

Early ontogeny shows the same interspecific variation as natural history parameters in the crested newt (*Triturus cristatus* superspecies) (Caudata, Salamandridae)

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Abstract

When the phenotypic divergence within a monophyletic group is characterised by parallel variation of different phenotypic traits, it is very likely that the environment through constraints and/or selection has affected the developmental pathways simultaneously. Such patterns of phenotypic divergence characterise the phenotypic evolution of the crested newts (*Triturus cristatus* superspecies). In this study, we have examined interspecific variations in the embryonic development of four crested newt species. The species are similar with respect to some basic developmental traits, some morphologically defined developmental stages and the survival rate during early embryogenesis. However, there is significant variation in the developmental rate, as well as differences in the pattern of correlation amongst analysed life-history and developmental traits. Consistent with previous studies, *T. dobrogicus* appears to be an outlier species, with the longest embryonic period and a significantly different correlation pattern for early life-history and developmental traits. We suggest that the invasion of a novel aquatic environment by *T. dobrogicus* resulted in large-scale directional changes in development, which could explain parallel change in numerous phenotypic and life-history traits with a high rate of evolution.

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Introduction

There is a common premise that evolutionarily related groups must show phenotypic similarity, including shared developmental basis, inherited from a common ancestor. When the phenotypic divergence within a monophyletic group shows parallel evolution of multiple phenotypic traits, it is most likely the case that the environment, through constraint and/or selection, affected the developmental pathways simultaneously, causing covariation between different phenotypic traits (see Hansen and Martins, 1996; Klingenberg, 2004; Revell *et al.*, 2008). This line of reasoning is especially strong in situations where the interspecific variations in ecological demands correlate to the variations in other phenotypic traits. Such a pattern of divergence characterises the evolution of crested newts (*Triturus cristatus* superspecies).

The crested newts form a well-supported monophyletic clade within the European newts (*e.g.*, Espregueira Themudo *et al.*, 2009). This group consists of six species: *T. cristatus* (Laurenti, 1768); *T. dobrogicus* (Kiritzescu, 1903); *T. karelinii* (Strauch, 1870); *T. arntzeni* Litvinchuk, Borkin, Džukić and Kalezić, 1999, which has been recently elevated to the species level (Espregueira Themudo *et al.*, 2009); and two more closely related species - *T. carnifex* (Laurenti, 1768) and *T. macedonicus* (Karaman, 1922) (see Arntzen *et al.*, 2007). Evolutionary splitting between these species is thought to have occurred within a short time span (Arntzen *et al.*, 2007; Espregueira Themudo *et al.*, 2009). The crested newt species differ in their ecological demands (Arntzen, 2003; Litvinchuk *et al.*, 2007), with *T. dobrogicus* at one

pole, *T. karelinii*, *T. arntzeni*, *T. carnifex* and *T. macedonicus* at the other pole, and *T. cristatus* as an intermediate species. Some morphological characteristics have been broadly used for the phenotypic differentiation of these species, such as body size and shape (Arntzen and Wallis, 1999), skull shape as inferred by geometric morphometrics (Ivanović *et al.*, 2008a), number of rib-bearing vertebrae (Crnobrnja-Isailović *et al.*, 1997; Arntzen and Wallis, 1999), relation between braincase and body size (Vukov *et al.*, 2007), egg size (Furtula *et al.*, 2008), limb skeleton (Ivanović *et al.*, 2008b), pattern of sexual size dimorphism (Ivanović *et al.*, 2008c), and life-history traits (Furtula *et al.*, 2009). All of these results point to the parallel evolution of a number of phenotypic and life-history traits, which follow a similar trend to species-specific ecological demands.

Thus far, the embryonic development of the crested newts has received very little attention. In particular, comparative embryology to date (*e.g.*, Griffiths and Wijer, 1994; D'Amen *et al.*, 2007; Litvinchuk *et al.*, 2007), especially quantitative data, has been insufficient to infer interspecific variation in early ontogeny. In this study, we applied a quantitative approach to investigate the embryonic development of four crested newt species (*T. dobrogicus*, *T. arntzeni*, *T. macedonicus* and *T. cristatus*), which represent the main phylogenetic clades (Espregueira Themudo *et al.*, 2009). We compared rates of development and the correlation patterns of several early life-history traits in order to quantify and explore variation in the early ontogeny of crested newts. The results of this study show that crested newts offer a unique paradigm in which to investigate the evolutionary basis for developmental changes within an ecological context.

Materials and methods

Experimental design and sampling

Two great advantages of using newts for experimental analysis are that European newt females, including crested newts, deposit their eggs one by one and embryonic development occurs in transparent mucoid capsules, making the tracing of developmental stages easy and accurate (Nieuwkoop, 1996).

We collected large, gravid females with inflated abdomens and swollen cloacae from natural populations of four species prior to the oviposition period. Females of *T. macedonicus* ($N = 7$) were collected in

March 2008: six originated from Ceklin (42°21'N; 18°59'E, 290 m above sea level (a.s.l.)) and one female from nearby Donji Ljubotinj (42°23'N, 19°07'E, 225 m a.s.l.), Montenegro. Females of *T. arntzeni* ($N = 8$) were collected in April 2008 from Borovsko polje, Serbia (42°58'N, 22°43'E, 890 m a.s.l.). Females of *T. dobrogicus* ($N = 4$) were collected near Kikinda town, Serbia (March 2006, 45°40'N, 20°29'E, 75 m a.s.l.). Females of *T. cristatus* females ($N = 6$) were collected in April 2006: five females were taken from Miroč, Serbia (44°29'N, 22°20'E, 440 m a.s.l.), and one female from Vršački breg, Korkana (45°06'N, 21°27'E, 180 m a.s.l.). The females were transferred to a laboratory at the Institute of Biological Research, University of Belgrade, within 24 h. All females were returned to their original population sites after the oviposition period ended.

Since crested newt species prefer different temperatures (Litvinchuk *et al.*, 2007), we provided two different experimental temperature conditions when examining interspecific differences in embryonic development. *Triturus dobrogicus* and *T. cristatus* were kept at 16–17°C, while the more thermophilic species *T. macedonicus* and *T. arntzeni* were kept at 18–19°C. Since the duration of European newt embryonic development correlates with temperature (Griffiths and Wijer, 1994; Bonacci *et al.*, 2005; D'Amen *et al.*, 2007), temperatures were kept constant to provide similar ecological settings for all individuals during embryonic development. In the laboratory, females were housed individually in 12-litre aquaria containing six litres of dechlorinated tap water. The captive females were fed every other day with worms and *Tubifex*. Plastic strips were provided for egg deposition and eggs were collected daily. Eggs were allowed to develop under laboratory conditions in Petri dishes (5 cm in diameter) containing a maximum of 10 eggs per Petri dish and filled with enough dechlorinated tap water to cover the eggs. The water was changed every second day.

Analysed traits

The eggs deposited by one female over a 24 h period were used as cohorts, and the mean values of analysed traits were calculated separately for each cohort. Eggs were photographed immediately after removal from the plastic strips for measurements. The morphologically defined developmental events (stages of embryonic development) were established according to a description of embryonic stages for *M. alpestris*

(Knight, 1938; see also Epperlein and Junginger, 1982). To estimate the variation in timing of developmental events within and between species, the developing embryos were examined under a binocular microscope by M. C. and N. T. K. at three different time points: at 7, 11 and 15 days following egg laying. The embryonic developmental stage was recorded at each particular checkpoint (at day 7, 11, and 15 of embryonic development), hereafter designated as S7, S11 and S15, respectively. Such experimental design provided a basis for analysing the variation in developmental events and developmental rates within and between species. Undeveloped eggs and dead embryos were removed regularly. Due to the possible effect of signalling between embryos on embryonic induction (Hall, 1999; 2003), median values for recorded stages of embryos per Petri dish (up to 10 embryos) were calculated; these data were used in further analyses. Hatched larvae were photographed with a digital camera (Nikon Coolpix 4500) and a 10-mm scale bar to measure the total length of larvae.

The following eight life-history traits were recorded: time of egg deposition relative to the oviposition period (DO), number of eggs laid per cohort (NE), mean vitellus diameter per cohort (RV), volume of galerta calculated as a difference among egg's volume and vitellus volume (VG), number of hatched larvae per cohort (NH), total length of hatched larvae per cohort (TL), duration of embryonic period of embryos per cohort (EP) and total hatchling survival rate of embryos per cohort (SR). Measurements of egg and vitellus diameter and TL of hatched larvae were taken by M. C. using UTHSCSA IMAGETOOL version 3.0 (<http://ddsdx.uthscsa.edu/dig/itdesc.html>).

Statistical analyses

To investigate the pattern of mortality during the embryonic period, we estimated survival rate (SR) as a proportion of live embryos recorded between two checkpoints. Survival rate confidence intervals were calculated according to the following formula:

$$CI = 2 \sqrt{\frac{q(1-q)}{n}}$$

where $q = 1 - SR$, SR is the survival rate and n is number of eggs at the beginning of the experiment. Two survival rates were considered significantly different (at 0.95 level) when respective confidence intervals did not overlap (Geller, 1983; Miaud, 1994).

To investigate the relationship between recorded life-history traits for each cohort (NE, RV, VG, SR, TL, EP), as well as between recorded developmental stages of embryos at each particular checkpoint (S7, S11, S15), we calculated Pearson correlation coefficients between these traits separately for each species. The pattern of correlation between recorded traits was analysed with matrix correlations. The similarity of these correlations between species was tested using Quadratic Assignment Procedures (Mantel's test) with 10,000 iterations. The significance test is based on the null hypothesis of no similarity in correlation patterns between compared matrices. A significant correlation between matrices would suggest a non-negligible concordance in correlation pattern between compared matrices.

Matrix correlations were used to estimate the degree of correspondence between interspecies observed correlation patterns and to estimate the robustness of observed correlations. We estimated the robustness and repeatability of observed correlation matrices using the resampling with replacement, or "bootstrapping", method (Cheverud *et al.*, 1989; Marroig and Cheverud, 2001). For each species of sample size n , n samples were randomly resampled with replacements from the original dataset. This procedure was repeated 500 times (Efron and Tibshirani, 1993), and 500 bootstrap datasets were generated separately for each species using Poptools 2.62 (Hood, 2004). For each generated dataset, correlation matrices were calculated and compared with the correlation matrix obtained from the original data using a matrix correlation. The frequency distributions of matrix self-correlations were used to estimate the robustness of correlation matrices. The repeatability of matrix self-correlations were used to estimate the theoretical maximum matrix correlation (R_{\max}) and to obtain an adjusted matrix correlation (R_{adj}) between two observed matrices (Marroig and Cheverud, 2001). The value of R_{\max} was calculated as $(t_A t_B)^{0.5}$, where t_A and t_B denoted the mean of the matrix self-correlations of matrices A and B, respectively. The adjusted matrix correlation (R_{adj}) was calculated as the observed correlation between two matrices (R_{obs}) divided by the maximum matrix correlation (R_{\max}).

Since temperature is one of the most important variables affecting amphibian embryonic development, direct comparisons of embryonic developmental timing between different species must be assayed at the animals' preferred developmental temperatures (Bonacci *et al.*, 2005; D'Amen *et al.*, 2007). Taking

Table 1. Mean values, standard deviations (mean \pm SD) and coefficients of variation (CV) for analysed life-history traits are presented for total number of eggs and larvae (N). Also given are the survival rate (r) and the confidence interval (CI) of survival rate at three checkpoints (I = 7 days, II = 11 days and III = 15 days) during embryonic development, as well as the total hatching success rate.

	<i>T. macedonicus</i>			<i>T. arntzeni</i>			<i>T. dobrogicus</i>			<i>T. cristatus</i>		
	N	mean \pm SD	CV %	N	mean \pm SD	CV %	N	mean \pm SD	CV %	N	mean \pm SD	CV %
Life history traits												
Diameter of vitellus (mm)	857	1.95 \pm 0.38	19.31	293	1.87 \pm 0.12	6.25	657	1.55 \pm 0.07	4.70	143	1.87 \pm 0.15	7.83
Volume of galerta (mm ³)	857	9.87 \pm 1.76	17.80	293	7.53 \pm 2.39	31.73	657	6.59 \pm 1.86	28.20	143	11.51 \pm 4.47	38.82
TL of hatched larvae (mm)	319	11.36 \pm 0.64	5.67	65	9.70 \pm 0.29	3.03	126	9.25 \pm 0.57	6.12	26	9.97 \pm 0.71	7.10
Embryonic period (days)	319	20.01 \pm 1.51	7.55	65	16.96 \pm 1.36	8.04	126	26.10 \pm 1.44	5.51	26	21.80 \pm 2.18	10.02
Survival rate												
		r	CI		r	CI		r	CI		r	CI
At checkpoint I		0.79	0.76-0.82		0.61	0.55-0.67		0.39	0.35-0.43		0.47	0.39-0.55
Between checkpoint I and II		0.98	0.97-0.99		0.86	0.81-0.91		0.95	0.93-0.98		0.94	0.88-0.99
Between checkpoint II and III		0.49	0.46-0.53		0.42	0.34-0.50		0.54	0.48-0.60		0.58	0.45-0.70
Between checkpoint III and hatching		0.97	0.95-0.99		1.00	/		0.90	0.85-0.95		0.70	0.55-0.85
Total hatching success rate		0.37	0.15-0.21		0.22	0.17-0.27		0.18	0.15-0.21		0.18	0.12-0.25

Table 2. Pearson's correlation coefficients between 11 analyzed traits. (a) *T. macedonicus*, number of cohorts $N = 91$ (upper diagonal) and *T. arntzeni*, number of cohorts $N = 34$ (lower diagonal) and (b) *T. cristatus*, number of cohorts $N = 19$ (upper diagonal) and *T. dobrogicus*, number of cohorts $N = 76$ (lower diagonal). Pearson's correlation coefficients were calculated from averaged data from each cohort (eggs laid by females during one day). Statistically significant correlation coefficients ($P < 0.05$) are in bold. Abbreviations are explained in the Materials and methods section.

a)											
	DO	NE	RV	VG	S7	S11	S15	NH	TL	EP	V
DO		-0.13	-0.38	0.54	0.54	0.18	0.24	0.07	-0.04	-0.36	0.22
NE	-0.17		0.12	0.15	0.09	-0.07	0.07	0.74	0.00	-0.10	-0.27
RV	0.42	-0.18		-0.12	0.14	-0.02	0.02	-0.02	0.01	-0.19	-0.20
VG	-0.55	0.43	0.03		0.53	0.03	0.01	0.21	-0.02	-0.39	0.03
S7	0.17	0.18	0.53	0.28		0.38	0.36	0.35	0.03	-0.44	0.24
S11	0.21	-0.16	0.34	0.11	0.52		0.67	0.20	0.00	-0.32	0.17
S15	0.70	0.04	0.34	-0.32	0.09	0.08		0.18	0.07	-0.33	0.14
NH	0.11	0.61	-0.06	0.00	0.40	0.28	0.23		-0.06	-0.19	0.23
TL	-0.46	-0.17	-0.41	0.15	-0.29	0.24	-0.32	-0.22		0.61	-0.03
EP	-0.70	0.02	-0.64	0.31	-0.62	-0.20	-0.53	-0.34	0.66		-0.11
V	0.30	-0.46	-0.19	-0.57	-0.49	0.26	0.07	0.05	0.21	0.13	
b)											
	DO	NE	RV	VG	S7	S11	S15	NH	TL	EP	V
DO		-0.50	0.21	-0.04	0.13	0.47	0.28	-0.63	0.38	-0.02	-0.33
NE	0.62		-0.33	0.37	0.38	-0.01	0.03	0.59	-0.52	-0.69	-0.29
RV	0.14	-0.24		0.17	0.29	0.11	0.43	-0.44	0.52	-0.08	-0.01
VG	0.42	0.42	-0.07		0.11	-0.13	0.37	0.14	-0.08	-0.34	-0.36
S7	-0.22	-0.38	0.27	-0.38		0.82	0.78	0.19	0.22	-0.88	0.10
S11	-0.50	-0.32	0.24	-0.14	0.24		0.76	0.12	0.50	-0.56	0.27
S15	0.36	0.07	0.28	0.09	0.23	0.21		0.22	0.62	-0.60	0.33
NH	-0.11	0.53	-0.50	0.33	-0.34	0.05	-0.32		0.05	-0.25	0.52
TL	-0.08	-0.28	0.32	-0.38	0.02	-0.08	-0.13	-0.24		0.14	0.54
EP	-0.31	-0.40	0.25	-0.36	0.05	0.09	-0.27	-0.09	0.77		0.20
V	-0.63	-0.37	-0.38	-0.02	0.04	0.32	-0.40	0.48	-0.10	0.19	

these limitations into account, we compared the rate and timing of developmental events of crested newt species at two temperatures. To analyse the differences in the embryonic developmental stages between

species at each particular checkpoint (S7, S11, S15), we performed Friedman and Kruskal-Wallis tests. We then used a Mann-Whitney analysis to test for statistical significance in pair-wise comparisons.

Results

Survival rate

Comparison of early life-history traits measured in this study revealed that all four species showed a similar pattern of survival rate with two critical periods (Table 1).

There was a large difference between the number of deposited eggs and the number of individuals that reached the late neurula stage (at the first checkpoint on day 7 of development). However, the highest peak of embryo mortality was recorded between the second and the third checkpoint (between 11 and 15 days of development). The examination of embryos at the third checkpoint revealed that in all four species, mortality occurred at the late tail bud stage. The high mortality at this stage is a result of the balanced lethal system that exists in crested newts, due to the homomorphism of heteromorphic chromosomes (Sims *et al.*, 1984). The observed mortality rate at this stage was around 50% in all four species, which corresponds to the previously reported data for crested newts (D'Amen *et al.*, 2006 and references therein). There were no differences in survival rate between *T. dobrogicus*, *T. cristatus* and *T. arntzeni*, while *T. macedonicus* had a significantly higher survival rate than the other three species (Table 1).

Developmental and early life-history correlation patterns

We investigated the pattern of correlation between all recorded life-history traits, including recorded devel-

Table 3. The analysis of correlation matrix patterns of eleven life-history and developmental traits. The observed coefficient of correlation between matrices (R_{obs}), and the robustness of correlation matrices and repeatability (the maximum possible correlation between matrix compared (R_{max}) and the values of adjusted matrix correlations (R_{adj}) are presented. Probabilities were derived using Mantel's test with 10,000 iterations. Asterisks denote probabilities of $P < 0.05$, indicating that two matrices were more similar to each other than expected by chance.

Species compared	R_{obs}	R_{max}	R_{adj}
<i>T. macedonicus</i> - <i>T. arntzeni</i>	0.465*	0.889	0.522
<i>T. macedonicus</i> - <i>T. dobrogicus</i>	0.304*	0.892	0.341
<i>T. macedonicus</i> - <i>T. cristatus</i>	0.520*	0.804	0.646
<i>T. arntzeni</i> - <i>T. dobrogicus</i>	0.189	0.851	0.222
<i>T. arntzeni</i> - <i>T. cristatus</i>	0.398*	0.767	0.519
<i>T. dobrogicus</i> - <i>T. cristatus</i>	0.327*	0.770	0.424

opmental stages of embryos at three checkpoints during embryonic development. We calculated a correlation matrix for all analysed traits (11×11) separately for each species (Table 2).

Even though the phenotypic correlation matrices for some species were calculated for a relatively small number of cohorts ($N = 19$ in *T. cristatus*), the estimated matrices were highly reproducible and reliable (Table 3).

We found no correlation between the time of egg deposition and the number of deposited eggs per cohort, with the exception of *T. dobrogicus*, which exhibited a significant positive correlation between oviposition time and the number of eggs laid per cohort (Table 2). There was no correlation between the number of deposited eggs and vitellus size in any of the four species. A significant negative correlation was found between the vitellus size (RV) and the duration of embryonic period in *T. macedonicus*, while there was no significant relation between these two traits for other species. However, there was a strong positive correlation between the duration of embryonic period and the size of hatched larvae, except in *T. cristatus* (Table 2).

We applied Mantel's test to explore interspecific similarities in the correlation of life-history traits. Based on this analysis, the correlations of life-history traits in *T. cristatus* were concordant with the pattern observed in *T. dobrogicus* ($R = 0.597$, $P < 0.05$), but were not similar to the other two species. The life-history trait correlation of *T. dobrogicus* also showed significant concordance with *T. macedonicus* ($R = 0.688$, $P < 0.01$), while the matrix correlation between *T. dobrogicus* and *T. arntzeni* was insignificant ($R = 0.266$, $P > 0.05$). *Triturus arntzeni* had a similar correlation pattern to *T. macedonicus* ($R = 0.450$, $P < 0.05$), but there were no similarities in the matrix correlation pattern compared to the other two species.

Interspecific variability in embryonic development

We performed a Kruskal-Wallis analysis to investigate interspecific variation in developmental rate (Figure 1). Based on our results, significant differences were found in all comparisons (at day 7, $H = 131.05$, $P < 0.0001$; at day 11, $H = 97.34$, $P < 0.0001$ and at day 15, $H = 81.52$, $P < 0.0001$).

We conducted a pair-wise comparison of recorded embryonic stages at each checkpoint using a Mann-Whitney test. The results revealed interspecific variability at early stages in all comparisons, but only *T. dobrogicus* was significantly different from the other spe-

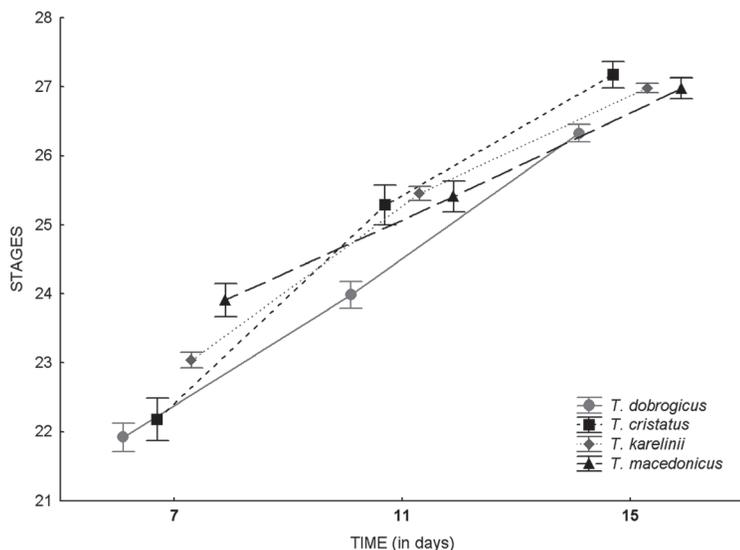


Fig. 1. Developmental stages at three different checkpoints (I = 7 days, II = 11 days and III = 15 days) during embryonic development in four crested newt species.

cies in later embryonic development. At day 7, all pair-wise comparisons showed highly significant differences, except in the *T. dobrogicus*/*T. cristatus* comparison where differences were only marginally significant (Table 4). By day 11 of embryonic development, significant differences were observed only between *T. dobrogicus* and other species. The same pattern was observed at day 15, and again only *T. dobrogicus* differed significantly in comparison to other species (Table 4).

Discussion

Although the embryonic development of all crested newts is similar in terms of the sequences of developmental stages and survival rate, species differ in the developmental rate and in the correlation pattern between analysed life-history and developmental traits. *Triturus dobrogicus* appears to be the outlier species, particularly in comparison to *T. arntzeni* and *T. macedonicus*, possessing the longest developmental period

and a different timing of morphologically defined developmental events. Previously, we found that this species is also divergent with regard to postembryonic development, showing the smallest larval size at hatching and the longest larval period, which gives rise to the largest metamorphosed juveniles compared to the other species (Furtula *et al.*, 2008, 2009). This suggests that the peculiarities of *T. dobrogicus* extend throughout ontogeny.

Differences in the sequence of developmental events, the timing and the rates of development are often invoked as causes that underlie observed phenotypic evolutionary changes (*e.g.* Ridley, 2003). In its most straightforward interpretation, our data suggest that heterochronic changes in early ontogeny can lead to lateral transposition of *T. dobrogicus* ontogenetic trajectories, as previously suggested for cranial shape (Ivanović *et al.*, 2007) and allometric limb skeleton trajectories (Ivanović *et al.*, 2008b). Additionally, two possible explanations can explain the differential embryonic pathway of *T. dobrogicus*. Since interspecific

Table 4. A pair-wise comparison of recorded embryonic stages at three checkpoints (S7, S11 and S15). The differences in developmental stages between species were tested using a Mann-Whitney test.

Species compared	S7		S11		S15	
	U	P	U	P	U	P
<i>T. macedonicus</i> - <i>T. arntzeni</i>	686.5	0.0001	1744	0.44	1212.0	0.86
<i>T. macedonicus</i> - <i>T. dobrogicus</i>	365.5	0.0001	421.5	0.0001	969.0	0.0001
<i>T. macedonicus</i> - <i>T. cristatus</i>	345.5	0.0001	587.0	0.18	587.0	0.18
<i>T. arntzeni</i> - <i>T. dobrogicus</i>	2.5	0.0001	160.0	0.0001	205.5	0.0001
<i>T. arntzeni</i> - <i>T. cristatus</i>	10.0	0.0001	281.0	0.76	123.5	0.24
<i>T. dobrogicus</i> - <i>T. cristatus</i>	261.5	0.0465	110.5	0.0001	157.0	0.0001

differences are present in the morphological traits of crested newts adults, it is possible that selection for adult traits have driven changes in developmental mechanisms at mid- and late embryonic stages, even though these stages are thought to be conserved in various vertebrate groups (Richardson, 1999; see also Richardson *et al.*, 1997). Alternatively, evolutionary changes in patterning mechanisms during embryogenesis may be driven largely by ecological determinants, rather than by the need to produce particular adult morphologies (Wray, 2000; Chipman *et al.*, 2000). This line of reasoning relates to the different ecological demands of crested newt species during embryonic and post-metamorphic stages. This hypothesis can be regarded as a likely starting point to explain the autapomorphies of *T. dobrogicus*, obtained by rapid evolution at the developmental, morphological and life-history levels. These changes have occurred much faster in *T. dobrogicus* than in other species, regardless of the historical biogeography scenarios proposed for the evolution of crested newts (see Crnobrnja-Isailović *et al.*, 1997; Arntzen *et al.*, 2007). It can be speculated that during the evolution of crested newts, the major shift in terms of ecological preferences occurred when *T. dobrogicus* invaded the floodplains along the present-day Danube river and its tributaries which are covered with extensive swamps and marshes. This event, given the appropriate ecological opportunity, might have facilitated *T. dobrogicus*' acquisition of phenotypic innovations. Local selection involving major developmental shifts can produce rapid changes in ecological preferences, morphology and life-history, especially for groups with complex life cycles like newts, and therefore may hasten divergence. Here we prove that *T. dobrogicus* ontogeny is subject to considerable evolutionary change as well. Therefore, the evolution of the crested newt could be another example of an ecological switch paralleled by dramatic changes in phenotype (see Sol *et al.*, 2005; and Herrel *et al.*, 2008). In this case, these changes follow, as summarised above, the same general interspecific patterns for large amounts of independent data gathered for various morphological traits, including for complex structures (*e.g.* skull), as well as life-history, ecological and developmental characteristics. Such patterns are most likely due to the same cause, more likely the high level of phenotypic integration in crested newts rather than homoplasies.

Mapping developmental traits onto the crested newts' phylogeny strongly suggests that developmental traits of *T. dobrogicus* are derived when compared to character traits from species sharing a common an-

cestor. In addition, the similarity of developmental traits of species from different clades (*T. macedonicus*, *T. cristatus* and *T. arntzeni*) might be due to the retention of ancestral characteristics rather than being independently acquired character states.

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