Post-metamorphic ontogenetic changes in head size and shape of the pool frog (*Pelophylax lessonae*, Ranidae)

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Abstract: Post-metamorphic ontogenetic variations of head size and shape were investigated in the pool frog (*Pelophylax lessonae*, Ranidae). We showed that allometry is an important aspect of the post-metamorphic ontogeny of the pool frog as most of the differences between the head shape of subadults and adults were size-related adjustments. The largest changes affected by size variations included the posterior part of the head and the snout and eye region. In comparison to subadults, adults have wider heads, shorter snouts and relatively smaller eyes. Analyses of the relationships between head size and shape and the maximum size of the prey indicated the absence of correlations in adults. A positive correlation was found in subadults, but only between head width and the maximum size of the prey. Further studies will allow us to draw more generalized conclusions about the ecological relevance of the variations in head size and shape during post-metamorphic development of the pool frog.

Key words: ontogeny; allometry; anurans; diet; maximum prey size

INTRODUCTION

The vertebrate head, as a complex morphological structure, has primary functions in protecting and supporting the brain, the main sense organs and the trophic apparatus [1]. Studies of variations in head size and shape should contribute to a better understanding of the relationship between form and function in developmental, ecological and evolutionary contexts.

The size and shape of the vertebrate head correlate with a number of biological functions such as feeding, intra- and interspecies social interactions, defense against predators, agonistic behavior related to territoriality, mate acquisition and locomotion [2-5]. As head morphology is under various selective pressures, the outcome of such evolutionary processes can be very distinct [6-12].

One of the factors with a profound impact on variations in head shape is ontogenetic scaling (increase in size during ontogeny) as an organism's size is one of the main components of fitness affecting ecology, behavior and physiology [13-17]. Changes in head form during ontogeny are allometric in tetrapods [15,18-22], and proximate causes of allometric scaling during cranial growth are not well known. One of the scenarios is that the ontogenetic shift in ecology can be a driver for adaptive changes in the scaling. Microhabitat utilization and therefore predator vulnerability, prey availability, physical exchange with the environment and social interactions often depend on body size [23].

Anurans are a good model system to elucidate the relationship between form and function through ontogenetic scaling, as anurans of different age (and size) partition their habitat. Ontogenetic habitat partitioning in different size classes was found specifically for the depth of water and distance from shore [24,25]. This implies that different ontogenetic stages are under different selective pressures due to differences in feeding, competition, predation and physiological constraints [26,27] that can affect head size and shape. However, studies of the ontogenetic scaling of anuran head morphology and those examining the impact of

diet on head size and shape are scarce, especially for post-metamorphic development [28-32].

In this study, we examined the changes in post-metamorphic head size and shape in subadults and adults of the pool frog (*Pelophylax lessonae*, Ranidae). Additionally, in both size classes, we explored the relationships between maximum prey size and head size and shape. The described pattern of head shape variation (in the light of ontogenetic scaling) could provide basic information for future investigations related to the functional basis of size and shape changes in the head of this species.

MATERIALS AND METHODS

Sampling

All animals were collected in accordance with permits provided by the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia (no. 353-01-554/2017-17). Pool frog females (*P. lessonae*, Serbia, Obedska bara (44°43' N, 19°53' E): 16 subadults, 36 adults) were obtained from the Batrachological Collections of the Institute for Biological Research "Siniša Stanković", Belgrade. To avoid bias due to pronounced sexual dimorphism in anurans [33,34], our sample was composed of one sex only. Sex was determined by inspection of the gonads. Specimens below 50-mm total body length were classified as subadults, and those above as adults [35]. The sample size in this geometric morphometric study was large enough for appropriate estimation of different parameters [36].

Data acquisition and processing

We used landmark-based geometric morphometrics to analyze the variations in head size and shape. High-resolution photographs of the dorsal head view were taken using a Sony DSC-F828 digital camera (resolution 8.0 MP; Sony Corp., Tokyo, Japan). The objective was set to be parallel to the head surface. Graph paper was placed under the frogs to record scale. To access variations in dorsal head size and shape across ontogenetic stadia, we chose a configuration of 13 two-dimensional landmarks. The landmarks were digitized by the same person (MK) using the program tpsDig2 [37]. To obtain shape variables, we performed Generalized Procrustes analysis (GPA), which eliminates differences due to position, scale and orientation [38,39]. We used a symmetric component of shape variation (the average of original and mirrored configurations of each specimen) in order to eliminate asymmetry and reduce error in the positioning of the head relative to the camera lens [40]. As a measure of the size we calculated the centroid size (CS), which represents the dispersion of landmarks from the center of the given configuration [41]. In addition, traditional morphometric measurements, with an accuracy of 0.01 mm, were taken with digital calipers as follows: total body size from the tip of the snout to the cloaca (L), head length (Lc) and head width (Ltc). All specimens were dissected and the size of the maximum prey (prey size, PS) was measured.

Statistical analysis

To explore variations in size, analysis of variance (ANOVA) with log CS as the dependent variable and ontogenetic stadium as the independent variable was performed. To test for differences in shape, multivariate analysis of variance (MANOVA) with shape variables as dependent variables and the ontogenetic stadium as the independent variable was performed. In addition, the difference in overall (allometric + non-allometric) shape variation between subadults and adults was quantified by Procrustes distance (Pd), a linear measure of shape differences between landmark configurations [42]. The statistical significance of shape differences between ontogenetic stadia was evaluated using a permutation test with 10000 iterations against the null hypothesis of no mean difference between subadults and adults [43,44].

To obtain the allometric component of shape variation and to estimate the impact of allometry on shape changes we used multivariate regression of symmetric component of shape variation onto log-transformed CS. The percentage of shape changes that can be predicted by size differences and statistical significance was tested with a permutation test against the null hypothesis of allometry independence [45]. To explore size-independent shape variations between subadults and adults, residuals from the multivariate regression were used. The difference in the non-allometric component of shape variation between subadults and adults was quantified by Procrustes distance.

Traditional morphometric measurements were tested for normality and were logarithmically transformed prior to further analyses to improve homoscedasticity and normality. As ontogenetic stadia differed in total body size (t-test, $P<0.05$), all traits were corrected for size by calculating the following ratios: Lc/L, Ltc/L, PS/L. Also, centroid size (CS) was corrected for total body size (CS/L). Differences between subadults and adults in size-corrected measures were tested by the t-test.

To access correlations between maximum prey size (PS/L) and head size and shape variables (CS/L, Lc/L, Ltc/L, symmetric component of shape), Pearson's correlation coefficients were calculated.

Procrustes superimposition, multivariate regression and visualization of shape changes were done using the software MorphoJ [46]; t-test, MANOVA, homogeneity of slope test, and Pearson correlations were performed in Statistica 10 (StatSoft Inc.).

RESULTS

Differences in body and head size

In comparison to subadults, the pool frog adults had significantly larger total body length (L), centroid size (CS), head length and head width (Lc and Ltc) (Table 1). After correction for size, there was no difference in head length and width (Lc/L, Ltc/L). However, head size (CS/L) was significantly larger in subadults indicating that they had larger heads for a given body size (Table 1).

Differences in head shape

MANOVA with ontogenetic stadium as a factor indicated significant head shape differences between subadults and adults (Wilks' Lambda = 0.2723, $F_{22,26}=2.2610$, $P<0.05$). Procrustes distance showed a significant difference between mean subadult and adult head shapes (Pd=0.0271, $P<0.05$). The main overall changes in head shape between subadults and adults involved a widening of the head (landmarks 1, 2, 3, 4), shortening of the snout (11, 12, 13) and relative reduction of eyes in adults (Fig. 2).

Table 1. The mean values and the standard deviations (SD) for Uncorrected variables (Total body length – L, Centroid size – CS, Head length – Lc and Head width – Ltc) and Size-corrected variables (Centroid size – CS/L, Head length – Lc/L and Head width – Ltc/L) of the pool frog *Pelophylax lessonae*.

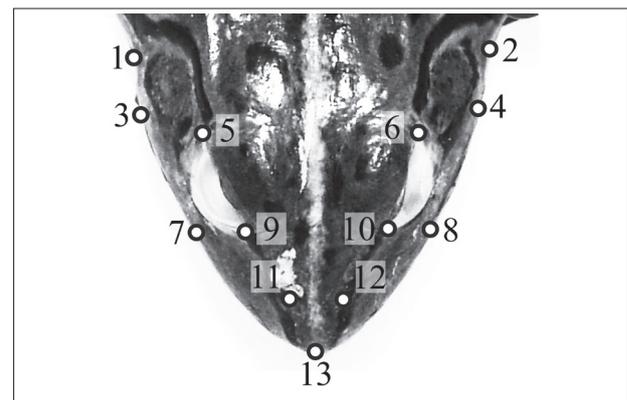|	Subadults			Adults			
	$\bar{x}$	±	SD	$\bar{x}$	±	SD	*P*
Uncorrected variables							
Total body length (L)	46.07	±	2.31	64.39	±	5.55	**<0.001**
Centroid size (CS)	28.85	±	1.75	38.61	±	3.43	**<0.001**
Head length (Lc)	15.65	±	1.88	20.90	±	2.72	**<0.001**
Head width (Ltc)	16.36	±	1.45	22.00	±	2.04	**<0.001**
Size-corrected variables							
Centroid size (CS/L)	0.63	±	0.03	0.60	±	0.02	**<0.001**
Head length (Lc/L)	0.34	±	0.05	0.33	±	0.03	>0.05
Head width (Ltc/L)	0.36	±	0.03	0.34	±	0.02	>0.05

Fig. 1. Landmarks recorded on the dorsal side of the head of the pool frog (*Pelophylax lessonae*). 1, 2 – jaw at the level of posterior edge of the tympanum; 3, 4 – jaw at the level of the anterior edge of the tympanum; 5, 6 – posterior corner of the eye; 7, 8 – jaw at the level of the anterior corner of the eye; 9, 10 – anterior corner of the eye; 11, 12 – nostril; 13 – tip of the snout.
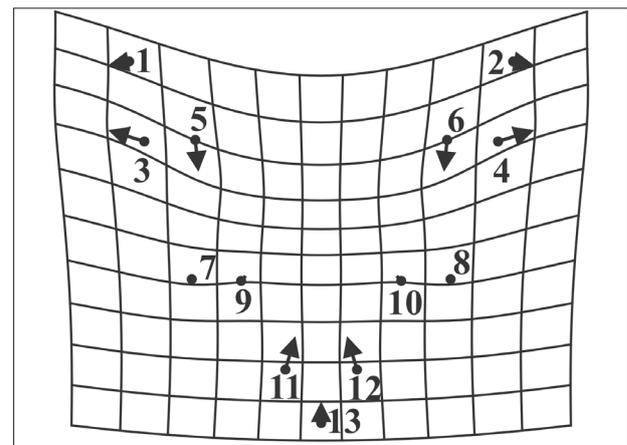

Fig. 2. Ontogenetic (from subadults to adults) overall change in head shape in the pool frog (*Pelophylax lessonae*). 1-13 – as in Fig. 1.

Allometric and non-allometric changes in head shape

Allometry had a significant impact on the changes in head shape in the pool frog (16.47%, $P<0.001$). The homogeneity of the slope test revealed that allometric slopes for subadults and adults were the same (Wilks' Lambda=0.3958, $F_{22,24}$=1.6647, $P>0.05$). Allometric changes in head shape correspond to overall changes in head shape between subadults and adults (widening of the head, shortening of the snout and relative reduction of eyes). After removing the effect of size on shape, Procrustes distance showed no significant difference between subadults and adults in the non-allometric component of head shape variation (Pd=0.0086, $P>0.05$).

Relation between head size and shape and maximum prey size

Adults consumed significantly larger prey than subadults (mean±SD, subadults: 9.74±2.43, adults: 14.18±5.82; $P<0.05$). However, after correction for size (PS/L), there was no difference in maximum prey size between subadults and adults (mean ± SD, subadults: 0.21±0.05, adults: 0.23±0.09; $P>0.05$). Prey size (PS/L) was not correlated with any of the analyzed variables (Lc/L, Ltc/L, CS/L, symmetric component of shape), except with head width in subadults (r=0.78, $P<0.05$).

DISCUSSION

The results of this study revealed differences in head shape between subadult and adult pool frogs, with adults characterized by wider heads and shorter snouts, but relatively smaller eyes. Since allometry had a profound impact on head shape variation, after removing the effect of size, subadults and adults did not differ in head shape. Considering relations between head size and shape and the maximum size of the prey, our results showed the absence of these correlations in adults. A significant positive correlation was found in subadults, but only between head width and maximum size of prey.

Ontogenetic head shape changes in anurans have been studied at larval stages [29,30,47], but data regarding the post-metamorphic development of head

are scarce. Ponssa and Candioti [32] showed that post-metamorphic ontogenetic shape variation in the skull of the *Leptodactylus fuscus* group is associated with elongation of maxillary bones, widening of the nasals and widening of the skull in the region of the squamosals. Like this study, we found that the most pronounced head shape changed during the post-metamorphic development of the pool frog, and encompassed the posterior part of the head (region of squamosals) and snout (region of nasals). In addition, our study showed the relative reduction of eye size during post-metamorphic development. This pattern is in agreement with general ontogenetic scaling in tetrapods [13], where development includes the reduction in the relative size of the sensory capsules and allometric and/or isometric growth of trophic structures. Allometry accounted for the main changes in head shape in our study. Isometry is generally unexpected in ontogenetic studies because different anatomical regions have to grow at different rates to maintain a function that would be lost if growth proceeded geometrically [13]. For instance, the relative increase in width of the posterior part of the head in pool frog adults could be related to their diet and maintenance of successful food acquisition, processing and ingestion.

One of the goals of this study was to establish whether maximum prey size is correlated with head size and shape during post-metamorphic ontogeny. Diet composition and the functional properties of prey are important factors that affect head size and shape in many animal groups [7,9,28,48-54]. Mechanical requirements of feeding are reflected through head morphology. Species consuming hard and large prey have taller and wider heads that can accommodate larger jaw muscles and increase the bite force [28,53,55]. However, studies of diet in European green frogs, including the pool frog, are limited as regards diet composition [56-58], with only one study analyzing prey size and its correlation with body size [59]. Studies in juvenile and adult specimens of water frogs from the *Pelophylax esculentus* complex showed that they have different diets [58,60], while the degree of trophic niche overlap between them indicated the existence of ontogenetic trophic niche partitioning [60]. For anurans it is well documented that different age groups, i.e. the age groups of different sizes, have different microhabitat preferences [24,25]. By occupying

different niches, they modify their exposure to food, predators and/or conspecifics that in turn determine their survival and fitness [23]. Although prey size may influence the head size and shape, our study showed that larger individuals do not eat larger prey, apart from subadults whose head width is positively correlated with maximum prey size. It should be noted that a larger sample size of both ontogenetic stadia is required to drawn more general conclusions. However, our results are nonetheless helpful for understanding the variations in head size and shape during the post-metamorphic development in the pool frog.

Studies of ontogenetic habitat partitioning are missing, with more in-depth access of ecological relevance of the ontogenetic head shape variation. Further studies are needed to improve our understanding of the relationships among ontogenetic shifts in form, function, and ecology in anurans.

Author contributions: Study conception and design: TDV, NTK. Acquisition of data: MK, MA. Analysis and interpretation of data: MK, TDV. Drafting of manuscript: TDV, NTK. Critical revision: TDV, MK, MA, NTK.

Conflict of interest disclosure: We have no conflicts of interest to declare.

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