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The ontogenetic origins of skull shape disparity in the *Triturus cristatus* group

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SUMMARY Comparative studies of ontogenies of closely related species provide insights into the mechanisms responsible for morphological diversification. Using geometric morphometrics, we investigated the ontogenetic dynamics of postlarval skull shape and disparity in three closely related crested newt species. The skull shapes of juveniles just after metamorphosis (hereafter metamorphs) and adult individuals were sampled by landmark configurations that describe the shape of the dorsal and ventral side of the newt skull, and analyzed separately. The three species differ in skull size and shape in metamorphs and adults. The ontogenies of dorsal and ventral skull differ in the orientation but not lengths of the ontogenetic trajectories. The disparity of dorsal skull shape increases over ontogeny, but that of ventral skull shape does not. Thus, modifications of ontogenetic trajectories can, but need not, increase the disparity of shape. In species with biphasic life-cycles, when ontogenetic trajectories for one stage can be decoupled from those of another, increases and decreases in disparity are feasible, but our results show that they need not occur.

INTRODUCTION

A major goal of evolutionary biology is to explain the origin and dynamics of morphological disparity, that is, morphological diversity (Gould 1991; Foote 1993a, 1997; Ciampaglio et al. 2001; Zelditch et al. 2003a). Explanations are typically classed into two broad categories, those that focus on external factors such as the available ecological space and the functional constraints imposed upon morphology by ecology, or internal factors, such as developmental and/or genetic constraints (e.g., Foote 1995; Valentine 1995; Eble 2000; Ciampaglio 2002; Zelditch et al. 2003a). By analyzing the ontogeny of form and the ontogenetic changes in disparity, the role of both internal and external factors can be dissected. The role of external factors can be illuminated when disparity changes over ontogeny due to ontogenetic niche shifts (e.g., Werner and Gilliam 1984; Claessen and Dieckmann 2002; La Croix et al. 2011). Ontogenetic studies illuminate the role of internal factors as well, by uncovering the evolutionary changes in ontogeny that determine the disparity of form.

Empirical studies have shown that evolutionary modifications of ontogeny can reduce as well as increase disparity (e.g., Zelditch et al. 2003a; Adams and Nistri 2010; Drake 2011; Frédérich and Vandewalle 2011; Gerber 2011; Ivanović et al. 2011; Piras et al. 2011; Urošević et al. 2013). Although it may seem intuitively obvious that developmental constraints should limit disparity, low disparity can even result from the *lack* of developmental constraints because, in the absence of constraints restricting modifications of ontogeny, two or more modifications of ontogeny can counteract each other; a phenomenon termed "counterbalancing" (Zelditch et al. 2003a) or "ontogenetic convergence" (Adams and Nistri 2010). In those cases, each modification, taken separately, can increase disparity but two (or more), taken together, decrease it (Zelditch et al. 2003a).

The most dynamic patterns of disparity may result from complex life-cycles because the decoupling of between phases can allow each one to adapt independently (e.g., Strauss and Altig 1992; Fisher-Rousseau et al. 2010; Frédérich and Vandewalle 2011; Ivanović et al. 2011). In effect, this decoupling makes developmental stages modular in that each stage can respond selection without interfering with the adaptations of another phase. That decoupling can increase morphological diversity, as it does in one group with a biphasic life-cycle, damselfishes which undergo a transition from homogeneous oceanic environment to the more ecologically heterogeneous coral reef environment (Frédérich and Vandewalle 2011). But that increase in disparity is also found in a group with a continuous life-cycle, lacertid lizards (Urošević et al. 2013). The decoupling between developmental phases can also reduce disparity as documented in two groups with biphasic life-cycles, including piranhas (Zelditch et al. 2003a) and frogs of the

Leptodactylus fuscus group (Ponssa and Candioti 2012) but also in an amphibian group with direct development, European cave salamanders (Adams and Nistri 2010) and one group of saxicolous lacertid lizards (Urošević et al. 2013). In crested newts, the focus of the present investigation, larval ontogenies diverge, concordant with patterns of interspecific differences in adult form, but larvae reach the juvenile stage with similar sizes and shapes, converging on a similar juvenile body form (Ivanović et al. 2011). Metamorphosis is hypothesized to reset the ontogenetic trajectories of crested newts, with post-metamorphic ontogeny producing the disparity seen in adults (Ivanović et al. 2011).

Amphibians are one of the best-known examples with complex life cycle, characterized by ontogenetic niche shifts and often dramatic morphological transformations such as the transformation of gilled, aquatic larvae into terrestrial juveniles. Crested newts (genus *Triturus*) have a complex life-cycle comprising an (1) aquatic larval stage, (2) metamorphosed, terrestrial juvenile stage, and (3) adult stage that annually return to aquatic habitat for breeding. Over the course of a few weeks during metamorphosis of crested newts, cranial morphology changes abruptly by resorption and remodeling of larval cranial bones (vomers, palato-pterygoids), and intensive ossification of dermal bones (maxillae, nasal, prefrontal) (Duellman and Trueb 1994; Rose 2003; Lebedkina 2004). After metamorphosis, juvenile ontogenies of ventral cranial shape are highly disparate in the direction of ontogeny but conservative in developmental rate, except, perhaps, in the case of *T. dobrogicus*, which develops most rapidly per unit change in size (Ivanović et al. 2007). Thus, spatial patterning rather than overall developmental rate appears to diverge in this group, and even the closely related species of the cristatus group diverge in the ontogenetic trajectory of shape. In this analysis, we extend that comparative study of ontogeny to the dorsal cranium as well, and measure disparity just after metamorphosis and at the adult stage using three species of crested newts (*Triturus dobrogicus, T. cristatus and T. macedonicus*). Extending the analysis to the dorsal skull is important because it might exhibit a different pattern as it does in lacertid lizards. In that group, ventral cranial disparity increased over ontogeny whereas dorsal cranial disparity is more conserved (Urošević et al. 2013). Ventral and dorsal cranial regions might be expected to differ in their patterns of both ontogeny and diversification because they serve different functional roles. The ventral cranium is formed by the upper jaw bones and palates well as the skull base; the premaxillae, maxillae and vomers are directly involved in feeding. Also, in newts, the ventral cranium appears to comprise functionally integrated modules, perhaps due to musculoskeletal interactions related to feeding (Ivanović and Kalezić 2010). The dorsal cranium comprises skeletal elements related to the brain and sensory organs that they support and, in crested newts, the dorsal cranium appears to lack predictable modules (Ivanović and Kalezić 2010).

The present study aims at a more complete understanding of cranial ontogeny and its impact on disparity by comparing ontogenetic trajectories, quantifying the degree of disparity and analyzing the structure of disparity. Because this analysis focuses on only three species, we can dissect the structure of disparity by considering not only summary statistics (like disparity) and summary plots (like principal components of the morphospace) but also the pairwise distances between species and the directions just after metamorphosis and as adults.

MATERIALS AND METHODS

Samples analyzed

Our sample contains two sister species (*T. dobrogicus*, *T. cristatus*) and one, *T. macedonicus*, from the lineage most closely related to *T. dobrogicus* and *T. cristatus* lineage (see Wielstra and Arntzen 2011). Of these species, *T. dobrogicus* is the most aquatic, inhabiting permanent and/or long-lasting, large, stagnant bodies of water. *T. cristatus* occupies mostly long-lasting, medium-sized bodies of water, and *T. macedonicus* is the most terrestrial of the three species (Arntzen 2003). For the investigation of the differences in skull shape, individuals just after metamorphosis (i.e., metamorphs) were obtained from laboratory experiments in which newts were reared under controlled laboratory conditions (for the experimental settings and origin of metamorphs see Cvijanović et al. 2009; Ivanović et al. 2011). All metamorphs are at the same age—7 days after metamorphosis (as determined by the full resorption of external gills and closure of gill slits). Adults were from osteological collection of the Institute for Biological research "Siniša Stanković." Specimens of *T. cristatus* were from Mt. Miroč (Serbia, 44° 29′N, 22° 20′E) collection numbers 20,042–20,045, 20,047, 20,049–20,059, 20,065–20,079, *T. dobrogicus* were from Ivanovo (Serbia, 44° 44′N, 20° 42′E) collection numbers 1C10–14C10, 16C10–18C10, 20C10, 23C10, 24C10, and *T. macedonicus* were from Rid (Montenegro, 42° 23′N, 18° 58′E) collection numbers 1C30–18C30. By rearing larvae in laboratory conditions we were able to obtain metamorphs at the same developmental stage for all three species. We assumed that laboratory reared metamorphs and those in natural populations do not differ in skull shape.

The skulls were cleared with trypsin and KOH and stained with Alizarin red S for bone depositions (e.g., Dingerkus and Uhler 1977). Prepared skulls were photographed with a 10-mm scale bar, with a Moticam 2000 camera connected to a Nikon SMZ800 stereozoom microscope (metamorphs), and with a Sony DSC-F828 digital camera (adults). The number of specimens for each species and stage is given in Table 1. The ventral skull shape is described by 27 two-dimensional landmarks and 24 landmarks describe the shape of the dorsal skull side (Fig. 1). All landmarks were digitized on both sides of the skull using TpsDig (Rohlf 2005) by the same person (M.C.).

Table 1. Sample size for each species and ontogenetic stage for each view

Species	Stages	Ventral, *n*	Dorsal, *n*
T. cristatus	Metamorphs	11	11
	Adults	29	29
T. dobrogicus	Metamorphs	23	22
	Adults	28	29
T. macedonicus	Metamorphs	30	30
	Adults	22	23

Coordinates of landmarks were superimposed using Generalized Procrustes Analysis (GPA), removing variation unrelated to shape, namely that due to variation in position, scale and orientation (Rohlf and Slice 1990; Dryden and Mardia 1998; Zelditch et al. 2012). Having digitized landmarks on both sides of the skull, we reflected and averaged the bilaterally symmetric landmarks to remove the redundancy of bilaterally homologous landmarks. To do this, we copied each configuration, reflecting one of them, and these were superimposed and the superimposed coordinates averaged, yielding the symmetric component of variation for object symmetry (Klingenberg et al. 2002). Size was measured by centroid size (CS), calculated as the square root of the summed squared distances of each landmark from the centroid of the form (Bookstein 1991). Reflection, superimposition and averaging of the configurations were done in Sage (Marquez 2008). Subsequent superimpositions, done when analyzing subsets of the data, were done using the gpagen function in the geomorph package (Adams and Otarola-Castillo 2013) in R (The R Foundation for Statistical Computing, vers. 3.01, 2013).

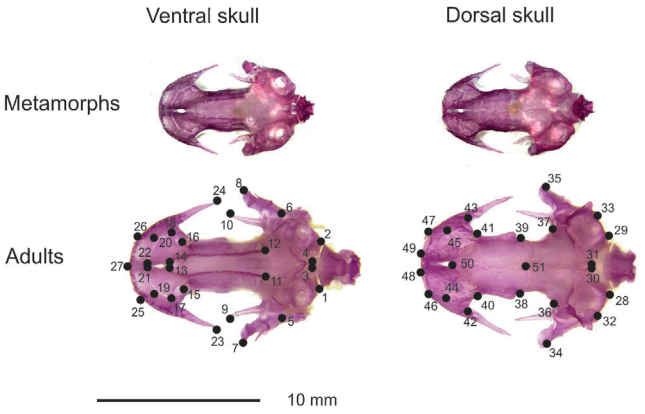

Fig. 1. Dorsal and ventral sides of adult's and skull of metamorphs. Symmetric landmarks digitized on the ventral and dorsal side of the adult's cranium.

Analyzing differences in skull shape and size

To compare skull sizes and shapes across species, we used a permutational analysis of variance (ANOVA) based on summed squared distances; this provides a very flexible approach that allows for direct additive partitioning of variation for complex models while retaining the flexibility and lack of formal assumptions of other non-parametric methods. This distance-based approach also has the advantage that the same method (and models) can be applied to both size and shape data, and the models can be fit to shape data even when the number of variables is large relative to sample size. This distance-based multivariate analysis of variance is equivalent to a Procrustes ANOVA (Zelditch et al. 2012; Adams and Otarola-Castillo 2013). In the case of shape data, the distance metric is the (partial) Procrustes distance between superimposed shapes, that is, the square root of the summed squared differences between homologous landmarks, summed over all landmarks (Rohlf and Slice 1990; Dryden and Mardia 1998; Zelditch et al. 2012). The statistical significance of the term in the models is tested by a permutation test (see Anderson 2001; Anderson and ter Braak 2003; Zelditch et al. 2012; Sheets and Zelditch 2013). In all analyses, sexes were pooled because a preliminary analysis found no differences between them in either dorsal or ventral skull shape (MANOVA, $P = 0.546$ and $P = 0.641$, respectively).

The permutational ANOVA was done using the adonis function in the vegan package (Oksanen et al. 2013) in R.

Analyzing and comparing ontogenetic trajectories

To describe the ontogeny of shape, we used a permutational ANOVA of the distance matrix. We used this approach rather than regression because we compared two developmental stages that differ substantially in mean size (see Results section, below); this design is better suited to ANOVA than regression. To depict the change in shape we used thin-plate spline deformation grids (Bookstein 1991). The length of each ontogenetic trajectory, like the distance between each species at metamorph and adult stages, was measured as the Procrustes distance between shapes. The statistical assessment of ontogenetic change was done using the adonis function in the vegan package in R; the depiction of the deformation used R code written by Claude (2008), modified by Adam Rountrey; the Procrustes distance between mean metamorph and mean shapes, and between the mean metamorph (and adult) shapes for each species was calculated using the riemdist function in the shapes package (Dryden 2013) in R.

To determine if species differ in their ontogenetic trajectories, we first performed a two-way, fully factorial, permutational ANOVA with "shape" as the dependent variable and "species" and "developmental stage" as the two independent variables. Differences in the ontogenies of species are inferred when the "species" × "developmental stage" interaction term is statistically significant. Those differences could result either from

differences in the direction of the ontogenetic trajectories and/or their length. To determine whether the trajectories differ in orientation and/or length, we compared the directions of ontogeny; the difference in those directions is quantified by the angle between the two trajectories. The angle is the arccosine of the signed inner products between the normalized ontogenetic vectors. If the two vectors do not differ in their orientation, the angle between them is zero degrees. To determine if the angle is greater than expected by chance, we used two approaches that differ in the design of their permutation test. According to one, the permuted units are the residuals of the reduced model, that is, a model lacking the intercept term. By permuting the residuals of the reduced model, the main effect(s) are held constant; the observed angle can then be compared to the distribution of the random values to assess statistical significance (Adams and Collyer 2009). The same approach can be used to test for a difference in length of the ontogenetic trajectory, which is measured by the Procrustes distance between each species' metamorph and adult mean shapes. These analyses were done using the trajectory.analysis function in the geomorph package (Adams and Otarola-Castillo 2013). An alternative approach for testing the difference in angles is to fit the model, draw *two* random samples of residuals (with replacement from each sample and add those residuals to the expected values; then to refit the model to each of these two samples and calculate the angle between them; the same procedure is done for the other sample, iterating the procedure (900 times) gives the distribution of the angles that can be obtained by chance. If the observed angle exceeds 95% of the angles obtained from both samples, the ontogenetic trajectories are inferred to be different. These analyses were done in VecCompare (Sheets 2010).

Quantifying and comparing disparity

To calculate morphological disparity, we used the variance of the species' means, measured as the summed squared distance between each species' mean shape and the grand mean, divided by $N-1$, where N represents the total number of species (Zelditch et al. 2003a, 2012). Disparity was calculated for both sides of the skull and at each developmental stage. To determine if disparity differs between developmental stages, we calculated the disparity and the standard error for the estimate, for each stage (metamorphs and adults) and skull side, and compared the disparities by a *t*-test. To examine the contribution that each species makes to the total disparity, we calculated the partial disparity for each species (Foote 1993b). Analyses of disparity and partial disparity were performed in DisparityBox6, IMP series (Sheets 2003).

To compare the structure of disparity, we used two approaches. One examines the axes of the space encompassing the three species, comparing these between metamorphs and adults; the other examines the dimensions that differ between pairs of species, comparing those between metamorphs and adults. To compare the morphospaces of metamorphs and adults, we used a method similar to common principal components analysis, CPCA (Flury 1988). This alternative, common subspace analysis, CSA (Flury 1987), based on a method devised by Krzanowski (1979, 1982), is more useful when the first and second principal components (PCs) explain nearly equal amounts of variation. The null hypothesis tested by CSA is that the samples do not differ in the set of eigenvectors spanning a given number of dimensions (two, in our comparisons). To determine if samples differ by more than expected by chance, the difference between sets of eigenvectors is measured by the minimum angle through which one subspace must be rotated to align it with the other (for more details on how that is done, see Krzanowski 1979; Zelditch et al. 2006; Appendix A). As the metric for the angle of rotation, Krzanowski used the sum of squared cosines of angles between the individual pairs of eigenvectors; the alternative is the total magnitude of the rotation. To determine whether the observed angle is larger than expected by chance, it is compared to the range of angles that obtained by resampling each developmental stage (metamorphs and adults) separately. From each sample, two random samples are drawn and the first two PCs are extracted for each sample, then the angle between the subspaces is calculated. This process is iterated 900 times, drawing two random samples from both samples at each iteration. The observed angle can then be compared to the distribution of angles that can be obtained by resampling a single developmental stage; if the observed angle exceeds 95% of the angles obtained by resampling each of the two developmental stages, the morphospaces are inferred to be different. These analyses were done in SpaceAngleThree6 (Sheets 2006).

As an alternative, we compared the directions in which species differ at the two developmental stages. This analysis is also a comparison of phenotype trajectories and thus uses the same methods as described above for comparing ontogenetic trajectories. In this case, the trajectory is interspecific, extending between the means of two species at single developmental stage. One trajectory extends between the means of the metamorphs, the other between the means of the adults. A decrease in the length of that trajectory would indicate "ontogenetic convergence" whereas an increase would indicate divergence. A change in the direction of the trajectory would indicate a change in the dimensions along which the species differ.

RESULTS

Skull size and shape

Mean centroid sizes for the dorsal skull of metamorphs are 15.02, 17.82, and 21.59 for *T. cristatus*, *T. dobrogicus* and *T. macedonicus*, respectively; for adults, the mean sizes are 25.6, 20.5, and 27.17. Mean centroid sizes for the ventral skull of metamorphs are 15.34, 18.17, and 22.35 for *T. cristatus*,

Table 2. Comparison of mean size and shape across species, done separately by developmental stage (metamorphs and adults) and skull region (ventral and dorsal cranium): (a) Centroid size; (b) shape

	df_{model}	df_{error}	F	P	R^2
(a)					
Metamorphs					
Ventral	2	61	151.9	0.0001	0.51
Dorsal	2	60	132.9	0.0001	0.82
Adult					
Ventral	2	76	200.6	0.0001	0.50
Dorsal	2	78	181.7	0.0001	0.83
(b)					
Metamorphs					
Ventral	2	61	31.85	0.0001	0.52
Dorsal	2	60	24.19	0.0001	0.45
Adult					
Ventral	2	76	38.55	0.0001	0.50
Dorsal	2	78	46.6	0.0001	0.54

T. dobrogicus, and *T. macedonicus*, respectively; for adults, the mean sizes are 26.92, 21.11 and 28.46. Not surprisingly, the species differ significantly in both ventral and dorsal skull size (Table 2a) and ventral and dorsal skull shape (Table 2b) at both developmental stages.

Ontogenetic shape change

Cranial shape, of both dorsal and ventral sides, changes over the course of ontogeny (Table 3). Ontogenetic changes account for approximately twice as much of the variance in shape of the ventral as dorsal skull in all three species; not surprisingly, the ontogenetic trajectories of ventral skull shape are nearly twice as long as those of dorsal skull shape. In units of Procrustes distance, lengths of the ontogenetic trajectories for ventral skull shape are 0.073, 0.076, and 0.072 (for *T. cristatus, T. dobrogicus*, and *T. macedonicus*, respectively) but only 0.042, 0.046, and 0.047 for dorsal skull shape (for *T. cristatus, T. dobrogicus* and *T. macedonicus*, respectively).

Table 3. Ontogenetic shape change, analyzed separately by species and skull region

Species	df	F	P	R^2
(a) Ventral				
T. cristatus	1	39.62	0.001	0.51
T. dobrogicus	1	44.64	0.001	0.48
T. macedonicus	1	56.37	0.001	0.53
(b) Dorsal				
T. cristatus	1	12.73	0.001	0.25
T. dobrogicus	1	23.9	0.001	0.33
T. macedonicus	1	30.45	0.001	0.37

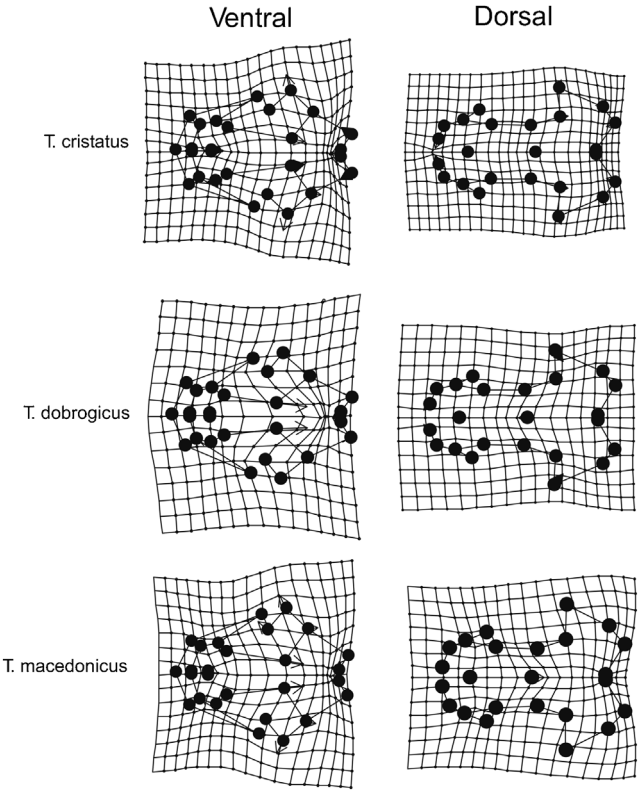

Fig. 2. The pattern of ontogenetic shape changes in three newt species. The deformation grids illustrate shape changes from metamorphs to adults.

The ontogenetic shape changes for both views are depicted in Figure 2. Regarding ventral skull side all species show a decrease in the relative size of the otico-occipital region and skull base (landmarks 1–4 and 11, 12), a relative elongation of the maxillae (landmarks 23, 24), vomers (landmarks 11–14) and pterygoids (landmarks 9 and 10). The relative elongation of vomers (vomerine teeth rows) are most pronounced in *T. dobrogicus*, while in *T. cristatus* and *T. macedonicus* the more marked change is a relative widening of the skull at the jaw articulation point (landmarks 7 and 8). Ontogenetic shape changes for the dorsal side common to all three species include a relative narrowing of the premaxillary (narial) process (landmarks 48, 49) and a relative elongation of the snout (landmarks 44–50), changes most pronounced in *T. cristatus*. In *T. cristatus*, the dominant changes are a widening of the skull at otico-occipital region (landmarks 28–33), and at the squamosals, which are further apart in adults (landmarks 34 and 35). In *T. dobrogicus*, ontogenetic changes in dorsal skull shape include an elongation of the parietal bones (36–39) and relative shortening of the medial suture of the frontal bones (landmarks 50, 51)—these changes, less pronounced, also occur in *T. cristatus*. In

Table 4. Comparisons of ontogenetic shape change by skull region

Effect	df	SS	MS	*F*	*P*	R^2
(a) Ventral						
Species	2	0.173	0.086	67.01	0.001	0.373
Stage	1	0.144	0.144	111.69	0.001	0.272
Species * stage	2	0.035	0.017	13.42	0.001	0.066
Total	143	0.529				
(b) Dorsal						
Species	2	0.123	0.061	61.04	0.001	0.374
Stage	1	0.037	0.037	37.12	0.001	0.114
Species * stage	2	0.029	0.015	14.62	0.001	0.090
Total	143	0.327				

T. dobrogicus, there are also notable changes in the relative position of squamosals (landmarks 34 and 35), which are positioned more posteriorly and closer to the midsagittal plane in adults. The major changes in *T. macedonicus* are a relative shortening/widening of the otico-occipital region (landmarks 28–33) and relative reduction of parietal bones together with the relative elongation of frontal bones.

Comparing ontogenetic shape changes

The species × developmental stage interaction was significant for both the dorsal and ventral skull shapes, indicating that species differ in their ontogenetic trajectories (Table 4). None of the pairwise comparisons reveal statistically significant differences in the length of their ontogenetic trajectories; for the comparisons of lengths, $P \geq 0.20$ for all pairwise comparisons. Species thus undergo statistically indistinguishable amounts of shape change. In contrast, all the pairwise comparisons reveal statistically significant differences in the direction of their ontogenetic trajectories; the comparisons of lengths, $P \leq 0.006$ for all pairwise comparisons. Each species has a unique ontogeny of skull shape.

Disparity

Disparity of ventral skull shape does not change over the course of ontogeny (Table 5); the disparity of metamorphs and adults do not differ statistically (*t*-test, $P = 0.1596$). The disparity of the adults lies within the confidence interval for the disparity of the metamorphs. The analysis of partial disparities shows that the three species make equal contributions to the disparity of metamorphs (*T. macedonicus* 34.42%, *T. dobrogicus* 35.81%, *T. cristatus* 29.77%), indicating that all three are nearly equidistant from the mean shape of metamorphs. As adults; *T cristatus* makes a smaller contribution to disparity than the other two species (18.18% for *T. cristatus* vs. 42.25% and 39.57% (for *T. macedonicus* and *T. dobrogicus*, respectively). In striking contrast to the constancy of disparity found for ventral skull shape, disparity of dorsal skull shape statistically significantly increases; (*t*-test, $P < 0.0001$); in this case, the confidence intervals for the two stages do not even overlap (Table 5). Based on the ontogenetic convergence test, these species neither converge nor diverge in terms of ventral cranial shape over the course of ontogeny (Table 6). Neither overall disparity, nor the pairwise distances change significantly over ontogeny. In contrast, the disparity of dorsal skull shape changes significantly over the course of ontogeny, with adults being more disparate than metamorphs (*t*-test, $P < 0.0001$). As metamorphs, *T. macedonicus* makes the largest contribution to overall disparity (54.78%) compared to *T. dobrogicus* (25.22%) and *T. cristatus* (20%). As adults, *T. dobrogicus* and *T. macedonicus* contribute nearly equally; their partial disparities are 47.98%, and 41.62% (for *T. dobrogicus* and *T. macedonicus*, respectively) and *T. cristatus* makes the smallest contribution to disparity, contributing merely 10.40%.

Figure 3 shows the first two dimensions of the morphospace of ventral and dorsal skull shape of metamorphs and adults. For ventral skull shape of metamorphs PC1 describes variation in the relative position of maxillae, pterygoids and quadrates (demarcated by landmarks 7–10, 23, and 24) and posterior part of the skull (otico-occipital region, landmarks 1–6). *Triturus dobrogicus* metamorphs differs from *T. macedonicus* by relatively shorter vomerine teeth rows, less developed (shorter) maxillar bones, quadrates positioned toward midsagittal plane and more elongated posterior part of the skull comparing to *T. macedonicus*. *T. cristatus* metamorphs occupies an intermediate position between *T. dobrogicus* and *T. macedonicus* along PC1, but clearly separate from these two species by having more elongated vomers (vomerine teeth rows) as described by shape changes along PC2 axis. In the morphospace of ventral skull

Table 5. Morphological disparity of different developmental stages for the ventral and dorsal skull shape, with confidence interval for disparity estimated by bootstrapping

Skull	Metamorphs-disparity	Bootstrap 95% CI of the within-species range	Adults-disparity	Bootstrap 95% CI of the within-species range
Ventral	0.0022	0.0019–0.0027	0.0019	0.0018–0.0022
Dorsal	0.0011	0.0010–0.0015	0.0017	0.0016–0.0020

Table 6. Comparing pairwise distances over ontogeny

	T. cristatus	*T. dobrogicus*	*T. macedonicus*
T. cristatus		0.217	0.298
T. dobrogicus	0.000		0.754
T. macedonicus	0.900	0.004	

Given are *P*-values for the null hypothesis that the distances are equal between metamorphs and adults. Above the diagonal are the comparisons of distances in ventral view; below are the comparisons of distances in dorsal view. All the statistically significant differences are limited to the dorsal view and all are significant increases in distance, indicating divergence.

shape of adults, the relative positions of species are similar and the major axes of variation appear to be at least moderately similar although not the same. However, the shape changes in adult stage described by PC1 and PC2 are related to relative changes in snout shape (landmarks 19–22 and 25–27) and position of jaw articulation point (landmarks 7 and 8). PC1 and PC2 of the adult morphospace are oriented at 51.9° and 50.45° relative to PC1 and PC2 of juvenile shape, respectively. The angle between the plane spanned by these two PCs, measured as the total angle of rotation is 69.65°.

In dorsal skull shape metamorphs of *T. cristatus* and *T. dobrogicus* cluster together in the morphospace described by first two principal axes (Fig. 3) and clearly separate from *T. macedonicus*. Compared to *T. macedonicus*, they have longer frontal suture (landmarks 50–51), relatively longer/narrower snout (landmarks 42–49), and generally narrower skull and anteriorly positioned squamosals. In the morphospace of adults, *Triturus dobrogicus*, compared to other species, has longer parietal bones (demarcated by landmarks 36–39) and more anteriorely positioned jaw articulation point (landmarks 34 and 35). PC2 describes variation in the size of parietal and otico-occipital region relative to frontal bones and snout. In the morphospace of adults, the major axis of adult variation is moderately similar to that of the metamorphs, being only at 53.3° to it, but the second axis (PC2) is not; PC of the adult

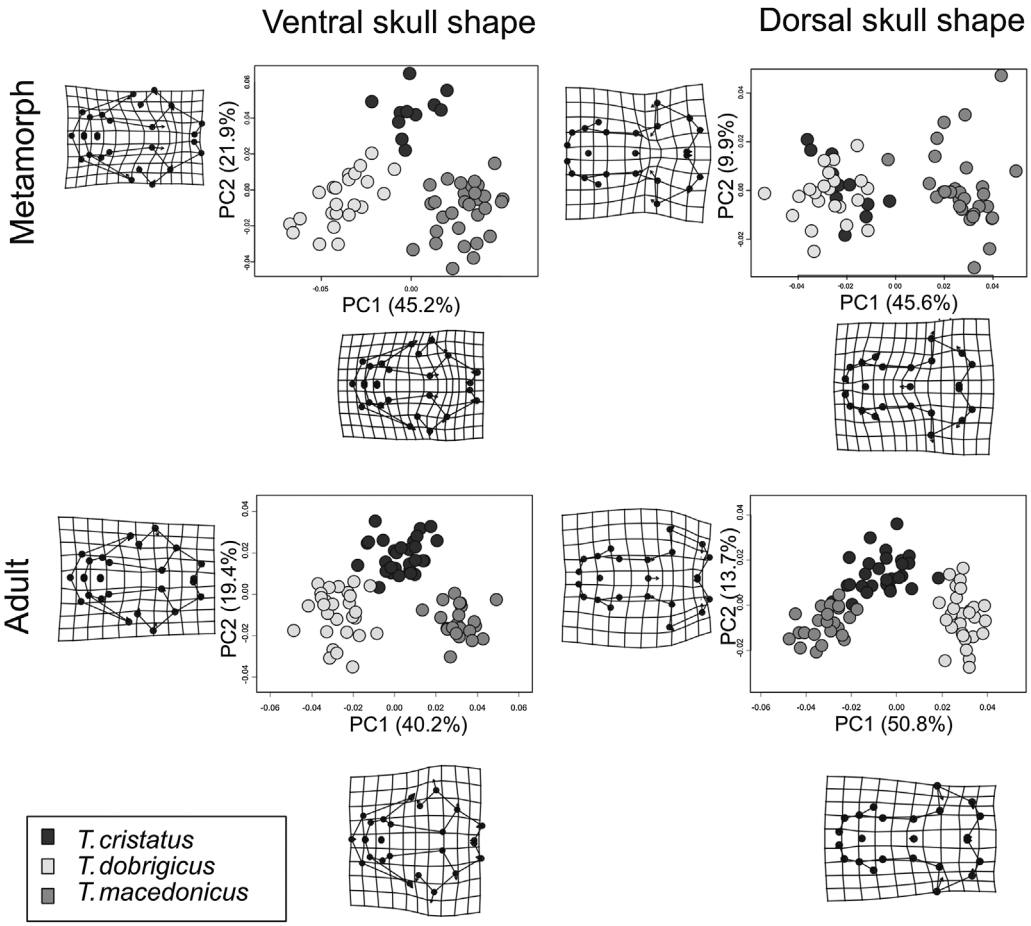

Fig. 3. Morphospaces of the ventral and dorsal skull shapes of metamorphs and adults obtained by principal components analysis (PCA). The deformation grids illustrate shape changes along PC1 in the direction of increasing scores.

morphospace is at 76.43° to that of the metamorphs. Comparing the plane of variation, the total angle of rotation is 91.65°, which exceeds, but only slightly, 95% of the angles obtained by the randomization procedure (89.24° and 36.72° for samples drawn from the metamorph and adult samples, respectively). Using Krzanowski's metric, the angle between planes is 0.471°.

Placing the ontogenetic trajectories in morphospaces comprising both metamorphs and adults suggests that, for ventral skull shape, species' means are merely translated along the major axes of the morphospace without altering the distances between means (Fig. 4). For dorsal skull shape, it is evident that species diverge over ontogeny. But both plots are low-dimensional summaries of variation in several dimensions. For example, the ontogenetic trajectories for dorsal cranial shape are nearly orthogonal to PC1 and PC2; those angles to PC1 are 89.9°, 79.8°, and 89.5° for *T. cristatus*, *T. dobrogicus* and *T. macedonicus*, respectively. Similarly, the angles between the ontogenetic trajectories and PC2 range from 83.4° to 88.4°.

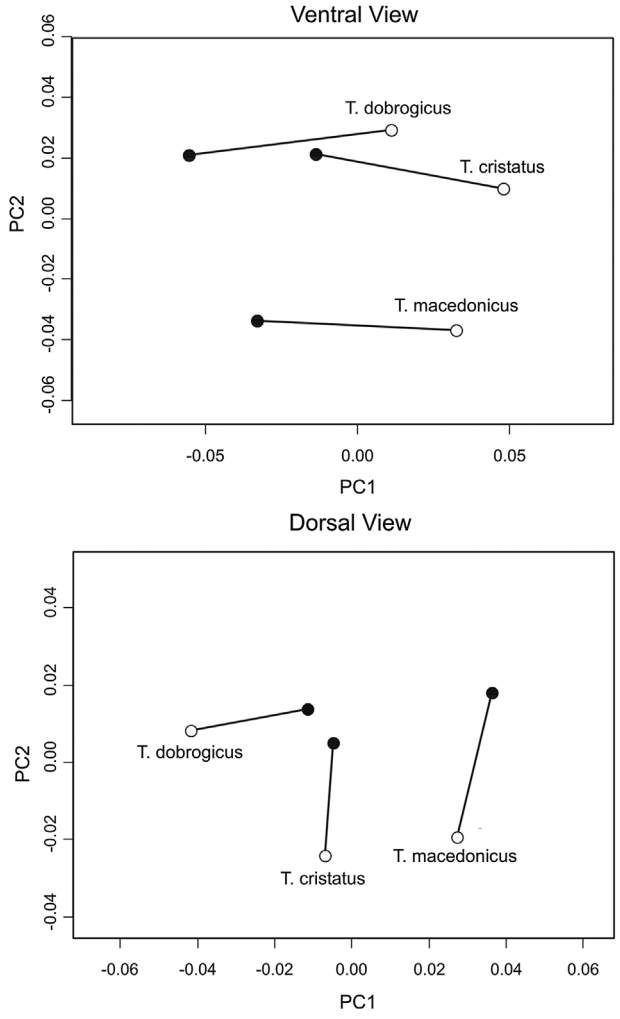

Fig. 4. Ontogenetic trajectories of ventral and dorsal skull shape in the space of the ontogenetic series for ventral and dorsal skull shape. The trajectories extend from the metamorph (white circle) to the adult (black circle).

To see in more detail how the patterns of disparity change over the course of ontogeny, we can compare the shape differences between species at the two developmental stages. In the comparison of ventral skull shapes between *T. cristatus* and *T. dobrogicus* at the two stages, the difference in orientation is visually striking (Fig. 5) and statistically significant (Table 7); this is the comparison that was done using both methods because, although very large, it was not inferred to be any greater than expected by chance using the permutation of residuals from the reduced model but was judged to be greater than expected by chance based on the distribution of angles between two random samples drawn from each developmental stage (metamorphs and adults). The distance between the species also changes, decreasing from 0.064 to 0.052. The direction of the interspecific difference between *T. cristatus* and *T. macedonicus* does not change over ontogeny (Fig. 5); the difference in the distance, which are 0.063 between metamorphs and 0.054 between adults, is just marginally non-significant (Fig. 5, Table 7). The direction of the difference between *T. dobrogicus* and *T. macedonicus* does change over ontogeny, but the change in distance, of 0.068 between metamorphs and 0.074 between adults, is not statistically significantly (Fig. 5, Table 7). Thus, in ventral skull shape, as adults, *T. cristatus* and *T. dobrogicus* are slightly more similar than they were as metamorphs, and *T. cristatus* and *T. macedonicus* are, perhaps, slightly more similar as adults than as metamorphs; *T. dobrogicus* and *T. macedonicus* are neither more nor less similar as adults than they are as metamorphs but the two developmental stages differ in the features that distinguish between them.

In dorsal view, the two distances to *T. dobrogicus* change significantly (Table 7). Between *T. cristatus* and *T. dobrogicus*, that distance nearly doubles, increasing from 0.0272 to 0.0521, and that between *T. dobrogicus* and *T. macedonicus* increases from 0.056 to 0.076. The distance between *T. cristatus* and *T. macedonicus* does not change (Table 7); the distance between the metamorphs is 0.63 and that between the adults is 0.54. All three interspecific trajectories change their orientation (Fig. 5, Table 7).

DISCUSSION

Just after metamorphosis, crested newts (*T. cristatus*, *T. macedonicus*, and *T. dobrogicus*) differ in their dorsal and ventral cranial morphology and the three species follow unique ontogenetic trajectories of shape, especially of ventral skull shape. Yet, disparity of adult ventral skull shape is no greater than that of metamorph ventral skull shape although disparity of adult dorsal skull shape is. That constancy of disparity of ventral skull shape is surprising, but it is only the level of disparity that is

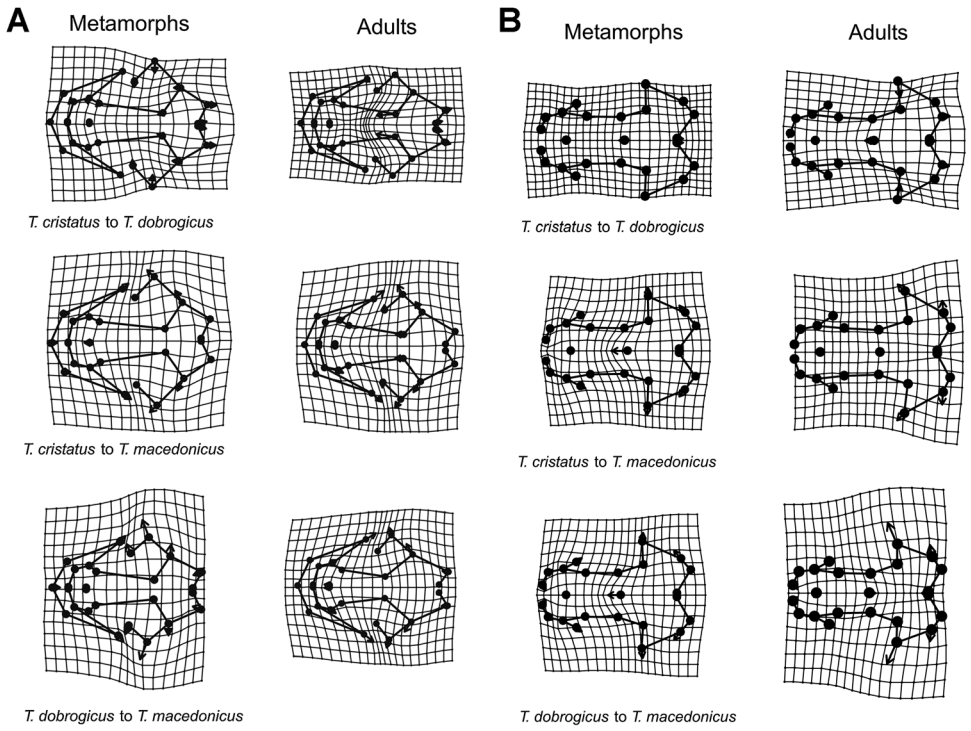

Fig. 5. Differences between pairs of species at metamorph and adult stages. A, ventral skull; B, dorsal skull.

constant. Even though the distances between individual species change modestly, what does change is the dimension along which *T. cristatus* and *T. dobrogicus* differ (Fig. 5). As metamorphs they differ in the relative size of vomers and vomerine teeth rows, the relative position of maxillary bones, pterygoids, and jaw articulation point (position of quadrates and squamosals), the size of frontal and parietal bones and in relative size of otico-occipital regions. As adults they differ in the relative position of jaw articulation point (position of quadrates and squamosals) and shape of otico-occipital regions. Such changes in directions of disparity might be anticipated when the level of disparity also changes and the dimensions along which dorsal skull shapes differ also change over ontogeny. In the case of ventral skull shape, disparate metamorphs, following disparate ontogenies of shape, remain equally disparate adults, but differ along different dimensions. In the case of dorsal skull shape, disparate metamorphs, following disparate ontogenies of shape, become more disparate adults, and they too differ along different dimensions. Our results thus support the hypotheses that metamorphosis resets ontogenetic trajectories, with post-metamorphic ontogeny producing the disparity seen in adults (Ivanović et al. 2011); the dimensions along which adults differ cannot be predicted from the dimensions along which metamorphs differ.

In these crested newts, the disparity of ventral skull shape is stable, whereas that of dorsal skull shape disparity increases,

Table 7. Pairwise comparisons of interspecific differences in shape ventral and dorsal skull of metamorphs and adults

Species	Direction	Length	*P* (direction)	*P* (distance)
(a) Ventral				
T. cristatus to *T. dobrogicus*	73.20	0.013	0.001	0.034
T. cristatus to *T. macedonicus*	31.99	0.008	0.001	0.054
T. dobrogicus to *T. macedonicus*	48.38	0.005	0.001	0.211
(b) Dorsal				
T. cristatus to *T. dobrogicus*	63.7	0.0237	0.001	0.001
T. cristatus to *T. macedonicus*	54.06	0.0089	0.001	0.044
T. dobrogicus to *T. macedonicus*	50.22	0.0198	0.001	0.001

a pattern at odds with that seen in both *Leptodactylus* frogs (Ponssa and Candioti 2012) and lacertid lizards (Urošević et al. 2013). In *Leptodactylus* frogs, disparity of ventral cranial shape decreases significantly over ontogeny, a decrease explained by functional constraints on both skeletal shape and associated musculature involved in adult feeding (Ponssa and Candioti 2012). In lacertid lizards, it is dorsal skull shape that changes little in disparity whereas ventral skull becomes increasingly disparate, because the ventral skull is shaped by the mechanics of jaw movement and feeding, increasing ecological disparity explains increasing disparity (Urošević et al. 2013). Although, we find different patterns in disparity, our results support the hypothesis that the modularity of biphasic life-cycles enables each developmental stage to adapt to its stage-specific ecological demands without interfering with the adaptations of other stages.

Abrupt changes of the cranial skeleton during metamorphosis lead to the transformation of the highly specialized skeleton of suction feeding, aquatic larvae, into crania of terrestrial juveniles that are faced with a new environment and different, terrestrial feeding mechanism (Deban and Wake 2000; Rose 2003; Lebedkina 2004). Significant ontogenetic niche shifts in skull morphology between these two, ecologically different stages is expected, and it is ”produced” by metamorphic changes. However, the observed divergence in ventral skull shape between two metamorphosed terrestrial stages, metamorphs and adults (without change in level of disparity), indicates another niche shift and possible species and stage-specific divergences in feeding performances, although both stages shared same general (terrestrial) ecological setting. As noted before, the changes in skull shape between metamorphs and adults are mostly related to the position of quadrates and squamosals, shape of the palate (position of vomeral teeth raws and pterygoids). Muscles directly involved in cranial kinesis are connected to these skeletal elements—the complex jaw adductor muscles (*m. adductor mandibulae externus* and *m. adductor mandibulae posterior*) are connected to the squamosals whereas *m. intehyoideus* is connected to the quadrate. Also, the muscle directly involved in swallowing (*m. levator bulbii*) is connected to the pterygoid (see Iordansky 1996 and references therein). Therefore, the changes in the relative position of these skeletal elements indicate differences in feeding performances and diet specialization. The study by Adams and Rohlf (2000) on *Plethodon* salamanders suggest that even small differences in skull shape can be indicative of change in feeding performance. Unfortunately, empirical data documenting differences in feeding performance due to differences in skull form are very rare (Deban and Wake 2000). Except for the major shift in biphasic life-cycles - from aquatic, larval suction feeding to terrestrial feeding (Deban and Wake 2000)there are no data on changes in feeding preferences between ontogenetic stages or among crested newt species.

The observed increase in disparity of dorsal skull shape is largely due to the changes in the shape of squamosals, in relative size of skull roofing bones and the shape of otico-occipital region, skull bones that are not directly related to the feeding performance. The two species that are most divergent in adult dorsal skull shape, and which therefore contribute most to disparity at that stage, are *T. dobrogicus* and *T. macedonicus*. These two species also show the most pronounced divergence in time that adults spend in water annually (Arntzen 2003). *Triturus dobrogicus* has the longest annual aquatic period (6 months), and, at adult stage, it differs most in skull shape due to its distinctively elongate skull and its more anteriorly positioned squamosal and quadrate bones (the jaw articulation point), both of which have been characterized as adaptations to aquatic conditions (Trueb 1993). The species with the shortest annual aquatic period, just four months, is *T. macedonicus*, which has a more robust skull, with a more posteriorly placed jaw articulation point. Intermediate between these two in both duration of the annual aquatic period (five months) and one dimension of shape variation is *T. cristatus*.

Complex postmetamorphic ontogenetic skull shape changes, transforming disparate metamorphs to disparate adults by divergent ontogenetic trajectories, are most likely shaped by the ecological factors that explain the evolutionary divergence in skull shape. The benefit of a biphasic life-cycle becomes most apparent when biphasic life-cycles are contrasted to continuous life-cycles such as those typical of mammals. Young mammals undergo substantial functional shifts, most notably, the ecological transition from suckling to chewing and they must maintain the functional integrity required for juvenile function while changing their shape to meet the demands of adult function. Because ontogenetic trajectories of species with continuous life-cycles are nearly linear, whether the age-specific ecological demands can be met throughout life depends on whether age-specific optima lie along a straight line. Ontogenetic trajectories do often curve (Bookstein 1991; Zelditch et al. 1992, 2003b; La Croix et al. 2011), as they may also do within the juvenile phase of a biphasic life-cycle (Walker 1993). But even when trajectories curve, juveniles may be functionally handicapped by juvenile morphology (La Croix et al. 2011). By decoupling stage-specific ontogenetic trajectories, each stage can adapt without interfering with the adaptations of another. Additionally, increases and decreases in disparity are possible, but, as we show here, such changes in disparity although need not occur.

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