Evolutionary diversification of the limb skeleton in crested newts (*Triturus cristatus* superspecies, Caudata, Salamandridae)

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We explored the interspecific variation patterns in the limb skeleton of four crested newts that underwent an extensive evolutionary diversification. All studied species shared the same basipodial pattern, within which the carpus exhibited much more variation than the tarsus. Morphometric analysis of stylopodium, zeugopodium, and the third metapodial element showed that the species differed in the size of skeletal elements, but they shared common allometric slopes. In comparison to the other crested newts, *T. dobrogicus* appeared as the outlier due to (1) significantly shorter stylopodium and zeugopodium relative to body size and (2) rather different anatomical designs regarding ossification level of the basipodium. Based on these results, it is suggested that the observed *T. dobrogicus* uniqueness originates from allometric heterochrony paralleled by a profound switch in habitat preference.

Introduction

Generally, the development of tetrapod limbs follows a conserved, predetermined pattern (*see* Cullinane 2000 for review) with a strong phylogenetical signal (Bininda-Emonds *et al.* 2007). Variation in basipodial pattern results from changes in the processes of chondrogenesis (Shubin & Alberch 1986) that occur during early development and indicate differences in species-specific developmental constraints and in the pattern of morphological integration (Vogl & Rienesl 1991, Rienesl & Wagner 1992). However, the available information about the basis of

limb variation is limited (e.g. Tickle 2002), and empirical data from different taxa are needed.

Tailed amphibian limbs represent a "natural laboratory for addressing problems of morphological evolution" (Shubin & Wake 2003) and have been used as a model system in numerous evolutionary and developmental research studies (Hanken 1983, Vogl & Rienesl 1991, Rienesl & Wagner 1992, Shubin & Wake 1996, Shubin 2002, Gvoždik & van Damme 2006). Intraspecific variation of limb pattern in adults covers most of the phylogenetic and morphological diversity within the salamander taxa (Rienesl & Wagner 1992, Zaffaroni *et al.* 1992, Shubin *et al.*

1995, Shubin & Wake 1996). Additionally, variation of the adult limb pattern reflects development- and life-history (Shubin 2002).

The crested newts (*Triturus cristatus* superspecies) experienced an evolutionary diversification and have been used as a model group for various aspects of evolutionary studies (e.g. Arntzen 2003, Arntzen *et al.* 2007). Five parapatric species of these large-bodied European newts differ in morphology, as well as in life history traits and aquatic habitat types (summarized in Arntzen 2003, Arntzen *et al.* 2007). The present study reports on limb skeletons of four crested newt species (*T. dobrogicus*, *T. cristatus*, *T. carnifex* and *T. karelinii*) that are still poorly understood. Basipodial variation patterns of some species have been analyzed previously (Rienesl & Wagner 1992, Zaffaroni *et al.* 1999), but the interspecific disparity pattern has never been fully assessed. More precisely, we explored the size differences of stylopodium, zeugopodium, and the third metapodial element, with the underlying allometry, as well as the basipodial variation pattern and ossification level, which might give insight into how changes in morphology could have occurred in these newts. The main question of our study was whether the intense evolutionary diversification of crested newts had a profound effect on the limb skeleton. If so, does the observed variation pattern reflect differences in the species' ecological preferences? In other words, will we find evidence that the morphological limb disparity correlates with ecological diversification?

Material and methods

Samples

We analyzed the population samples of four crested newt species. To minimize possible effects of geographical variation, each species was represented by specimens collected from a single breeding unit only. The sample of *T. cristatus* came from Mt. Miroč (Serbia: 44°29´N, 22°20´E, 440 a.m.s.l.), *T. dobrogicus* from the locality Svetozar Miletić (Serbia: 20°41´E, 19°12´E, 100 a.m.s.l.), *T. karelinii* from Vlasi (Serbia: 42°59´N, 22°38´E, 470 a.m.s.l.), and *T. carnifex* from Podstrmec (Slovenia: 45°48´N, 14°34´E, 556 a.m.s.l.) (*see* Table 1 for sample review). The taxonomical affiliations of chosen populations were confirmed by their morphology, distribution, and mitochondrial DNA sequences (e.g. Kalezić *et al.* 1997, Arntzen & Wallis 1999, Arntzen *et al.* 2007, Ivanović *et al.* 2008). Only adult individuals with well-developed secondary sexual characteristics were included in the analysis. All specimens were from Georg Džukić's Bartachological Collection (Institute for Biological Research "Siniša Stanković", Belgrade) and were previously used for studies of ontogeny in the cranial shape (Ivanović *et al.* 2007) and cranial shape disparity (Ivanović *et al.* 2008).

Skeletal preparation and data collection

The limbs were preserved in 75% ethanol and prepared as cleared whole mounts, differentially stained for bone with alizarin red and for cartilage with alcian blue, according to Dingerkus and Uhler (1977). Limb skeletons, together with the scale in millimeters, were scanned using a flatbed scanner (CanoScan 5200F, Canon) in 24-bit color at 1200 dpi (Young & Hallgrimsson 2005). The scanned images were then used for analyzing skeletal variation. Each limb was inspected for the presence of fused, missing, or supernumerary mesopodial or phalangeal elements. Limbs damaged during the prepara-

Table 1. Sample sizes.

	T. carnifex	*T. cristatus*	*T. dobrogicus*	*T. karelinii*	Total
Females	13	16	10	7	46
Males	15	15	16	13	59
Front limbs	58	64	56	20	198
Hind limbs	58	64	58	40	220

tion procedure were not used in the analysis of the basipodial pattern. Also, the phalanges with changed shape were classified as damaged by mutilation and excluded from the analysis. Homologous two-dimensional landmarks (Fig. 1) were scored by the same person (N.T.) using the TpsDig software (Rohlf 2005). The landmark points were defined as endpoints perpendicular to the long axis of the bone. The dimensions of skeletal elements (in mm) were calculated as Euclidian distances between the given landmarks using IMP software (Sheets 2000). The percentage of bone in the basipodium was estimated by subtracting the ossified area from the total area of the basipodium (*see* Fig. 1). Unfortunately, we could not estimate the percentage of cartilage in the basipodium due to poor or different intensity of cartilage staining in the sample.

We used the snout–vent length (SVL) measured from the tip of the snout to the posterior end of the cloaca as a commonly used proxy for body size.

Statistical analyses

Following the methodology for studies of skeletal (basipodial) variation pattern (Rienesl & Wagner 1992, Zaffaroni *et al*. 1999), the pattern was examined and scored for the total sample (*see* Table 1). Frequency distributions were compared using a *G*-test with Williams' correction (Sokal & Rohlf 1981).

For morphometric analysis, measures of the front and hindlimb skeletal elements were taken only on the left side of each specimen. Morphometric datasets (except the data of ossification percentages) met the assumptions of normality and homogeneity of variance. To determine the differences in dimensions of skeletal elements and body size between the sexes and species, a two-way ANOVA was performed for each variable separately. We used the contrast analysis to test for pairwise comparisons with SAS statistical software (SAS package, SAS 9.1.3 Inst. 2006).

To estimate allometric relationships and the strength of the linear relationship between the size of the limb skeletal elements and body size, we performed the standardized major-axis
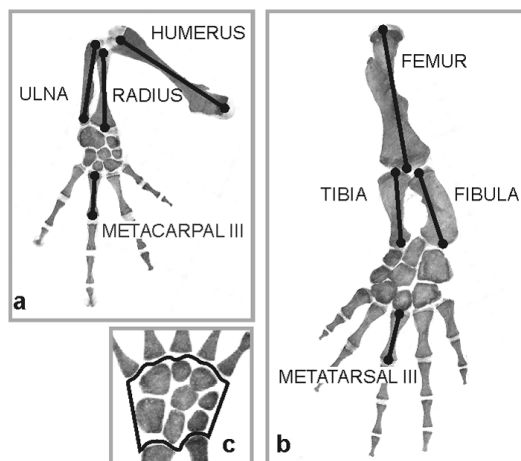

Fig. 1. The homologous landmark points, distances measured, and the estimation of the basipodium ossification level.

(SMA) analysis, recommended as most appropriate for allometric analyses (Warton *et al*. 2006). The difference in allometric relationships among the species and comparisons of the relative size of analyzed skeletal elements (tests for difference in elevation of fitted slopes) were performed using the SMATR program (SMATR software, ver. 2.0) (Falster *et al*. 2006).

Results

Basipodial variation pattern

As in other European newts (genera *Triturus*, *Mesotriton* and *Lissotriton*), the carpus of the crested newts consists of seven separate elements: ulnare/intermedium, radiale, centrale, centrale 1, distal carpals 1-2, 3 and 4 (*see* Shubin & Alberch 1986). In the analyzed samples, we found five distinct carpal fusions, one supernumerary element, and two cases of missing/lack of elements (Fig. 2). Variable combinations of observed fusions, supernumerary, and missing elements led to the seven variant carpal patterns, each containing as few as five and as many as eight separate carpal units (Fig. 2). From 196 individual manuses examined, 14 (7.1%) differed from the typical pattern. No statistically significant differences in the carpal pattern of the
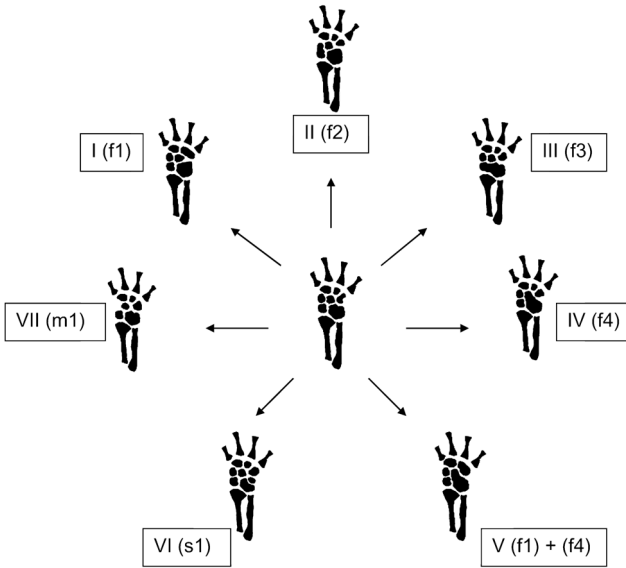
Fig. 2. Typical and observed variants of carpal patterns. Fusion f(1) consisted of two anterio- posteriorly sited distal carpals 3 and 4. Fusion f(2) was an inter-element fusion between two proximo-distally radiale and centrale 1. Fusion f(3) combined the anterio-poster neighboring radiale and ulnare/intermedium. Fusion f(4) consisted of ulnare/intermedium and centrale. Supernumerary element s(1) was likely a result of a secondary separation of the normally fused ulnare/intermedium. Missing element m(1) relate to (absence of) centrale 1.

four crested newt species were found ($G = 3.710$, $P > 0.294$). The tarsus of the crested newts consists of eight separate elements: fibulare, intermedium, tibiale, centrale, centrale 1, distal tarsals 1-2, 3 and 4 (Fig. 3). Only two of 213 tarsals examined (0.9%) showed a pattern that was different from typical, leading to two variant tarsal patterns (Fig. 3). Although both variations were found with *T. carnifex*, no statistically significant differences in tarsal variation pattern were observed among the species. In our sample, the typical patterns of fore- and hind limbs were observed in 90.5% of cases. The exception was a population of *T. karelinii*, as neither variation in carpus nor in tarsus was found. Cases of missing elements only occurred in *T. dobrogicus*. In
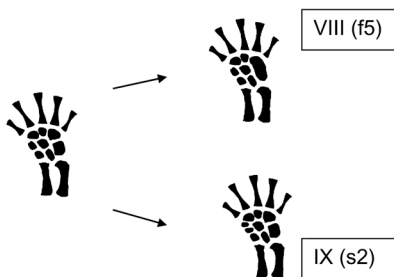
addition, fusion 2 and missing element 1 were coupled and found in four symmetrical cases in *T. dobrogicus*. The crested newt manus, which appeared first during development, was much more variable than the pes in all four species, as previously reported for *Triturus carnifex* and *T. cristatus* populations (Rienesl & Wagner 1992, Zaffaroni *et al*. 1999), as well as for some other salamander species (*Plethodon cinereus*, Hanken & Dismore 1986).

The typical phalangeal formulae were 2-2-3-2 for forelimbs and 2-2-3-3-2 for hindlimbs (Rabl 1910). Out of 323 limbs, 29 (9.0%) showed variation in the phalangeal formulae, which consisted mostly of digital reduction; however, supernumerary phalanges also occurred in fore- and hind limbs. Two individuals of *T. karelinii* had a branching pattern with phalangeal formulae of 2/3-3/3-4-3 and 2-2-3/2-2. Such serious malformations were absent from the rest of the sample. No statistically significant difference was found among species in the phalangeal formula for both the forelimbs ($G = 4.088$, $P > 0.252$) and the hind limbs ($G = 1.625$, $P > 0.654$).

Morphometric variation of limb skeletal elements

A two-way ANOVA showed that significant variation in body size existed between the sexes and

Fig. 3. Typical and observed variants of tarsal patterns. Tarsal fusions (f5) between two proximo-distal fibulare and distal tarsal 4 and one supernumerary elements (s2) lay between distal tarsal 1-2 and centrale 1.

among the species of crested newts (Table 2). Also, significant differences in the size of limb skeletal elements were found between species in the stylopodium and zeugopodium skeletal components (Table 2); though no differences between sexes in the size of these skeletal elements were found in either species. Therefore, the strength of the relationship between the size of a particular skeletal element and body size was examined for each species and sex separately using the SMA analysis (Table 3). We observed a weaker correlation between the size of particular bones of the limb skeleton and body size in *T. dobrogicus* compared with other crested newts species (Table 3). A multiple comparison of species- and sex-specific slopes revealed that the species shared common allometric slopes for each of the analyzed traits. To examine differences in the size of the limb skeleton relative to body size, we examined whether the species and the sex specific slopes had a common elevation. This analysis revealed significant differences in the size of the limb skeleton relative to body size among the newt species (ANOVA: $P < 0.05$ in all comparisons). Pairwise comparisons showed a similar pattern in the differences among the

species in respect to relative size of stylopodium and zeugopodium for both sexes. No differences between *T. carnifex* and *T. karelinii* were found; however, as compared with other crested newt species, *T. dobrogicus* had the shortest limbs, while *T. cristatus* had an intermediate position, with significantly longer bones than *T. dobrogicus*, but significantly shorter bones than *T. carnifex* and *T. karelinii*. The analyzed species were more similar in the relative size of the length of metatarsal III. However, a weak correlation between the size of metacarpal III and body size was found for both females and males of *T. dobrogicus*. When comparing allometric slopes for metatarsals among females, significant differences in relative size were found only for *T. dobrogicus*. For males, the differences in the relative size of metatarsals were found among *T. karelinii*, with longer relative sizes of metatarsals than those of *T. carnifex* and *T. dobrogicus*.

The ossification level

Visual examination of the basipodial variation pattern showed that specimens varied largely in

Table 2. The variability in body size and limb skeletal dimensions among crested newts as analyzed using two-way ANOVA with species and sex (nested within species) as factors.

Trait	Source of variation	df	SS	F	P
Body size					
SVL	species	3	0.1128	29.66	0.0001
	sex	4	0.0212	4.17	0.0037
Frontlimb skeleton					
humerus	species	3	0.6150	92.97	0.0001
	sex	4	0.0099	1.12	0.3523
radius	species	3	0.6382	127.39	0.0001
	sex	4	0.0127	1.91	0.1155
ulna	species	3	0.7300	108.24	0.0001
	sex	4	0.0125	1.39	0.2419
metacarpal III	species	3	0.5736	75.19	0.0001
	sex	4	0.0899	8.84	0.0001
Hindlimb skeleton					
femur	species	3	0.5288	102.8	0.0001
	sex	4	0.0078	1.13	0.3451
tibia	species	3	0.5566	107.8	0.0001
	sex	4	0.0118	1.71	0.1544
fibula	species	3	0.5598	107.06	0.0001
	sex	4	0.0147	2.11	0.0861
metatarsal III	species	3	0.3618	51.51	0.0001
	sex	4	0.0929	9.92	0.0001

Table 3. Relationship between the size of the limb skeletal elements and body size obtained from the standardized major axis (SMA) analysis: coefficient of determination (r^2) is calculated for each sex and species separately (the sample sizes for each species and sex are given), the common allometric slope and the obtained significance levels are given. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Trait	Sex	T. carnifex			T. cristatus			T. dobrogicus			T. karelinii			Common slope (CI)	
		n	r^2	P	n	r^2	P	n	r^2	P	n	r^2	P	(CI)	P
Frontlimb															
Humerus	f	13	0.773	***	16	0.695	***	7	0.740	*	7	0.571	*	1.133 (0.949–1.353)	ns
	m	15	0.86	***	13	0.809	***	13	0.334	*	13	0.791	***	1.502 (1.305–1.727)	ns
Ulna	f	13	0.826	***	16	0.772	***	10	0.262	ns	7	0.338	ns	1.283 (1.069–1.542)	ns
	m	15	0.889	***	15	0.683	***	14	0.093	ns	13	0.816	***	1.488 (1.278–1.732)	ns
Radius	f	13	0.847	***	16	0.762	***	10	0.380	ns	7	0.442	ns	1.095 (0.922–1.302)	ns
	m	15	0.902	***	15	0.657	***	15	0.086	ns	13	0.858	***	1.297 (1.118–1.505)	ns
Metacarpal III	f	13	0.912	***	16	0.619	***	10	0.433	*	7	0.638	*	1.093 (0.894–1.332)	ns
	m	12	0.871	***	15	0.758	***	15	0.203	ns	12	0.694	***	1.728 (1.456–2.051)	ns
Hindlimb															
Femur	f	13	0.765	***	16	0.677	***	10	0.791	***	7	0.463	ns	1.047 (0.878–1.249)	ns
	m	15	0.823	***	15	0.74	***	15	0.525	**	13	0.89	***	1.290 (1.130–1.474)	ns
Tibia	f	13	0.734	***	16	0.609	***	10	0.642	**	7	0.409	ns	1.024 (0.840–1.248)	ns
	m	15	0.795	***	15	0.738	***	15	0.243	ns	12	0.796	***	1.293 (1.098–1.521)	ns
Fibula	f	13	0.737	***	16	0.685	***	10	0.683	**	7	0.426	ns	1.032 (0.852–1.249)	ns
	m	15	0.767	***	15	0.821	***	15	0.277	*	12	0.896	***	1.192 (1.007–1.409)	ns
Metatarsal III	f	12	0.474	*	16	0.756	***	10	0.461	*	7	0.595	*	1.189 (0.965–1.466)	ns
	m	14	0.63	***	15	0.825	***	14	0.168	ns	13	0.734	***	1.551 (1.294–1.858)	ns

relative size and in the amount of "bone packaging" in the basipodium (*see* Fig. 4). To quantify this trait, we estimated the total basipodial area and the area of each ossified basipodial element for each specimen (Fig. 1). "Bone packaging" was estimated as the ratio of ossified tissue in the basipodium and was given as percentage. The non-parametric, Kruskal-Wallis test was used to estimate differences in the ossification level between sexes and among species. We found no statistically significant difference in the percentage of ossification between the sexes in any of the analyzed crested newt species for both front and hind limbs ($P > 0.05$ in all comparisons). Therefore, the sexes were pooled for interspecific comparisons.

Statistically significant differences in ossification level were found among the species. Pairwise comparisons showed that *T. dobrogicus* (front limb: mean = 56.1%, range = 23.2%–74.4%; hind limb: mean = 61.2%, range = 43.5%–77.4%) had the lowest ossification level and limper basipodial "packaging" as compared with those in all other crested newt species ($P < 0.01$ in all comparisons). *Triturus cristatus* (front limbs: mean = 72.8%, range = 60.2%–82.8%; hind limbs: mean = 72.4%, range = 62.1%–82.4%) and *T. carnifex* (front limbs: mean = 75.0%, range = 55.8%–83.6%; hind limbs: mean 70.2%, range = 41.5%–82.8%) had the highest ossification level and stiffer "packaging" of basipodial elements, while *T. karelinii* had a somewhat lower percentages of ossification level (front limbs: mean = 69.9%, range = 32.7%–82.3%; hind limbs: mean = 66.0%, range = 28.8%–76.6%). A statistically significant difference among these three species was found only between *T. cristatus* and *T. karelinii* for the hind limb ossification level ($P < 0.01$). In all other pairwise comparisons, no difference in ossification level was found among *T. carnifex*, *T. cristatus*, and *T. karelinii* ($P > 0.05$). Therefore, in contrast to *T. dobrogicus*, other crested newt species have a well-ossified basipodium.

Discussion

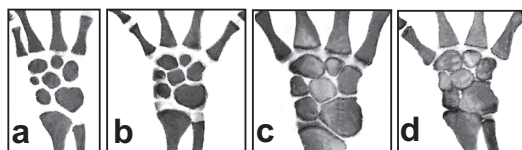The morphometric analysis showed that between crested newts, the sizes of stylopodium and

Fig. 4. The forelimbs of four crested newt species that illustrate differences in "bone-packaging". Bones are grey, cartilage and surrounding soft tissue are clear or semi-transparent. (**a**) *T. dobrogicus*, (**b**) *T. karelinii*, (**c**) *T. cristatus* and (**d**) *T. carnifex*.

zeugopodium have a congruent, cline-like interspecific variation pattern. Comparing species-specific allometries, we found that these skeletal elements share a common allometry, which most likely indicates a preserved conservative direction of ontogenetic trajectories of proximal parts of limb skeletons in these newts. In contrast, the observed lateral shift in species-specific allometries of *T. dobrogicus* and *T. cristatus*, as compared with that of the other two species, indicates evolutionary changes in allometric trajectories. The observed significant lateral shift of allometric trajectories indicates that heterochronic changes could underlie the observed morphological variation. In line with this is that the tailed amphibians are particularly prone to heterochrony during their limb evolution (Hinchliffe 2002). We rely here on static allometry, which only limited our analysis for evolutionary issues, although such allometry could be used as a substitute for ontogenetic allometry (*see* Klingenberg, 1998 for review).

The other two aspects of morphological limb variation also indicate heterochronic changes and support previous findings. The patterns uncovered in this study suggest the observed basipodial variants in the crested newt species are concordant with the described patterns of variation in other salamander species (Shubin & Wake 2003), as well as with previously reported basipodial variation in *T. carnifex* (Zaffaroni *et al*. 1999). A notable finding of our study is that the *T. dobrogicus* manus has lost one of the metacarpal elements (centrale 1), which could be a result of the retention of embryonic connections into the adult stage (Shubin & Alberch 1986, Shubin 2002). The exceptional nature of *T. dobrogicus* extends to the basipodium skeleton

design as well, with a low ossification level and concomitant loose "bone packaging".

Based on our results, we can speculate that the proximate mechanism of morphological modeling in *T. dobrogicus* could be heterochronic changes. However, the influence of growth on the evolution of the crested newt limbs could only be inferred from the study of ontogenetic and phylogenetic interplay. Unfortunately, such data are currently not available, but they seem promising for future study.

The observed limb skeleton morphology, including extension ossification and "bone packaging", can give some clues about its possible adaptability to a particular environment. As amphibians with a biphasic life cycle where larvae and breeding adults are aquatic and juveniles and non-breeding adults live on land, the crested newts use both aquatic and terrestrial locomotion. The four crested newt species differ considerably in their preferences for aquatic habitats. The most aquatic species is *T. dobrogicus*, whose adults spend at least six months annually in water, followed by *T. cristatus* (five months), *T. carnifex* (four months), and *T. karelinii* (three months) (Andreone & Giacoma 1989, Arntzen 2003). *Triturus dobrogicus*, in contrast to other crested newt species, is exclusively restricted in its distribution to relatively large, open bodies of water (e.g. stagnant swamps and marshes and channels with slow running water) in flood lowlands. Conversely, *T. carnifex* and *T. karelinii*, as predominantly pond-dwellers, spend their breeding periods exclusively in small lentic ponds, most often at relatively high altitudes. The well-ossified manus and pes and longer bones of the proximal parts of limbs, which have to sustain body weight when on land, can be regarded as adaptations of these newts that are produced by the mechanical demands of terrestrial locomotion. The most aquatic *T. dobrogicus* has short limbs and a much lower ossification level in the basipodium. The slender body and reduced limbs of *T. dobrogicus* should favor undulatory swimming while they are in water, while the stout body, short trunk and the larger stride lengths of well developed limbs (*T. karelinii*, *T. carnifex* and *T. cristatus*) are adaptations for terrestrial locomotion (Arntzen & Wallis 1999, Arntzen *et al.* 2007). However, contrary to expectations, no

evidence of an evolutionary trade-off between the aquatic and terrestrial performance of these newts has been found (Gvoždík & van Damme 2006).

Nevertheless, we suggest that the limb skeleton traits in crested newts are correlated with their ecological diversifications. Namely, the adaptive shift in habitat preference during the evolution of crested newts occurred when the *dobrogicus* clade invaded the lowland, large, freshwater bodies of the Pannonian and Dobrogean plains, having become adapted for a much more aquatic lifestyle (e.g. Crnobrnja-Isailović *et al.* 1997, Arntzen *et al.* 2007). The change was so drastic that *T. dobrogicus* could be considered to have a different ecological niche than the other crested newt species (e.g. Arntzen & Wallis 1999).

Acknowledgements

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