



Geographic (in-)variability of gender-specific traits in Hermann's tortoise

Sonja Djordjević¹, Ljiljana Tomović^{1,2}, Ana Golubović¹, Aleksandar Simović¹, Bogoljub Sterijovski³, Marko Djurakic⁴ & Xavier Bonnet⁵

¹Institute of Zoology, Faculty of Biology, University of Belgrade, Belgrade, Serbia, ²Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia,

³Herpetology Group, Biodiversity Section, Macedonian Ecological Society, Skopje, FYR of Macedonia, ⁴Department of Biology and Ecology, Faculty of Natural Sciences, University of

Novi Sad, Novi Sad, Serbia, ⁵Centre d'Etudes Biologiques de Chizé, CNRS-UPR 1934, Villiers en Bois, France

We processed 40 morphological characteristics of 676 adult Hermann's tortoises (*Testudo hermanni*) (310 males and 366 females) from four localities in the central parts of the Balkan Peninsula. Analyses of variance and covariance showed significant differences between males and females in 38 and 35 traits, respectively. On the other hand, analyses of geographic variability within each gender, i.e. analyses of variance and covariance of separate traits between localities showed "constant" dimensions and proportions of several body parts. Most of the invariable traits were the elements of the rear portion of the tortoises' shell and free body parts, in both genders. Among these are some of the most prominent sexually dimorphic traits in *T. hermanni*. Therefore, we speculate that "standard" sizes of certain superficial bodily attributes in this species are conditioned by their role in gender discrimination, as well as courting and mating endeavours.

Key words: central Balkans, Hermann's tortoise, invariable dimensions of body parts

INTRODUCTION

Major selective forces (i.e. ecological selection, fecundity and sexual selection) do not equally influence the variability of various morphological traits. For instance, sexual selection can favour the development of peculiar features such as bulky ornamentations in one sex (e.g., antlers in red deer), opposite to the influence of ecological selection (Zahavi, 1975; Andersson, 1994). The resulting assortment of morphological traits that characterize each species and sex is thus the outcome of complex interplay. Nevertheless, the respective reproductive role of each sex represents a major axis of variations (Fairbairn et al., 2007; Bonduriansky, 2007; Ceballos & Valenzuela, 2011; Östman & Stuart-Fox, 2011).

Among species, reproductive traits tend to diverge more markedly compared to non-reproductive traits, hence the widespread use of genital morphology to distinguish species otherwise similar in appearance (many arthropods: e.g., Mutanen et al., 2006; Costa-Schmidt & Araujo, 2010). An expected corollary is that within species, traits shaped by sexual selection, including genitalia, should vary less among populations (i.e. within species) compared to other traits (Anderson, 2000; Tatsuta et al., 2001; Mutanen et al., 2006). For instance, wide ranges of body sizes are observed across arthropods, fish, amphibian or reptile populations (e.g.,

Willemsen & Hailey, 1999) in response to local factors such as food availability, whereas less variation is expected for traits shaped by sexual selection (e.g., size and shape of genitalia, mating calls, pheromone composition; Teder, 1998; Jennions & Kelly, 2002). To our knowledge, this hypothesis has not been tested in reptiles.

The morphology of chelonians is well suited to examine the ideas presented above. The shell provides protection against predators but constrains locomotion and reproduction (e.g., it limits males mobility during mate searching, courting and mating). In tortoises and turtles, the general shell shape (i.e. bodily proportions) is influenced by fecundity selection in females and by sexual selection in males (Bonnet et al., 2001, 2010; Mann et al., 2006; Zuffi & Plaitano, 2007; Kaddour et al., 2008; Djordjević et al., 2011). Besides the investigations focusing on general shell shape, tortoises offer an opportunity to examine how discrete parts of the body vary in response to the main selective forces. Indeed, their external morphology can be described as a continuous set of sutured shell plates and free body parts covered with horny scales. All these elements can be measured separately and precisely, whereas a role in mating and non-mating activities can be attributed to most of them (McRae et al., 1981). For instance, adult males utilize their tail to inseminate females (a trait influencing mating success), whilst a relative enlargement of the costal scutes of the shell is associated with an increased abdominal

Correspondence: Sonja Djordjević (sonjadj@bio.bg.ac.rs)

volume and an enhanced capacity to hold large clutches in females (a trait largely unrelated to mating success). Furthermore, chelonians exhibit continuous growth patterns, a wide range of adult body sizes and thus offer a means to assess the relationship between growth and body shape (Chiari & Claude, 2011).

Total body size is also a key morphological trait influenced by ecological and sexual selection in chelonians (Berry & Shine, 1980; Stubbs et al., 1984). Both mean body size and the degree of sexual size dimorphism can vary geographically (Willemsen & Hailey, 1999, 2003; Ashton & Feldman, 2003; Ashton et al., 2007; Sacchi et al., 2007; Kaddour et al., 2008; Litzgus & Smith, 2010; Lovich et al., 2010). Considering that body size can be examined independently from body shape, we propose the following hypothesis: at the species level, total body size and the relative dimensions of non-genital body parts should be prone to substantial variation in response to ecological factors, while the proportions of the structures directly involved in mating should be less affected (Mutanen et al., 2006). Inter-population comparisons of the same species provide an opportunity to examine which parts of the body are the most variable in both sexes.

In the current study we examined sexual dimorphism in body size and body shape among disjunct populations of Hermann's tortoise, a species that displays substantial geographical variations in body size (Djordjević et al., 2011). The aims of this study were to analyze inter-population variability of main morphological traits (general and those directly involved in sexual interactions) in males and females, and to propose functional significance for the variability, or invariability, of the traits under focus.

MATERIALS AND METHODS

Study species

Hermann's tortoise, *Testudo hermanni*, is a medium-sized terrestrial species (Vetter, 2006; Bertolero et al., 2011) found in southern Europe and Turkey (Türkozan et al., 2005; Fritz et al., 2006; Bertolero et al., 2011). The eastern part of its range is occupied by *T. h. boettgeri* (Bertolero et al., 2011).

In *T. hermanni*, females are larger (e.g., Willemsen & Hailey, 1999, 2003; Bertolero et al., 2011; Djordjević et al., 2011), and sexes in the genus *Testudo* are generally dimorphic with regards to shell shape (Bonnet et al., 2001; Willemsen & Hailey, 2003; Kaddour et al., 2008). The most prominent sexually dimorphic traits are a longer and wider tail, wider anal notch, a more concave plastron, incurved supracaudal scutes, shorter bridges between carapace and plastron and a wider rear part of the carapace in males compared to females (Stubbs et al., 1984; Willemsen & Hailey, 2003; Zuffi & Plaitano, 2007; Djordjević et al., 2011).

Study sites and sampled populations

We sampled four populations: three in the Republic of Serbia and one in the Former Yugoslav Republic of Macedonia (FYROM), roughly along the 21st parallel,

covering approximately 3° latitude. General information about the localities and sample sizes are provided in Table 1. The populations are separated by about 100 km. Although the spatial genetic structure of the studied populations is currently unknown (Fritz et al., 2006), the limited dispersal ability of the species and the presence of many obstacles (topography, rivers, fragmented and unsuitable habitats, roads, etc.) suggest that populations are mutually isolated with low possibility of gene flow.

The three Serbian study sites (Trstenik, Prolom Banja, Pčinja River valley) are similar in altitude, topography, vegetation cover and climatic conditions (Republic Hydrometeorological Service of Serbia). The Macedonian site (Konjsko) lies on the north-western coast of the Prespa Lake (near Greece and Albania); it is situated further south and at higher altitude (accurate climatic data for this area are not available).

Samples and measurements

We recorded morphometric traits previously used in the literature (Bonnet et al., 2001; Willemsen & Hailey, 2003; Kaddour et al., 2008), including three recently introduced measurements (Djordjević et al., 2011). "General" measurements refer to overall body size and shape, whereas "Additional" measurements were used to depict subtle body shape details (Bonnet et al., 2001; Carretero et al., 2005; Kaddour et al., 2008; Djordjević et al., 2011). Among these, we focused on distinctive traits directly related to mating activities. Names, abbreviations, and short descriptions of the traits measured are provided below:

1) General measurements: Straight Carapace Length, SCL (straight distance from the cervical scute to the tip/tangent of the supracaudal); Curvilinear Carapace Length, CCL (curved distance from the tip of the cervical to the tip of the supracaudal scute); Mid-body Carapace Width, MCW (width of the carapace at the 6th marginal scutes); Maximal Carapace Width, MaxCW (maximal width of the posterior part of the carapace); Curvilinear Carapace Width, CCW (distance between left and right seam between carapacial and plastral scutes at the 6th marginal scutes); Midline Plastron Length, MPL (from the notch between gulars to the notch between anal scutes); Maximal Plastron Length, MaxPL (from the tips of gulars to the tips of anals); Plastron Width at the 6th pair of marginal scutes, PW₆; Maximal Plastron Width, MaxPW; Total Longitudinal Circumference, TLC (along the longer axis of the shell); Mid-body Circumference, MBC (along the shorter shell axis, at the 6th marginals); Shell Height, SH (at the highest point of the shell, at the level of 3rd vertebral scute); Body Mass, BM; Front and Hind Limb Length, Left and Right, FLL_L, FLL_R, HLL_L, HLL_R; Bridge Length, Left and Right, BL_L, BL_R (length of the bridge between carapace and plastron); Head Length, HL (from the tip of the snout to the rear edge of jaw joint); Skull Length, SL (from the tip of the snout to the rear edge of the cranium); Head Width, HW (at the widest point, at the level of orbits); Head Height, HH (at the highest point); Tail Length, TL (from the anterior edge of cloaca to the tip of the tail).

2) Additional measurements: Width of the 2nd, 3rd and 4th Vertebral scutes (WV₂, WV₃, WV₄); Length of midline sutures of the plastral scutes: Gulars, Humeral, Pectorals, Abdominals, Femorals and Anals (GSL, HSL, PSL, AbSL, FSL, AnSL); Anal Notch Width, ANW (distance between the tips of anal scutes); Analia to Supracaudal Junction, ASJ (distance from the bottom of the anal notch to the tip of supracaudal); Plastron Concavity, PC (depth of the concavity at the centre of the plastron); Shell Height Posterior, SHP (from the cross-junction of femoral and anal scutes to the anterior edge of the areola on the 5th vertebral scute - see figure 1 in Djordjević et al., 2011); Curviness of the 5th vertebral scute (horizontal distances from the tip of, i.e. tangent to supracaudal to the, equally vertically distributed, four points on the 5th vertebral scute, C₁, C₂, C₃, C₄ - see figure 1 in Djordjević et al., 2011); Supracaudal Curviness, ScC (from the tip of, to the tangent to supracaudal scute - see figure 1 in Djordjević et al., 2011); Claw Length, CL (length of the horny claw on the tip of the tail).

We measured straight-line measurements using a dial calliper (precision 0.1 mm) and curvilinear traits with flexible tape (precision 1 mm). Body mass was measured with a digital scale (precision 0.1 g). For the symmetrical body parts (shell bridges and limbs) we averaged the two values.

We considered only adults: males larger than 130 mm (straight carapace length, SCL) and females larger than 150 mm (Hailey & Loumbourdis, 1990; Willemsen & Hailey, 1999, 2003). Tortoises were processed in the field and released immediately after measuring at the place of capture. Each individual was permanently marked for future identification using a modified method of Stubbs et al. (1984). No animals were injured or mistreated during the study.

Statistical analyses

Our goal was not to provide a single descriptor of body size or body shape, but to examine a set of discrete traits. Therefore, we did not rely on integrative measurements (e.g., PCA, geometric mean). Similarly, we did not implement all morphological variables into integrative models in an attempt to extract the main contributors of body size and shape to assess sexual dimorphism or population divergences. Instead, we performed *ad hoc* analyses on each trait (and thus generated relatively long tables of results, see Appendix). This somewhat fastidious approach was essential to limit the influence of undesired source of variance associated to highly variable traits (e.g., body mass, size) and to examine subtle variations or poorly variable traits (see Results). Finite element analysis based on landmarks on the shell, plastron, head, tail and legs would have provided an excellent alternative to explore which parts of the shell are the most variable in each sex and across populations (e.g., Stayton, 2009). Unfortunately, this method is not easily applicable in the field, is time consuming and thus precludes the use of a large sample size ($n > 650$ tortoises in the current study).

Overall, we relied on analyses of variance and covariance. This approach also offered the possibility for comparisons with previous studies (Djordjević et al.,

2011 and references therein). Straight Carapace Length (SCL) was used as co-variate for all the measurements; for the elements of the rear part of the shell we repeated the analyses with tail length (TL) as a co-variate. For example, scaling ANW (distance between the tips of anal scutes) relative to body size would indicate if the space to move the tail is proportionally larger in one sex, or different among populations.

We firstly assessed sexual dimorphism in size and shape, and then we focused on inter-population variability. To limit the influence of variance associated with sex or population differences, we followed a systematic approach. We first compared absolute and size-corrected dimensions of each measured trait between sexes by pooling all individuals; then, we re-performed the same analyses on each population separately.

As we focused on a simple question (do the traits involved in mating vary more/less than the other traits?), we considered that significant *versus* non-significant differences across populations would provide a robust index for such an assessment. Reproductive roles are clearly separated in tortoises; therefore we performed the analyses in each gender separately. Consequently, a lack of difference across populations could be attributed to a strong stability of the trait examined, especially if body size and body shape (size-corrected traits) varied significantly. We performed analyses of variance and covariance (SCL as the covariate) in each sex. Body size greatly overlapped between sexes and across populations (see Results), allowing us to perform analyses of variance easily. Slopes of all traits against SCL were checked for homogeneity.

Finally, we calculated a Sexual Dimorphism Index (SDI) for the pooled sample and for each population separately, both for mean values (dimorphism in size) and for adjusted means (SCL as the covariate, shape dimorphism) to facilitate comparisons across samples. We used the formula from Willemsen & Hailey (2003): $SDI = [(F-M)/M] \times 100$. Statistics were performed using Statistica 5.1 (Statsoft Inc.).

RESULTS

Sexual dimorphism in body size and body shape

Analysis of variance (40 traits) and covariance (SCL as covariate, 39 traits) revealed consistent patterns of sexual size and sexual shape dimorphism. In the pooled sample, we found significant differences between the genders in almost all traits (except FSL and C₄ in ANOVAs and CCW, MBC, GSL and C₁ in ANCOVAs: Appendix, Tables A1 and A2). Females were larger than conspecific males in all but five (12.5%) measured traits, namely tail length (TL), anal notch width (ANW), plastron concavity (PC), supracaudal curviness (ScC) and length of the horny claw on the tail (CL), which were larger in males (Appendix, Table A1). Body shape analyses showed a different pattern: 23 traits were significantly larger in males (almost 59%), while 12 (31%) were significantly larger in females, and four (approximately 10%) were not significantly different between the sexes (Appendix, Table A2). Analyses

Table 1. Characteristics of the four localities and corresponding sample sizes of adult tortoises. The first three localities are in Serbia, the fourth is situated in FYR of Macedonia.

Locality	Pčinja River valley	Prolom Banja	Trstenik	Konjsko village
Coordinates	42°19'N 21°53'E	43°20'N 21°25'E	43°37'N 21°00'E	40°54'N 20°59'E
Altitude (m a.s.l.)	600	600–800	300–500	800–900
Dominant relief	Hills, mild slopes; flat river valley	Hills, mild slopes	Hills, mild slopes	Hills, mild slopes; flat lake shore
Males	162	23	62	63
Females	122	80	100	64
Total	284	103	162	127

performed separately in each population led to similar trends, with minor differences (significant *versus* non-significant effects), without conflicts (Appendix, Tables A1 and A2).

Overall, we observed significant sexual size and shape dimorphism for almost all traits examined. SDI analyses provided convergent results and revealed that some of the most pronounced sex differences (% of divergence) were related to morphological traits involved in mating (tail size or plastron concavity for instance) (Appendix, Table A3).

Geographic (in-)variability in morphology of males and females

Analyses of variance performed separately in males and females revealed that most traits varied across populations (Appendix, Table A4). In males, the absolute size of most traits differed substantially among populations ($n=36$, 90%), except anal notch width (ANW), tail length (TL) claw length (CL) and plastron concavity (PC) (Table 2). Similarly, in females, most traits were variable ($n=38$, 95%) and two traits were not (Table 2; Appendix, Table A4): the length of the suture between anal scutes (AnSL) and curviness of the supracaudal scutes (ScC).

Body shape analyses (ANCOVAs) showed convergent trends, although the number of invariant traits substantially increased when SCL was used as covariate (Table 2; Appendices, Table A5). Fifteen traits (38%) were invariant in males and eleven (28%) were invariant in females (Table 2). The proportions of invariant *versus* variable traits across populations were not different between sexes ($\chi^2=0.62$, $p=0.43$ and $\chi^2=0.92$, $p=0.34$, respectively, for absolute dimensions and size-corrected traits).

In tortoises, the tail can be functionally considered as an important element of the “complex” of body parts involved in mating; therefore, we performed further analyses with this organ’s features. When tail length was used as a covariate in the analyses of the traits directly involved in copulation, no significant difference among the samples was found for anal notch width (ANW) in males and length of anal scutes suture (AnSL) in females (Table 2; Appendix, Table A5).

DISCUSSION

The prominent sexual dimorphism we describe in *Testudo hermanni* from the central parts of the Balkans (Tables in the Appendix) is in accordance with previous findings (e.g., Willemsen & Hailey, 2003; Djordjević et al., 2011). However, the causes of variation in body sizes and shape, and in the degree of sexual dimorphism were not the main topic of the current study; instead we focused on the inter-population variability of a set of discrete traits. Variations across populations have been studied previously, but they were limited to taxonomic purposes (e.g., Brophy, 2004; Türkozan et al., 2010).

Inter-population morphological stability of males

In general, this study showed that amongst males from different populations, both absolute and relative values of several morphological traits of the rear part of the shell and body were invariant (i.e. stable: Table 2). All these traits are directly involved in mating activities of males. The male tail sheaths the penis and supports it during courtship and mating, two distinct activities that involve substantial physical contact between mates (Hailey, 1990; Willemsen & Hailey, 2003). The distance between the tips of anal scutes (ANW), combined with the length of suture between them (AnSL) and the distance to the rear part of the carapace (ASJ), delimits the space available for ventral and lateral movements of the tail plus penis (Mosimann & Bider, 1960; McRae et al., 1981; Bonnet et al., 2001; Willemsen & Hailey, 2003). We assume that dimensions of the space available for tail movements in males could be determined by the dimensions of the tail itself, (i.e. the penis positioned in its basis). The claw on the tip of the tail and curved supracaudal scutes are rigid structures that additionally support the tail and contribute to intromission, while plastron concavity enhances mounting (Willemsen & Hailey, 2003; Pritchard, 2008). In addition, a prominent tail claw is used to stimulate females (Hailey, 1990). Therefore, the combination of the aforementioned traits could be viewed as a complex of traits that facilitates copulation.

Among the three Balkan *Testudo* species, two of them, *T. graeca* and *T. marginata* lack the long “claw” on the tail tip, and their courtship and mating differ from these

Table 2. List of geographically stable morphological traits in females and males of *T. hermanni* (ANOVA and ANCOVA of the separate characters within the two genders, among four localities).

	Females	Males
ANOVA		
Rear shell and body parts	AnSL	ANW
	ScC	TL
		CL
		PC
ANCOVA, SCL as the covariate		
Shell domedness	CCL	CCL
	TLC	TLC
		MBC
Plastron		PC
		MPL
	MaxPL	MaxPL
	PW ₆	
	GSL	GSL
	HSL	HSL
	AbSL	AbSL
	FSL	
Rear shell parts	AnSL	
	ANW	
	ScC	ScC
Head and legs		HW
		HH
		FLL
	HLL	HLL
ANCOVA, TL as the covariate		
	AnSL	ANW

in *T. hermanni* (Hailey, 1990; Willemsen & Hailey, 2003). Therefore, we can suppose that the complex of mating-related traits serves as an isolating mechanism among these species, notably as they are syntopic in various places (Willemsen & Hailey, 2003).

It was previously shown that certain genital and non-genital structures involved in tactile communication between males and females of arthropods are sexually selected and comparatively stable in size - a "one-size-fits-all" hypothesis (Eberhard et al., 1998; Eberhard, 2010). Subsequent studies on several other taxa, including vertebrates, confirmed that the sizes of external male genital structures do not co-vary with male body size (Anderson, 2000; Bernstein & Bernstein, 2002; Bertin & Fairbairn, 2007; Ramm et al., 2010). On the contrary, body parts which are being advertized from some distance (visual or auditory) are prone to exaggeration in size/colouration/overall appearance in numerous animal groups (Eberhard et al., 1998). Thus, it is supposed that various, functionally different males' genital and non-genital traits are differently perceived and therefore under different modes of female choice and sexual selection (Eberhard et al., 1998; Song & Wenzel, 2008). Therefore, the invariable sizes of several elements of the rear part of tortoise males' body and

shell could be viewed in the light of the aforementioned hypotheses. We can assume that the penis and/or supportive muscular and rigid horny/bony structures in *T. hermanni* are under stabilizing sexual selection.

Results of ANCOVA also showed that several general shell dimensions (CCL, TLC, MPL, MaxPL) did not differ among males from the four populations (Table 2, Appendix, Table A5). Invariable relative values of curved carapace length and total longitudinal circumference imply a certain "constant" degree of shell domedness. Previous studies (e.g., Sacchi et al., 2007) showed that shell domedness varies with latitude, suggesting its significance in thermoregulation. If the roundness of the shell is highly important in maintaining body temperature, it is not surprising that it was weakly variable in thermally similar environments. Concerning plastron length (both midline and maximal), similar mean values across populations may be the result of similar pressures of natural and sexual selection that influence the dimensions of the plastron (Bonnet et al., 2001).

In addition to similarities of males rigid structures (shell elements), we showed that certain less rigid, i.e. free body parts were stable as well. Relative dimensions of the limbs displayed no geographic variability in males. We assume that these locomotory structures, which play important roles in reproductive behaviour (chasing mates, fighting, mounting, supporting during copulation: Hailey, 1990), can also be under sexual selection which acts in stabilizing manner considering that the broad shape of female shell does not vary geographically. Limb size invariability across populations may also reflect similarities of the habitats (e.g., Molina-Borja et al., 2010): all the tortoises sampled live in areas with similar topography. Head dimensions (height and width) were also invariable among our samples of males. These traits could also be under sexual selection, as biting and head bobbing play an important role in chelonian reproductive behaviour (Auffenberg, 1965, 1977; Schafer & Krekorian, 1983; Willemsen & Hailey, 2003; AG personal observation). Alternatively, natural selection could stabilize head dimensions in similar environments (food quality and quantity).

Inter-population stability in morphology of females

In females, several general shell dimensions (CCL, TLC, MaxPL, PW₆) did not differ across populations (Table 2; Appendix, Table 4). However, in females, the invariable parts of the body are not exactly the same compared to males. The low variability of the maximal plastron length and midline plastron width may be the result of similar pressure of fecundity selection, which enlarges the plastron, and thus increases the size of a belly-protective structure and the space available for body reserves and for the clutches (e.g., Bonnet et al., 2001; Schwarzkopf, 2005; Kaddour et al., 2008). The rear part of the shell was also invariable (stable) (Table 2). All these traits are related to reproductive activity in females. Anal notch width (ANW), length of the suture between anal scutes (AnSL) and supracaudal curviness (ScC) limit the space for intromission and for eggs passing. However, in the absence of data on the size of the eggs amongst

populations, we cannot adequately address this question at the moment.

In addition to similarities among females in rigid structures (shell parts), we showed that relative dimensions of hind legs (HLL) displayed no geographic variability. This finding can be related to the important role of hind legs in reproduction (digging nests), and can also be under strong stabilizing natural selection.

It is extremely difficult to obtain accurate measurements of the genitalia of living animals. The testing of hypotheses proposed to date (lock and key, sexual selection by female choice, post-copulatory male competition, i.e. sperm competition, etc.), which explain the causes and consequences of inter- and intraspecific variation in genital structures, might be possible in the future, after the comparisons of a wider array of taxa.

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