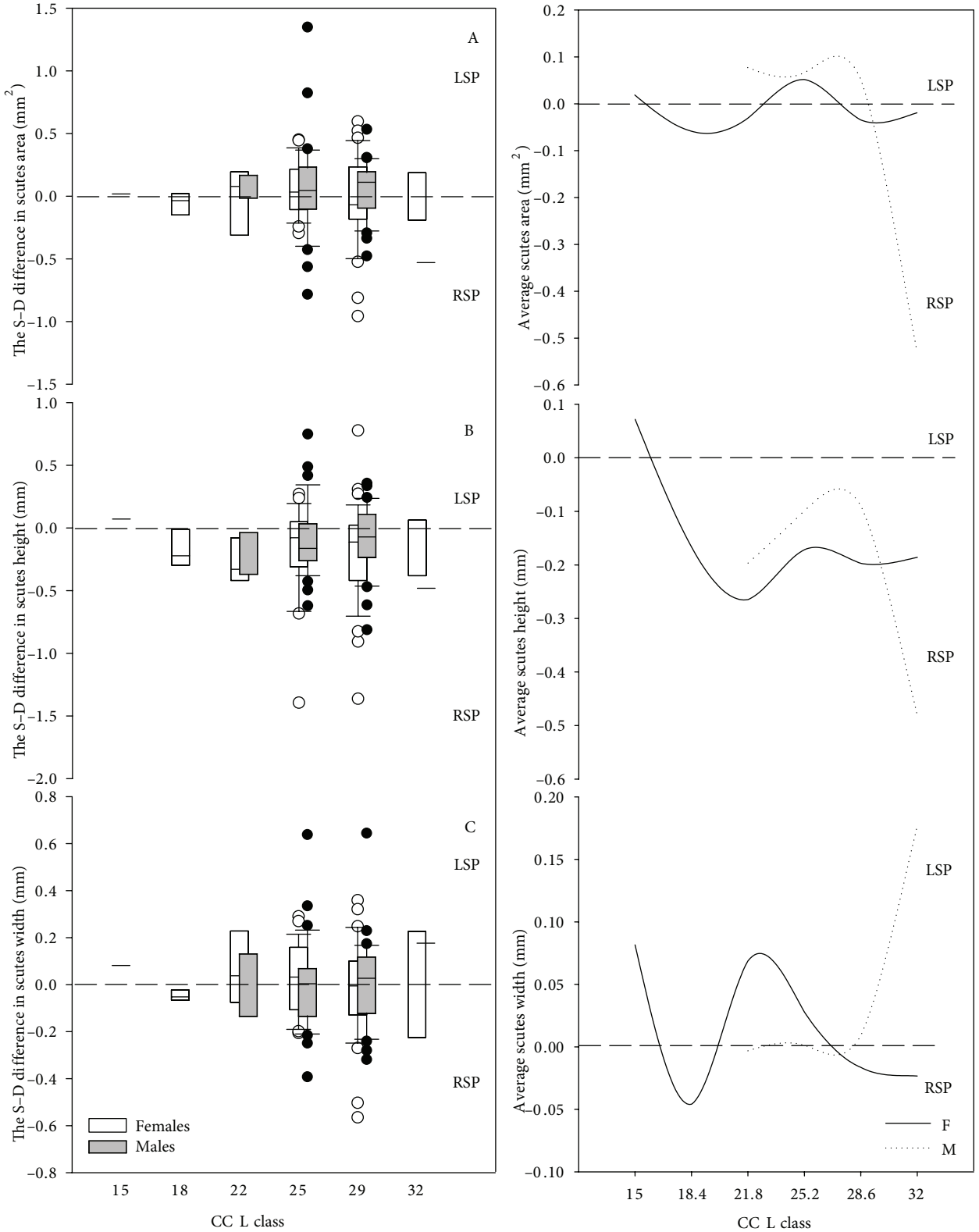

Figure 5. The distribution of the differences between the average values for area (A), height (B), and width (C) for the left (LSP) and right (RSP) sides of the plastron relative to straight carapace length (SCL) and the corresponding average value in each CCL class (females n = 79, male n = 76).

(Figure 6). This does not influence the general left–right symmetry of the plastron but does affect the difference of the area for the 2 analysed scutes. Individual variations were also recorded for humeral scutes in males, with the right side humeral scute having a larger area, height, or width. The differences were reduced, on average under 1 mm^2 for area and less than 1.5 mm for height and width. In females the left humeral scute had larger values for the 3 parameters, with the same average values. The femoral and anal scutes' variation was extremely reduced in both males and females. This result indicates a form of directional asymmetry (DA) (Razzetti et al., 2007).

4. Discussion

The results show reduced differences in FA in all 3 studied parameters for the 3 selected scutes. The influences of sex and body size (SCL, CCL) are not significant. The FA analysis may be influenced by anomalies in the development of the scutes.

The missing correlation for body size and any of the parameters leaves the observed variations in asymmetry open to speculation. A growth period is the most likely explanation, because a slight increase in asymmetry was observed with an increase in body size.

A decrease of FA with increasing size was observed only in males for the area and height of scutes. This trend is linked to the increase in body size and may be explained by the reduction in growth after sexual maturity (Willemsen and Hailey, 2003). This trend was not observed for scute width in relation to SCL.

The lack of relationship between FA and body size has been observed in studies on other populations of *T. graeca* (Băncilă et al., 2012). The differences in the scutes from the left and right sides are consistent with the data obtained by Davis and Grosse (2008).

The results of this study represent an estimate of the fitness status of the studied population at present. A similar study in the future, correlated with an estimate of human impact, could provide irrefutable proof of the usefulness of FA in estimating the status of populations of concern, or the absence of causality between FA and environmental stressors. The evolution of anthropogenic activities and changes in environmental factors are dynamics that must be considered in future studies that test the hypothesis of plastron deformation with an increase in size, and to highlight relationships with other features of the body of tortoises and environmental or hereditary factors (Møller and Swaddle, 1997).

While the general plastron asymmetry may be reduced, an internal asymmetry can exist in cases of abnormal scute growth or an abnormal number of scutes, resulting in an increased DA at individual level. Only in the case of height were there differences in all 3 parameters for males and females. This is probably related to the more diverse morphology of these scutes.

The method used in this study for estimating FA revealed results close to those in the literature. Although this method is time-consuming, the bias is reduced and it provides data for 3 parameters of the plastron, and implicitly for the left and right sides of the plastron, allowing a better perspective for the understanding of FA.

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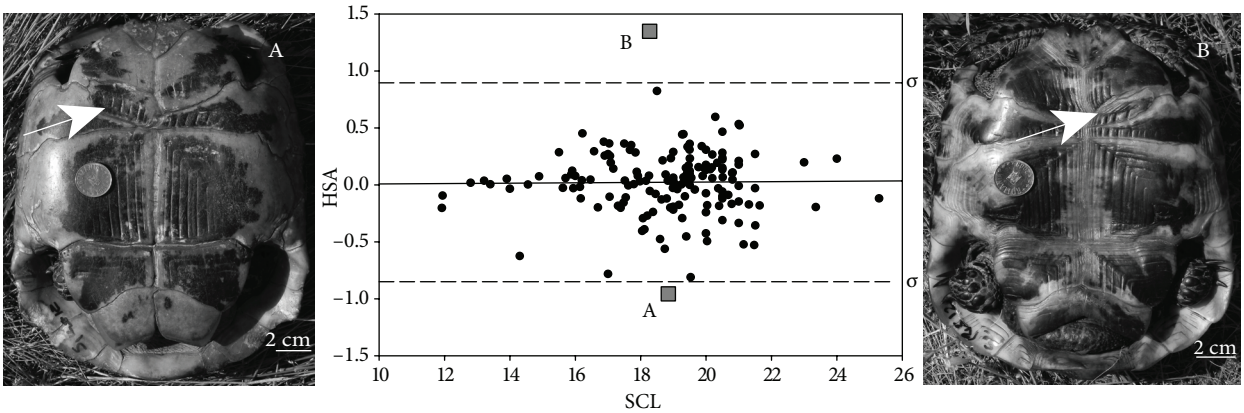

Figure 6. Regression plot with extreme variation in humeral scute area (HSA) for differences in left–right sides of the plastron due to malformations indicated by white arrows (SCL: straight carapace length; dashed line: standard deviation).

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