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## Ontogeny of pileus shape in Natrix natrix and N. tessellata

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We examined the divergence in pileus shape and ontogenetic allometric changes in pileus shape in two closely related species of snakes - *Natrix natrix* and *N. tessellata*. These two species have similar ecology, but different microhabitat and diet preferences. We analysed the ontogenetic series from hatchlings to adults. Hatchlings of two species significantly diverged in pileus size and shape. At the adult stage, significant sexual dimorphism in pileus size and shape was found within both species. Adults of *N. natrix* and *N. tessellata* (females and males) diverged in pileus shape, but not in size. Allometric shape changes accounted for significant amount of variance in pileus shape among different age classes, and appear to be species- and sex-specific. Our results suggested that allometries of pileus shape are highly evolvable traits, which may result from natural selection for functional optimisation of head shape in Natricine species.

Key words: geometric morphometrics, head scales, Natricinae, ontogenetic trajectories, sexual dimorphism

## **INTRODUCTION**

he ontogenetic changes in form (size and shape) of related species provides an insight into the processes of evolutionary diversification and helps us to understand mechanisms and constraints underlying morphological variability (Alberch et al., 1979; Klingenberg, 2010; McNamara, 2012; Pélabon et al., 2014). In closely related taxa, it is expected that the directions of the ontogenetic trajectories are conserved (i.e. the allometric slopes do not diverge), due to developmental constraints inherited from the common ancestor (Weston, 2003; Frederich & Sheets, 2010; Wilson & Sanchez-Villagra, 2010; Pélabon et al., 2014). However, ontogenetic trajectories of shape can considerably diverge between closely related species (Gidaszewski et al., 2009; Urošević et al., 2013), and between ecological morphs within species (Kaliontzopoulou et al., 2008). To understand the relationship between evolutionary changes in morphology and allometry, empirical data on various groups and morphological structures are still needed (Pélabon et al., 2014).

In this study, we analysed ontogenetic variation in the dorsal head scalation (pileus) in two closely related species from the genus *Natrix*. The pileus is generally rather conserved in the Natricine, and thus has taxonomical relevance. The two analysed *Natrix* species (the grass snake *Natrix natrix* Linnaeus, 1758; and the dice snake *N. tessellata* Laurenti, 1768) diverged from the common ancestor 13–22 my ago (Guicking et al., 2006), and have the same basic organisation of the pileus. The grass snake is typically associated with lentic water habitats such as ponds and lakes, where it feeds mostly on anurans (Beebee & Griffiths, 2000; Latifi, 2000; Szczerbak, 2003). The dice snake prefers relatively open, often rocky shore habitats close to water and feeds mostly on fish (Mebert, 2011).

The goals of this study are to address whether i) species or sexes within species differ in the size and shape of the pileus, ii) the shape of the pileus changes during ontogeny, and iii) whether the differences in pileus shape resulted from the allometric changes or changes in the direction of ontogenetic trajectories. Although *N. natrix* and *N. tessellata* are closely related sister species, a divergence in pileus shape and ontogenetic allometries could be explained by different hunting strategies and prey preferences. We also expected similar allometries of pileus shape in females and males, and assumed that divergence in pileus shape between sexes could be explained by allometric shape changes.

## MATERIALS AND METHODS

### Analysed locality and samples

A sample of 423 individuals of both species and across a range of life stages was collected at the locality of Pančevački rit (Serbia, vicinity of Belgrade – 44°50'N, 20°29'E, Table 1). We used syntopic populations of grass and dice snakes to eliminate possible geographic variation (Ajtić et al., 2013; Luiselli et al., 2005). All snakes were captured by hand and stored in calico bags until they were measured. The snakes were fixed on a panel with a camera (Olympus SP 590 uz, 12 MP). We kept the head of the snake in the centre of the optical field, with the

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Fig. 1. Landmarks of the pileus used in this study. Medial landmarks: 1 – point of maximum curvature on the tip of the rostral scale, 3 – the point of connection between internasal and rostral scales, 6 - the point of connection between prefrontal and internasal scales, 10 - the point of connection between prefrontal and frontal scale, 16 - the point of connection between frontal and parietal scales, 21 – the posterior point of connection of parietal scales. Bilaterally symmetric landmarks: 2 (left side) and 4 (right side) – point of connection between internasal, rostral and nasal scales, 5 (left side) and 7 (right side) - point of connection between internasal, prefrontal and nasal scales, 8 (left side) and 12 (right side) / point of connection between prefrontal, supraocular and preocular scales, 9 (left side) and 11 (right side) - point of connection between prefrontal, frontal and supraocular scales, 13 (left side) and 19 (right side) - point of connection between supraocular, postocular scale and the edge of the eye, 14 (left side) and 18 (right side) point of connection between supraocular, postocular and parietal scales, 15 (left side) and 17 (right side) / point of connection between supraocular, frontal and parietal scales, 20 (left side) and 22 (right side) - posterior-most point on the curve of the parietals scales.

pileus oriented in parallel to the photographic plane. All animals were then released at the capture site. Sex was determined by the presence of a hemipenis, determined by squeezing the tail in immature individuals, or by examining the shape of the tail base in adults (Mebert, 1993). We measured snout-to-vent length (SVL, following

**Table 1.** Sample size for analysed species (*N. natrix* and *N. tessellata*) separated by sex (males and females) and age classes.

Sex	Age	Natrix natrix	Natrix tessellata
Females	Hatchlings	5	6
	Immatures	60	88
	Adults	39	24
Males	Hatchlings	6	8
	Immatures	46	73
	Adults	33	60
	Total	178	245

Beuttell & Losos, 1999; Calsbeek & Smith, 2007), and classified males above 48cm SVL and female above 55cm SVL as adults (Luiselli & Rugiero, 2005), where individuals under these threshold values were consider as immatures. The data for hatchlings were obtained from transferring eggs to the laboratory until hatching, before animals were re-released at the capture site.

#### **Data analyses**

To analyse variation in size and shape of the pileus, we applied a geometric morphometric approach (Zelditch et al., 2012), by quantifying allometry (the statistical relationship between size and shape) by regression of the Procrustes shape coordinates on size variables (centroid size). This approach was previously used to explore allometric shape changes of the pileus in various lacertid lizard species (Kaliontzopoulou et al., 2008; Ljubisavljević et al., 2011). For the analysis of the shape and size of the pileus, we chose a configuration of 22 landmarks (Fig. 1). The landmarks were digitised using the program tpsDig2 (Rohlf, 2010) by the same person (MA). Each specimen was digitised once because preliminary analyses revealed no variation due to measurement error (<4%).

To obtain shape variables we performed Generalised Procrustes Analysis (GPA), which eliminates the differences caused by size, position and orientation, by scaling, translating and rotating the configuration of landmarks (Rohlf & Slice, 1990; Dryden & Mardia, 1998). As a measure of size we calculated the centroid size (CS), which represents a measure of dispersion of landmarks from the centre (centroid) of the given configuration (Zelditch et al., 2012). Mathematically, CS is the square root of the summed squared distances of each landmark from the centroid of the configuration. To eliminate asymmetry and reduce the error in positioning of the pileus relative to camera lens, the average of original and mirrored configurations of each specimen (Klingenberg et al., 2002) were obtained (symmetric component of shape variation). A principal component analysis (PCA) on covariance matrices of the symmetric component was performed for hatchlings and adults separately, and for the entire sample (hatchlings, immature and adult individuals) of both species. In MANOVA and MANCOVA tests, PC scores were used as shape variables (Webster & Sheets, 2010). We selected the scores on the first ten PC axes that describe >90% of variance in pileus shape in all



**Fig. 2.** The warped outline graphs illustrate pileus shape differences between species for hatchlings. Grey lines present pileus shape of *N. natrix,* black lines present pileus shape of *N. tessellata*.

Significancei								
	Size				Shape			
A)	SS	Df	F	p	Wilks' lambda	Df1/Df2	F	р
Species	0.0028	1	6.97	0.015	0.0175	10/12	67.33	0.001
Sex	0.0002	1	0.60	0.447	0.4232	10/12	1.64	0.208
Species x Sex	0.0002	1	0.46	0.503	0.6709	10/12	0.59	0.796
В)								
Species	0.0028	1	1.6	0.205	0.0537	10/143	251.91	0.001
Sex	0.2147	1	122.6	0.001	0.6223	10/143	8.68	0.001
Species x Sex	0.0072	1	4.1	0.045	0.7229	10/143	5.48	0.001

**Table 2.** Tests for differences in pileus size (ANOVA on log CS) and shape (MANOVA on PC scores) between *N. natrix* and *N. tessellata* hatchlings (A) and adults (B). Df: degrees of freedom, SS: sum of squares; *F*: F test statistic, *p*: statistical significance.

analysed groups (Table A1, Online Appendix), assuming that remaining variance is related to measurement error.

The differences in CS between species, sexes and developmental stages were investigated using analysis of variance (Two-Way ANOVA, factors species, sex and their interaction). Divergence in pileus shape between hatchlings and adults was analysed by multivariate analysis of variance (Two-Way MANOVA, factors species, sex and their interaction). In order to test whether differences in pileus shape between species and/or sexes are a consequence of differences in ontogenetic trajectories, we performed a multivariate analysis of covariance (MANCOVA) with shape as a dependent variable, species and sex as factors and log CS as a covariable. Statistically significant interactions between species or sex and log CS would indicate that the onto genetic trajectories of the species or sexes are different.

The relationships between shape and size for each species and sex were estimated with a multivariate regression of shape (symmetric component) onto log CS, and visualised using warped outline drawings (Klingenberg, 2013). The amount of size-dependent

**Table 3.** Tests for differences in pileus shape - MANCOVA on shape variables (PC scores), A) with species and sex as a factor and size (Log CS) as a covariable B) with sex as a factor and Log CS as a covariable for both species *N. natrix* and *N. tessellata*.

Wilks' lambda	Df1/Df2	F	р		
0.6724	10/406	19.78	0.001		
0.8963	10/406	4.70	0.001		
0.2005	10/406	161.88	0.001		
0.7111	10/406	16.49	0.001		
0.8777	10/406	5.66	0.001		
0.9504	10/406	2.12	0.022		
0.9473	10/406	2.26	0.014		
Wilks' lambda	Df1/Df2	F	p		
Natrix natrix					
0.8049	10/165	4.00	0.001		
0.1951	10/165	68.05	0.001		
0.7821	10/165	4.60	0.001		
Natrix tessellata					
0.9297	10/232	1.75	0.070		
0.1886	10/232	99.78	0.001		
0.9171	10/232	2.10	0.025		
	Wilks' lambda 0.6724 0.8963 0.2005 0.7111 0.8777 0.9504 0.9473 Wilks' lambda 0.8049 0.1951 0.7821 0.9297 0.1886 0.9171	Wilks' lambda Df1/Df2   0.6724 10/406   0.8963 10/406   0.2005 10/406   0.7111 10/406   0.8777 10/406   0.9504 10/406   0.9473 10/406   Wilks' lambda Df1/Df2   Natrix na 0.8049   0.1951 10/165   0.7821 10/165   0.9297 10/232   0.1886 10/232   0.9171 10/232	Wilks' lambda Df1/Df2 F   0.6724 10/406 19.78   0.8963 10/406 4.70   0.2005 10/406 161.88   0.7111 10/406 5.66   0.9504 10/406 2.12   0.9473 10/406 2.26   Wilks' lambda Df1/Df2 F   0.8049 10/165 4.00   0.1951 10/165 4.60   0.7821 10/165 4.60   Natrix tessellata 0.9297 10/232 1.75   0.1886 10/232 99.78 0.9171 10/232 2.10		



**Fig. 3.** The warped outline graphs illustrate pileus shape differences between : A) females *N. natrix* (grey lines) and *N. tessellata* (black lines); B) males *N. natrix* (grey lines) and *N. tessellata* (black lines); C) females (grey lines) and males (black lines) in *N. natrix*: D) females (grey lines) and males (black lines) in *N. tessellata*. All the shape changes are exaggerated 2-fold for better visibility.

shape variation was quantified as a percentage of the total variation explained by size, and its statistical significance was estimated using a permutation test with 10,000 iterations against the null hypothesis of independence between size and shape (Good, 1994). Procrustes superimposition, multivariate regression and visualisation of shape changes were carried out using the software MorphoJ (Klingenberg, 2011), while ANOVA, MANOVA and MANCOVA were performed in SAS v.9.1.3 (SAS Institute Inc).

### RESULTS

## Differences in pileus size and shape of hatchlings and adults of *N. natrix* and *N. tessellata*

Centroid sizes of the pileus for hatchlings were 14.7±0.7 (females) and 14.7±0.8 (males) for N. natrix, as well as 15.6±0.6 (females) and 15.2±0.6 (males) for N. tessellata. Centroid sizes of the pileus for adults were 34.9±3.9 (females) and 28.1±1.9 (males) for N. natrix, as well as 33.2±4.2 (females) and 28.5±2.5 (males) for N. tessellata. The pileus size is significantly different between the species in hatchlings, but not in adults (Table 2). Only adults showed a differences in pileus size between the sexes, and there is marginal interaction between species and sex (Table 2). Grass snakes and dice snakes significantly differed in pileus shape at both hatchling and adult stage. Sexual dimorphism in pileus shape was found at the adult stage only, when the interaction between sex and species was statistically significant (Table 2). The shape changes between hatchlings of N. natrix and N. tessellata are presented in Fig. 2. *Natrix natrix* have a proportionally shorter and wider pileus than *N. tessellata* (especially for internasal and prefrontal scales).

At the adult stage, females and males of N. natrix had a proportionally shorter and wider pileus than N. tessellata (Fig. 3A, B). The shortening and widening of the pileus of N. natrix are largely due to shape changes (shortening and widening of the internasal, prefrontal, parietal and widening of supraocular scales). Also, the frontal scale appears to be relatively larger in N. natrix comparing to N. tessellata. Within species, males had a slightly shorter and wider pileus than females (Fig. 3C, D). In both species, parietal scales were overall shorter in males than in females. In males of N. natrix, the frontal scale is more elongated than in females. A MANCOVA with species and sex as factors, and log CS as covariable showed a significant interaction between species, sex and log CS (Table 3A). A MANCOVA with sex as factor and log CS as covariable showed significant interaction between sex and log CS in both species, indicating that the ontogenetic trajectories of the species and sexes are different (Table 3B).

#### Ontogenetic changes of pileus shape

In *N. natrix,* allometry accounts for 31% (females) and 41% (males) of the variation in pileus shape. These shape changes are expressed as elongating and widening of internasal and prefrontal scales, and narrowing of frontal and supraocular scales in both sexes. Changes in the length of parietal scales display opposite trends in females and males (elongation in females and shortening in males).

In *N. tessellata*, allometry accounts for 33% (females) and 30% (males) of the variation in pileus shape. These shape changes are related to an increase in the relative size of the anterior part of the pileus (especially the internasal scales) and change in shape of frontal and supraocular scales, which become triangular in adult individuals. The shape changes of the frontal and supraoculars are related mostly to the reduction in the length of the suture between the frontal and parietal scales. The shape changes in the posterior part of the pileus are similar to those described for *N. natrix*, and related to a reduction in size of the suture between the parietals in adults, while the posterior part becomes slightly elongated.

### DISCUSSION

Our results indicated that two closely related snake species, *N. natrix* and *N. tessellata*, significantly differ in ontogenetic trajectories of the pileus shape, which also diverge between the sexes in *N. natrix*. Previous studies on closely related species have revealed both conserved allometric trajectories (Marroig, 2007; Üzüm et al., 2014), but also that allometric trajectories of closely related species significantly diverge from each other (Gidaszewski et al., 2009; Cvijanović et al., 2014), even between consecutive ontogenetic stages that differ in feeding modes (Frederich et al., 2008). Our data indicate that changes in pileus shape from hatchlings to adult individuals have the same direction in the two study species, which both have marked shifts in feeding preferences during ontogeny (Mushinsky et al., 1982; Fillippi et al., 1996; Luiselli et al., 1997, 2007; Velensky et al., 2011).

Examining the ontogenetic sources of morphological variation can shed light on evolutionary processes, for instance by pinpointing the age at which interspecific differences first appear (Klingenberg, 1996). Hatchlings of *N. natrix* and *N. tessellata* have different pileus shapes, indicating that ontogenetic trajectories already start to diverge at a prenatal state. A further divergence during ontogeny suggests that ontogenetic allometric shape changes are highly evolvable traits.

Interspecific differences in pileus shape could reflect general differences in head shape, and divergence in head shape in Natricine snakes can be explained by diversity in foraging ecology and correlations among form, function and diet (Shine, 1991; Herrel et al., 2008). Dice snakes have an elongated head and feed on fish (Mebert, 2011), while grass snakes have a more robust head and predominantly feed on frogs (Beebee & Griffiths, 2000). Also, differences in feeding environment (aquatic or terrestrial; De Queiroz, 1992; Werler & Dixon, 2000; Hibbitts & Fitzgerald, 2005) and predatory behaviour (Herrel et al., 2008; Van Wassenbergh et al.,



**Fig. 4.** The ontogenetic shape changes of the pileus. The represented shape changes are from juveniles to adult for females and males; A) *N. natrix,* B) *N. tessellata*. The significance of the multivariate regression of shape variable on log CS, and the proportion of variation in shape for which the regression accounts is provided for each group separately. The warped outline graphs illustrate the pileus shape changes during ontogeny (hatchlings – grey outline; adults – black outline).

### 7

2010) could affect head size and shape. The observed sexual dimorphism in pileus shape is further reflected in general differences in head shape between the sexes in snakes (Arnold, 1993; Shine, 1994). Further studies on morphological structures more tightly linked to feeding (such as head shape and shape of cranial skeleton), would provide more information about the effects of natural selection on functional optimisation and evolvability of allometry in the Natricine group.

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