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Fluctuating Asymmetry in the Eurasian Spur-Thighed Tortoise, *Testudo graeca ibera* Linneaus, 1758 (Testudines: Testudinidae)

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ABSTRACT. – **We studied plastron-shaped asymmetry of the Eurasian Spur-Thighed Tortoise, *Testudo graeca ibera*, as related to life stage, gender, and distribution. Our analyses of 523 individuals showed that fluctuating asymmetry (FA) in plastron shape varied with gender (males exhibited higher levels of FA than did females) and across populations, whereas life stage had no significant effect. Although we could not identify the potential sources of variation responsible for the observed patterns of developmental instability, our study shows the value of FA as a method for studies of developmental instability in turtles.**

One of the most important tasks in evolutionary biology, population ecology, or conservation is estimating the fitness of an individual or a population. A simple measure of fitness that can be easily assessed by nondestructive methods is needed to identify populations subject to environmental stress before these populations are irreversibly affected. The developmental stability, (i.e., the ability of an individual to withstand random perturbations during its development) has been proposed as an index of individual fitness (Soulé 1967; Møller 1994) because it is considered an indicator of environmental and genetic stress. A common means of assessing developmental stability is through analysis of fluctuating asymmetry (FA) in bilateral traits (Van Valen 1962; Leary and Allendorf 1989; Palmer 1994). FA is defined as the minor random deviations from perfect bilateral symmetry as evidenced by measurable differences between the right and left sides (Van Valen 1962).

Table 1. Sampling localities and sample sizes. M = male, F = female, J = juvenile.

Country	Population	Coordinates		Sample size			
		Long	Lat	F	M	J	Total
Romania	Măcin (Măc)	E28.30	N45.06	92	177	51	320
Romania	Dumbrăveni (Dum)	E27.98	N43.94	59	83	16	158
Bulgaria	Cape Kaliakra (Kal)	E28.46	N43.38	8	14	6	28
Turkey	Efes (Efe)	E27.34	N37.94	5	12	0	17
Total				164	286	73	523

Tortoises are good model species for the study of FA because they have a shell that exhibits bilateral symmetry and provides a record of all major and minor growth perturbations occurring during their long life span (Lynn and Ullrich 1950). The plastron is made up of bony plates covered by scutes and can be important taxonomically to characterize turtle species (Lovich and Ernst 1989; Lovich et al. 1991; Ernst et al. 1997). Plastron scutes

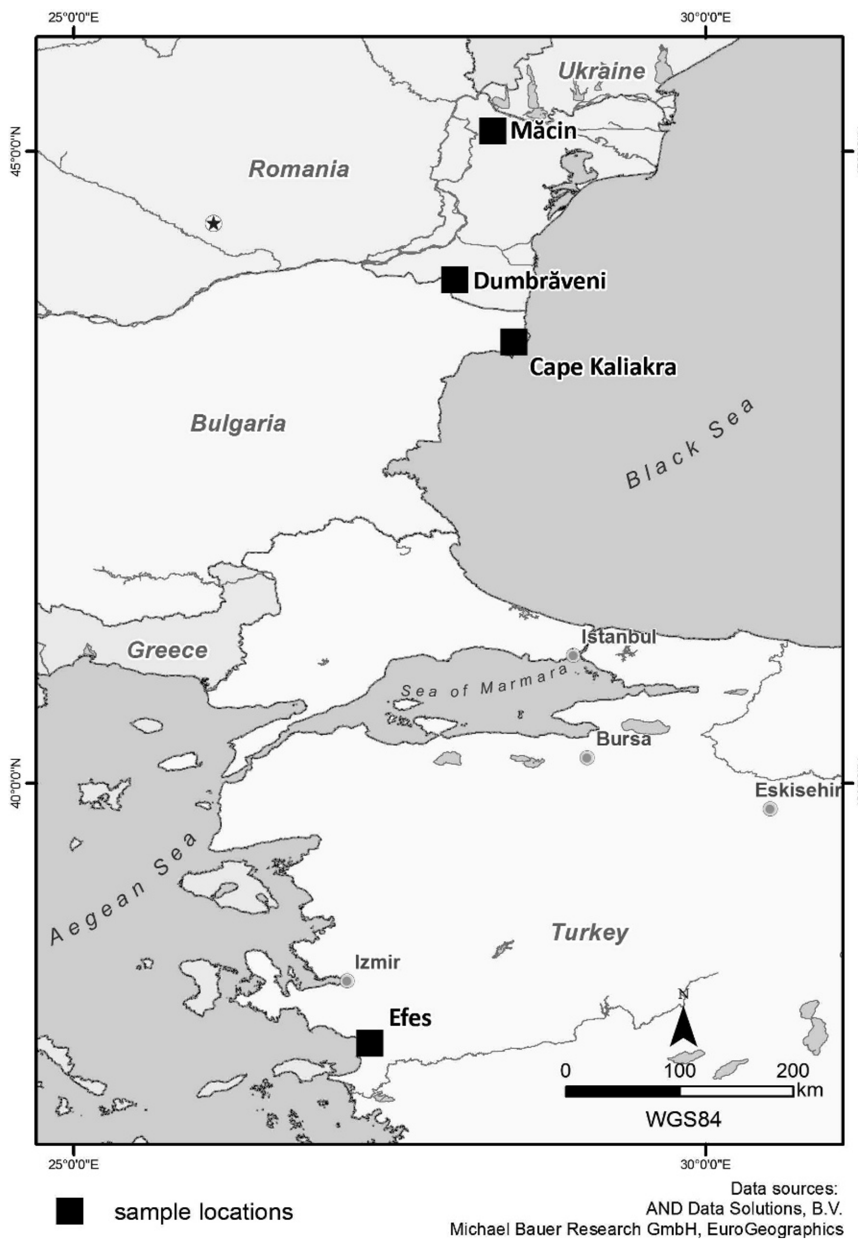

Figure 1. Study area showing the four sampling sites (black squares): Măcin (Măc, Romania), Dumbrăveni (Dum, Romania), Cape Kaliakra (Kal, Bulgaria), and Efes (Efe, Turkey).

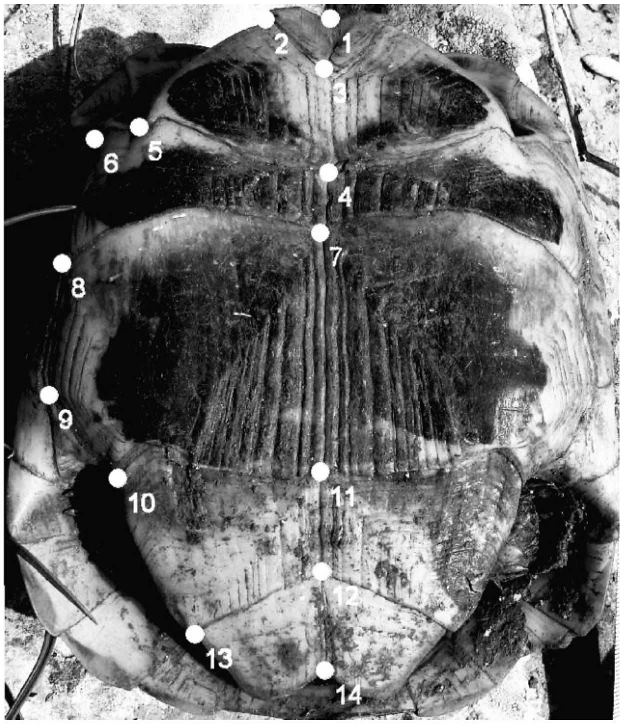

Figure 2. Definition of landmarks on the right side of the Eurasian Spur-Thighed Tortoise plastron.

grow larger by forming new tissues along the central suture line (Magwene 2001). This may result in a pressure from the opposing scutes directed toward the center, and as a tortoise ages, it may cause a deviation from the straight line normally seen in younger tortoises. Changes in plastron scute asymmetry with size (as a proxy for age) were demonstrated in the Yellow-Bellied Slider *Trachemys scripta* (Davis and Grosse 2008). FA has been recently studied using the landmark superimposition method (the so-called Procrustes superimposition method; Debat et al. 2000), a powerful geometric morphometric approach to the study of integrative morphological variation. FA can be accurately quantified using fixed landmarks on tortoise plastrons.

Turtles show sexual dimorphism with adult females attaining larger body sizes than do males (Congdon and Gibbons 1983; Gibbons and Lovich 1990; Rowe 1997; Aresco and Dobie 2000) in many species, possibly because females have a somewhat higher growth rate than have males (Chaloupka and Limpus 1997; Chaloupka et al. 2004). This variation in growth rates may result in symmetry differences between sexes. Females could be more symmetrical than males attributable to faster and consequently more efficient shell growth or, on the contrary, more asymmetrical than males attributable to the more frequent accumulation of errors in symmetrical growth (Davis and Grosse 2008).

In this study, we used FA to determine whether age (estimated from size), gender, and geographic location have an influence on plastron shape asymmetry in the Eurasian Spur-Thighed Tortoise (*Testudo graeca ibera*).

METHODS

Studied Populations. — The fieldwork was done during the period of 2006–2010. A total of 523 specimens of *T. g. ibera* (164 females, 286 males, and 73 juveniles) without any detectable abnormalities (such as injuries by predators or unusual additional scutes or plates) were used in the study. The specimens were sampled across a north–south gradient from the Măcin Mountains National Park, in Northern Dobruja, Romania, the northern limit of this species' range, down to Efes in Turkey (Table 1; Fig. 1).

Data Collection. — In the field, each live tortoise was assigned a number, which was temporarily written with a marker on the plastron, measured for the straight carapace length along the midline (MCL) and weighed on a portable electronic balance (KERN, model ABJ 220-4M). Sex was assigned with certainty for individuals with MCL $>$ 180 mm in Măcin population, MCL $>$ 154 mm in Dumbrăveni population, and MCL $>$ 110 mm in Cape Kaliakra population. Both the carapace and the plastron were photographed. Twenty-eight landmarks (14 symmetric pairs) were digitized on each plastron picture. The landmarks were digitized (two replicates per specimen) with the TpsDig 1.18 software (Rohlf 1999; Fig. 2).

Data Analysis. — The least-square Procrustes superimposition algorithm was used to obtain the coordinates of optimally superimposed landmark configurations of left and right sides scaled to a centroid size of 1. The superimposed landmark configurations were then entered into Procrustes ANOVA (Klingenberg and McIntyre 1998) to analyze: 1) the overall asymmetry (FA) of plastron shape, 2) directional asymmetry (DA; Van Dongen et al. 1999), and 3) measurement error (ME). The individuals and the plastron side were entered as random and fixed effects, respectively. FA is measured by the

Table 2. Results of Procrustes ANOVA with individual as a random effect.[a]

Source	SS	MS × 10,000	df	*F*
Side	0.000092	0.0383	24	1.956 (NS)
Individual	0.173	0.14	12,360	7.144*
Side × individual	0.024	0.0196	12,288	87.346***
Error	0.015	0.0002	668,808	

[a] SS = sum of squares; MS = mean square; df = degrees of freedom; NS = not significant, * $p < 0.05$, and *** $p < 0.001$.

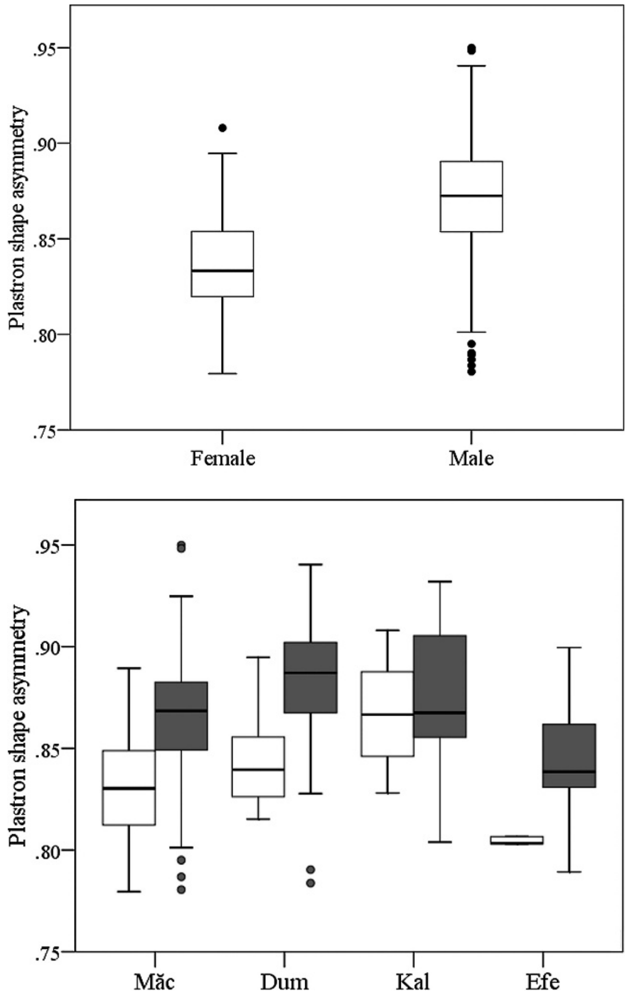

Figure 3. (Top) Influence of gender on mean plastron shape asymmetry in Eurasian Spur-Thighed Tortoise. Box plots display the interquantile range and outliers around the median. Errors bars represent 95% confidence intervals. (Bottom) Mean plastron shape asymmetry variation among populations separately on sexes (female—white boxes, male—gray boxes) in Eurasian Spur-Thighed Tortoise. Box plots display the interquantile range and outliers around the median. Errors bars represent 95% confidence intervals.

side × individual interaction; DA is expressed by the main effect for sides; and ME is expressed by the residual term. The degrees of freedom are those for ordinary ANOVA multiplied by the shape dimensions, which is twice the number of the landmarks minus the four degrees of freedom that are lost during superimposition (two degrees lost during translation, one degree for each of the two dimensions, and one each for scaling and rotation).

Antisymmetry (AS; Van Dongen et al. 1999) was determined by visually examining the scatter plots for each landmark of the left and right vectors after superimposition by the Procrustes algorithm. The clustering of vectors would suggest antisymmetry and is the equivalent to a binomial distribution of left/right differences. To test whether size dependency would affect the asymmetry of shape, regression of size (calculated as the centroid size of landmarks) against asymmetry (i.e., the absolute differences between the sides) was examined with multivariate regression (Jobson 1992). To analyze the subtle shape asymmetry differences between gender/life stages and populations, we used linear mixed models (LMM). Prior to analysis, the Kolmogorov-Smirnov *D*-test was used to check for normality and the Levene's test to ensure that the assumption of homogeneity of variance was not violated. Because there was no difference in FA between adults and juveniles ($F_{1,516} = 0.226$, $p = 0.798$), we analyzed only two groups: females and males. In LMM analysis, the Procrustes distance was entered as a dependent variable and gender as fixed factor. The population was treated as a random factor. We compared the plastron shape asymmetry among populations for each sex using one-way ANOVA. When significant differences were found, the least significant difference (LSD) was used for post-hoc multiple comparisons. All analyses were done using SPSS version 17.0 (SPSS, Inc., Chicago, 1999).

RESULTS

The Procrustes ANOVA of plastron shape variation showed that FA was statistically significant and higher than measurement error, whereas no significant directional asymmetry was found (Table 2). The examinations of scatter plots revealed no evidence for clustering of vectors of shape asymmetry that would have suggested antisymmetry (results not shown). The regression of unsigned shape asymmetry on mean centroid size was not significant ($r^2 = 0.079$, $p = 0.069$).

The level of asymmetry differed significantly between sexes ($F_{1,442} = 29.261$, $p < 0.001$). FA was higher in males compared to females (LSD; mean differences ± SE = 0.030 ± 0.006, $p < 0.001$; Fig. 3, top).

The results suggested significant differences in plastron shape asymmetry among populations in both sexes (females: $F_{3,163} = 5.881$, $p < 0.01$; males: $F_{3,285} = 9.153$, $p < 0.001$). There were significant differences in mean plastron shape asymmetry, after Bonferroni corrections, among female populations from Efes and Cape Kaliakra (0.061 ± 0.016, $p < 0.001$), Efes and Dumbrăveni (0.037 ± 0.015, $p < 0.05$), and Cape Kaliakra and Măcin (0.034 ± 0.012, $p < 0.05$) (Table 3; Fig. 3, bottom). The post-hoc comparisons showed significant differences in mean plastron shape asymmetry, after Bonferroni corrections, among male populations from Efes and Dumbrăveni (−0.04 ± 0.009, $p < 0.001$) and Cape Kaliakra and Efes (0.031 ± 0.023, $p < 0.05$) (Table 3; Fig. 3, bottom).

DISCUSSION

This study showed that FA in the plastron shape of the Eurasian Spur-Thighed Tortoise, *T. g. ibera* varied with gender and across populations, whereas carapace length had no significant effect on asymmetry. We found no significant differences in the levels of plastron shape

Table 3. The results of post-hoc comparisons showing the differences in the mean plastron shape asymmetry between the four studied populations of the Eurasian Spur-Thighed Tortoise, *Testudo graeca ibera*, separated on sexes: females (upper part of the matrix) and males (lower part of the matrix); in parentheses the significance level (*p*).

	Măc	Dum	Kal	Efe
Măc	0	0.010 ± 0.007 (0.139)	0.034 ± 0.012 (0.036)	0.027 ± 0.015 (0.390)
Dum	0.002 ± 0.004 (0.356)	0	0.024 ± 0.013 (0.318)	0.037 ± 0.015 (0.034)
Kal	0.008 ± 0.003 (0.238)	0.010 ± 0.016 (0.463)	0	0.061 ± 0.016 (0.006)
Efe	0.023 ± 0.005 (0.341)	0.041 ± 0.009 (0.001)	0.031 ± 0.023 (0.042)	0

asymmetry between adults and juveniles tortoises. A previous study (Davis and Grosse 2008) reported that plastron scutes were more asymmetrical in adults than in juveniles, assuming that adults of long-lived animals have more opportunities for erroneous growth because of longer exposure or accumulation of the stress effects. A possible explanation of our results may be that asymmetry in Eurasian Spur-Thighed Tortoise is the product of a stress during a critical stage of tortoise development and not one of cumulated effects from previous stresses over the entire growth history. Furthermore, the functionally integrated traits accumulate fewer developmental errors than do nonintegrated (linear measurements) traits (Badyaev et al. 2005). The integration of developmental noise of a functional group of traits will increase as they develop. This may be explained by the compensatory and constraining interactions among a great number of linked components (Swaddle and Witter 1997; Badyaev 1998; Hallgrımsson 1999; Aparicio and Bonal 2002; Hallgrımsson et al. 2004; Foresman and Badyaev 2005), which can accommodate the effects of stress.

Female tortoises have a faster growth rate than do males (Znari et al. 2005). This may be the main reason for the sexual differences in the FA. The faster growth in females could be argued as an indication of more ''efficient'' (i.e., more symmetrical) growth, with fewer bilateral errors. However, Lagarde et al. (2001) found a slight decrease in growth rate prior to maturation especially for females suggesting a reverse pattern of growth rate.

In conclusion, we found that levels of asymmetry varied significantly among populations in both males and females. It is difficult to discriminate between the natural levels of FA and those determined by stress. However, we suggest three possible reasons for the asymmetry differences observed among populations. The variation may be attributable to differences in environmental conditions, genetic variation (Møller and Swaddle 1997), or differences in sample size among populations (Palmer 1994). Although our study could not differentiate among the potential sources of variation responsible for the observed patterns of developmental instability, these efforts support the value of FA as a useful method for further studies of developmental instability in tortoises.

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