

Egg Size Variation in Crested Newts from the Western Balkans (Caudata: Salamandridae: *Triturus cristatus* Superspecies)

Milena Furtula^{1,*}, Ana Ivanović², Georg Džukić¹, and Miloš L. Kalezić^{1,2}

¹Institute for Biological Research, Siniša Stanković, Department for Evolutionary Biology, Bulevar despota Stefana 142, Belgrade 11060, Serbia

²Institute for Zoology, Faculty of Biology, Studentski trg 16, Belgrade 11000, Serbia

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Milena Furtula, Ana Ivanović, Georg Džukić, and Miloš L. Kalezić (2008) Egg size variation in crested newts from the western Balkans (Caudata: Salamandridae: *Triturus cristatus* superspecies). *Zoological Studies* 47(5): 585-590. We report herein on egg size characteristics (vitellus volume and jelly/vitellus ratio) of 4 crested newt species (*Triturus cristatus* superspecies). The key results were as follows: (1) maternal and population effects on these characteristics were detected, however, the species effect was much more pronounced; (2) in terms of the vitellus volume, *T. karelinii* and *T. macedonicus* had the largest eggs, *T. dobrogicus* had the smallest, and *T. cristatus* had eggs of intermediate size; (3) all species significantly differed in their jelly/vitellus ratio values, with the lowest ratio in *T. macedonicus* followed by *T. karelinii*, *T. cristatus*, and *T. dobrogicus* with the highest ratio value; and (4) the species shared a common allometric slope of the egg volume/body size relationship. The egg size traits appeared to follow a evolutionary trend similar to that of the morphological and ecological diversity characteristics. <http://zoolstud.sinica.edu.tw/Journals/47.5/585.pdf>

Key words: Interspecific diversity patterns, Life history, Allometry.

One of the model groups for various aspects of evolutionary diversification is the monophyletic clade of crested newts (*Triturus cristatus* superspecies) (e.g., Arntzen et al. 2007). This group includes 5 parapatric species of *T. dobrogicus* (Kiritzescu, 1903), *T. cristatus* (Laurenti, 1768), *T. karelinii* (Strauch, 1870), *T. carnifex* (Laurenti, 1768) and *T. macedonicus* (Karaman, 1922), the latter two of which are closely related as they have only recently been recognized as separate species (Arntzen et al. 2007). Morphological, biogeographical, and ecological aspects of crested newts have been intensively studied (summarized in Arntzen 2003). The interspecific diversity patterns of morphological (e.g., body size and shape) and ecological traits (e.g., duration of the aquatic period) are a feature of crested newt evolution that is characterized by a general cline, with *T. dobrogicus* and *T. karelinii*

at opposite poles, and *T. cristatus*, and to a lesser extent *T. carnifex* and *T. macedonicus*, as intermediate species. Thus, *T. dobrogicus* has an elongated trunk with short legs, *T. karelinii* has a stocky trunk with sturdy legs; whereas *T. cristatus*, *T. carnifex*, and *T. macedonicus* are intermediate. In terms of ecology, the annual period of aquatic life follows a decreasing order (see Fig. 1) of *T. dobrogicus* to *T. karelinii* (Arntzen and Wallis 1999). To date, only scant data on a few of the crested newt life history traits are available (e.g., Kalezić et al. 1994, Griffiths 1996, Arntzen 2003). In this study of interspecific variation patterns of egg size characteristics, we wanted to gain additional insights into crested newt evolutionary diversification. Since "major changes in life history traits may represent key innovations that can spur diversification" (Givnish and Sytsma 1997), we searched for species-specific diversity patterns

*To whom correspondence and reprint requests should be addressed. Tel: 381-11-2078378. Fax: 381-11-2761433. E-mail: milena.furtula@ibiss.bg.ac.yu

of size characteristics of laid eggs of 4 crested newt species (*T. macedonicus*, *T. cristatus*, *T. dobrogicus* and *T. karelinii*).

The vitellus size only reflects the maternal nutrient input, whereas the size of the mucoid capsule gives protection from injury, fungal infestation, and ultraviolet-B radiation (UVB), and facilitates fastening onto objects (Duellman and

Trueb 1994). In the present study, we asked whether egg size traits follow an observable trend of ecological variation patterns in crested newts. In other words, is there any correlation between egg traits and habitat preferences by species that could be relevant in identifying general evolutionary factors that contribute the most to the observed interspecific patterns of egg characteristics?

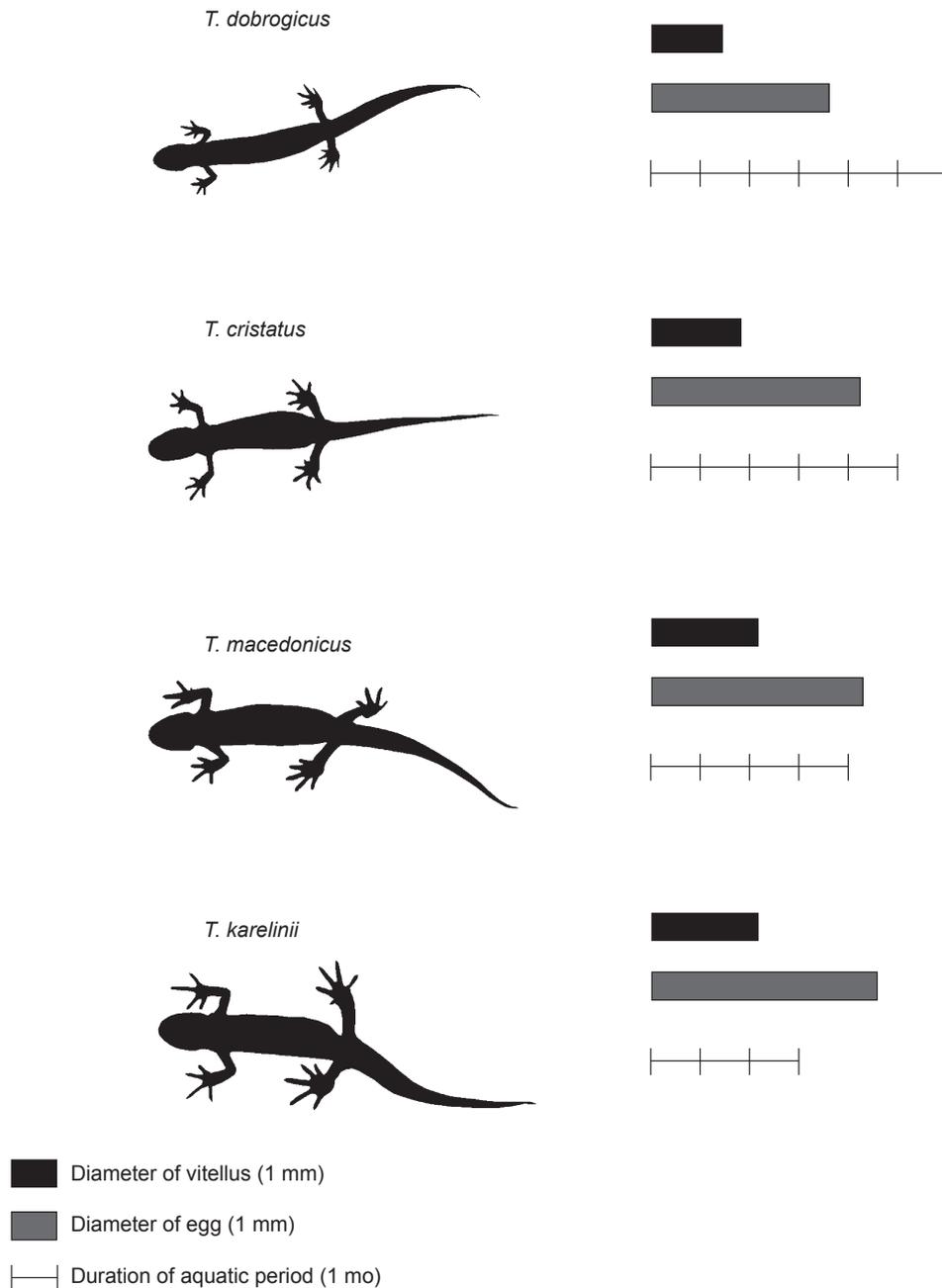


Fig. 1. Morphotypes, average egg measurements, and durations of the annual aquatic phase (months) of crested newts.

MATERIALS AND METHODS

In view of our primary goal of exploring between-species patterns of variation of a number of early life-history traits, we collected gravid females from natural populations and transferred them to the laboratory within 24 h to lay eggs. The collected adult females of 4 crested newt species were well above the size at metamorphosis (within the range of body sizes); they all had an inflated abdomen (due to the accumulation of ripe eggs) and a swollen cloaca. Females were gathered during early spring, presumably before the oviposition period began.

In the laboratory, females were individually housed in 12 L aquariums filled halfway with dechlorinated tap water under natural lighting and a controlled room temperature. The water was changed once a week. The captive females were fed every other day with worms and *Tubifex* sp. Plastic strips were provided for egg deposition, and eggs were collected and counted twice per day, early in the morning and in the evening, over the course of the entire oviposition period (Table 1). Since egg dimensions increase substantially during the 1st few cleavages, thereby introducing a potentially large source of error into the estimates of egg dimensions, the eggs were photographed with a digital camera (Nikon Coolpix 4500 alongside a 10 mm scale bar) immediately after they were removed from the plastic strips. The sizes of the eggs (maximum length and width of the jelly and maximum width of the vitellus) were measured from the photographs using the computer graphics analysis program, UTHSCSA

ImageTool version 3.0 (<http://ddsdx.uthscsa.edu/dig/itdesc.html>). The snout-vent length (SVL) of females was measured with digital calipers at the end of the oviposition period.

We calculated the vitellus volume (in mm³) using the equation for the volume of a sphere: $V = 4/3\pi r^3$, where r is the radius of the vitellus. Because the jelly capsules have an ellipsoid shape, we calculated their volume (in mm³) using the equation for an ellipsoid volume: $V = 4/3 \pi (R_1/2)(R_2/2)^2$, where R_1 is the length of the long axis and R_2 of the short diameter of the ellipsoid.

The population data, number of females, number of deposited eggs, duration of the oviposition period, and mean egg size are given in table 1.

Statistical analyses

To achieve the assumptions of normality and homogeneity of variance, the data were log-transformed as the most appropriate method for the given dataset (Fernandez 1992) and for the allometric analyses. To analyze the variability in egg sizes between species and females, we performed a two-way nested analysis of variance (ANOVA) with species, populations, and females as factors. Furthermore, we used contrast analysis from one-way ANOVA to test for statistical significance of pairwise comparisons between species (SAS statistical package vers. 9.1.3, SAS Institute 2006). Considering that the reproductive output, including egg size, can be positively correlated to female size at both the intraspecific and interspecific levels (Kaplan and

Table 1. Overview of the analyzed samples of *Triturus*: species, geographic populations, number of females, their size (snout-vent length, SVL), duration of oviposition period, and number of deposited eggs

Species	Locality	Coordinates	Elevation (m)	Date of collection	No. of females	SVL of females (mm)	Total no. of eggs	Oviposition period (d)
<i>T. macedonicus</i>	Ceklin	42°21'N 18°58'E	315	Mar. 2007	4			
	Rid	42°23'N 19°03'E	300	Mar. 2007	1	67.1-77.8	1336	21-30
	Progonovići	42°25'N 19°04'E	443	Mar. 2007	2			
<i>T. cristatus</i>	Miroč	44°29'N 22°20'E	440	Apr. 2007	8	60.1-80.7	371	1-15
	Vršacki breg	45°07'N 21°27'E	300	Apr. 2006	4			
<i>T. dobrogicus</i>	Kikinda	45°49'N 20°27'E	73	Mar. 2006	5	53.1-65.7	735	17-41
	Opovo ^a	45°03'N 20°25'E	67	Mar. 2005	6	48.9-67.2	141	4-19
<i>T. karelinii</i>	Pirot	43°09'N 22°35'E	367	Apr. 2005	5	63.4-68.9	450	4-12

^aPopulation only used for analysis of intraspecific variability.

Salthe 1979), we first estimated the strength of the linear relationship between egg and female body sizes by a standardized major axis (SMA) analysis (Warton et al. 2006). To examine the general relationship of vitellus dimensions relative to the body size in crested newts, the SMA analysis was performed on a pooled dataset (mean egg measures for each female were regressed to the SVL). The vitellus/body size relationships among the analyzed groups (species) and comparisons of relative vitellus sizes (tested for differences in elevation and a shift in the fitted slopes) were performed using the SMATR program, vers. 2.0 (Falster et al. 2006).

RESULTS AND DISCUSSION

Egg size and egg number in amphibians vary among taxonomic groups (e.g., Kaplan and Salthe 1979), among different populations of the same species (e.g., Kalezić et al. 1994), and among and within females of the same population (e.g., Rafińska 1991). Our main focus in this study was to explore interspecific egg size patterns.

To investigate the level of variation among females within populations, as well as variation among populations, 2 two-way ANOVAs were performed for samples of *T. cristatus* and *T. macedonicus*, for which samples from different populations were available. In the case of *T. dobrogicus*, we compared the data of 2 population samples. One sample (Opovo) came from another experiment that was undertaken under uncontrolled laboratory conditions; it was therefore used for interpolation comparison only (Table 1). Since variations in vitellus size among these 3 species were much greater than interpopulational variations (Table 2), further analyses were performed on the data consisting of pooled population samples of *T. macedonicus* and *T. cristatus* and single populations of *T. dobrogicus* and *T. karelinii* obtained under the same laboratory conditions. Since only 1 population sample of *T. karelinii* was available for the study, we therefore had to presume that this species followed the same pattern of intraspecific variability as the other 3 species analyzed.

Highly significant differences in vitellus volume were found for all interspecific comparisons (ANOVA, contrast statement, $p < 0.0001$), except for the *T. macedonicus* and *T. karelinii* pair. The smallest eggs by far were those of *T. dobrogicus* (with a mean of 1.936 mm^3 and a standard

deviation (SD) of 0.423 mm^3), followed by *T. cristatus* (mean of 3.502 mm^3 and an SD of 0.782 mm^3), *T. karelinii* (mean of 5.002 mm^3 and an SD of 1.023 mm^3), and *T. macedonicus* (mean of 5.022 mm^3 and an SD of 0.998 mm^3). For the jelly/vitellus ratio, significant differences in all pairwise comparisons were detected (ANOVA, contrast statement, $p < 0.0001$). The lowest jelly/vitellus ratio was from *T. macedonicus* (mean of 2.829 and an SD of 0.648), followed by *T. karelinii* (mean of 3.508 and an SD of 0.796) and *T. cristatus* (mean of 4.132 and an SD of 1.150); *T. dobrogicus* had the highest value (mean of 4.753 and an SD of 1.430).

Our results indicate that larger crested newt females tend to produce larger eggs, since a statistically significant relationship was detected between the female SVL and vitellus volume ($r^2 = 0.295$, $p = 0.0023$), but not between the jelly/vitellus ratio and SVL ($r^2 = 0.035$, $p = 0.3315$). The SMA analysis showed that the studied species shared a common allometric slope of the egg size vs. body size (SVL) relationship. There were significant differences in the elevation shift (Table 3). Relative to body size, *T. dobrogicus* had the smallest eggs in comparison to the other species ($p < 0.0001$, to *T. cristatus* $p = 0.0217$). Eggs of *T. cristatus* were also smaller in comparison to those of *T. karelinii* ($p = 0.0003$) and *T. macedonicus* ($p = 0.0157$).

The most notable result of our analyses is that the egg size was found to be a species-specific life-history trait with a cline-like distribution of specific egg size values (Fig. 1). In terms of egg size, *T. dobrogicus* has the smallest eggs, *T. karelinii* and *T. macedonicus* have the largest eggs, and *T. cristatus* has intermediate-sized eggs. Another cline concerns the jelly/vitellus ratio values, the only non-size-related egg measure; *T. dobrogicus* had the highest jelly/vitellus ratio, *T. macedonicus* had the lowest, and *T. cristatus* and *T. karelinii* had intermediate values.

In contrast to other crested newt species, *T. dobrogicus* has an exclusively restricted distribution to relatively large open bodies of water in flooded lowlands of the Pannonian and Dobrogean plains, while *T. macedonicus* and *T. karelinii* are predominantly pond-dwellers; they spend their breeding periods exclusively in small lentic ponds at relatively high elevations. We hypothesized that an adaptive shift in *T. dobrogicus* habitat preference during the evolution of crested newts (Crnobrnja-Isailović et al. 1997, Arntzen et al. 2007) was paralleled by a shift in life-history

traits, including egg-size characteristics, as well as in body morphology and ecology.

Further research should gather more life history data in an effort to understand the mechanisms underlying the observed clines. Nevertheless, when looking for possible selective agents among the natural environmental

features, the key may be in the existing habitat preferences of these crested newts. In terms of the hydroperiod, temperature, and oxygen concentration, *T. dobrogicus* occupies more-stable water bodies, whereas *T. macedonicus* and *T. karelinii* live in conditions of much more variable temperatures and oxygen concentrations.

Table 2. Results of two-way ANOVAs for vitellus volume and the egg/vitellus ratio of *Triturus* spp. The analyses of population’s effect and the effects of females nested within populations were performed separately for each species

Variable	Source of variation	d.f.	MS	F	p
Vitellus volume					
<i>T. macedonicus</i>	Populations	2	0.8554	157.27	***
	Females (population)	4	0.3453	63.49	***
<i>T. cristatus</i>	Populations	1	0.0376	6.93	**
	Females (population)	10	0.1388	25.62	***
<i>T. dobrogicus</i>	Populations	1	1.7272	191.9	***
	Females (population)	9	0.3198	35.53	***
Pooled data	Species	2	13.5647	1545.67	***
	Populations (species)	4	2.842	323.84	***
Jelly/vitellus ratio					
<i>T. macedonicus</i>	Populations	2	0.6065	85.62	***
	Females (population)	4	0.4126	58.25	***
<i>T. cristatus</i>	Populations	1	0.0968	8.68	**
	Females (population)	10	0.0922	8.27	***
<i>T. dobrogicus</i>	Populations	1	0.0078	0.63	ns
	Females (population)	9	0.2692	21.64	***
Pooled data	Species	2	7.625	973.34	***
	Populations (species)	4	0.417	36.83	***

ns, not significant; $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Results of standardized major axis (SMA) regression analysis for vitellus volume and snout-vent length (SVL) of *Triturus* spp.

Trait pair (X and Y)	Species	n	r ²	p	Slope	Intercept	Slope homogeneity (p)	Shift in elevation (p)	Shift along slope (p)
Vitellus volume and SVL	<i>T. macedonicus</i>	7	0.160	0.373	2.26	-3.50	ns	***	***
	<i>T. cristatus</i>	12	0.048	0.495	2.02	-3.19			
	<i>T. dobrogicus</i>	5	0.065	0.679	-1.10	2.22			
	<i>T. karelinii</i>	5	0.047	0.727	-4.46	8.83			

ns, not significant; $p > 0.05$; *** $p < 0.001$.

These general habitat features and the observed relationship to the size of the mucoid capsule (egg volume/vitellus volume) seem to be concordant with the relationship between the jelly coat and oxygen consumption (the mucoid layer is a significant barrier to oxygen uptake, i.e., Seymour 1999) and the possible role in protecting eggs from UVB (i.e., Adams et al. 2001, Palen et al. 2005).

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REFERENCES

- Adams MJ, DE Schindler, RB Bury. 2001. Association of amphibians with attenuation of ultraviolet-B radiation in montane ponds. *Oecologia* **128**: 519-525.
- Arntzen JW. 2003. *Triturus cristatus Superspecies* – Kammolch-Artenkreis. (*Triturus cristatus* (Laurenti, 1768) – Nirdlicher kammolch, *Triturus macedonicus* (Laurenti, 1768) – Italienischer kammolch, *Triturus dobrogicus* (Kiritzescu, 1903) – Donau-Kammolch, *Triturus karelinii* (Strauch, 1870) – Sudlicher kammolch). In W. Böhme, ed. *Handbuch der Reptilien und Amphibien Europas*. Band 4/IIA: Schwanzlurche (Urodela) IIA. Wiebelsheim: Aula-Verlag, pp. 421-514.
- Arntzen JW, GE Themudo, B Wielstra. 2007. The phylogeny of crested newts (*Triturus cristatus* superspecies): nuclear and mitochondrial genetic characters suggest a hard polytomy, in line with the paleogeography of the centre of origin. *Contrib. Zool.* **76**: 261-278.
- Arntzen JW, GP Wallis. 1999. Geographic variation and taxonomy of crested newts (*Triturus cristatus* superspecies): morphological and mitochondrial DNA data. *Contrib. Zool.* **68**: 181-203.
- Crnobrnja-Isailović J, G Džukić, N Krstić, ML Kalezić. 1997. Evolutionary and paleogeographical effects on the distribution of the *Triturus cristatus* superspecies in the central Balkans. *Amphibia-Reptilia* **18**: 321-332.
- Duellman WE, L Trueb. 1994. *Biology of amphibians*. Baltimore, MD: Johns Hopkins Univ. Press.
- Falster DS, DI Warton, IJ Wright. 2006. SMATR: Standardised Major Axis Tests and Routines, vers. 2.0. Available at <http://www.bio.mq.edu.au/ecology/SMATR/>.
- Fernandez CJ. 1992. Residual analysis and data transformations: important tools in statistical analysis. *HortScience* **27**: 297-300.
- Givnish TJ, KJ Sytsma. 1997. *Molecular evolution and adaptive radiation*. Cambridge, UK: Cambridge Univ. Press.
- Griffiths R. 1996. *Newts and salamanders of Europe*. London: T & AD Poyser.
- Kalezić ML, D Cvetković, A Đorović, G Džukić. 1994. Paedomorphosis and differences in life-history traits of two neighbouring crested newt (*Triturus carnifex*) populations. *Herpetol. J.* **4**: 151-158.
- Kaplan RH, SN Salthé. 1979. The allometry of reproduction: an empirical view in salamanders. *Am. Nat.* **113**: 671-689.
- Palen WJ, CE Williamson, AA Clauser, DE Schindler. 2005. Impact of UV-B exposure on amphibian embryos: linking species physiology and oviposition behavior. *Proc. R. Soc. B.* **272**: 1227-1234.
- Rafińska A. 1991. Reproductive biology of the fire-bellied toads, *Bombina bombina* and *B. variegata* (Anura: Discoglossidae): egg size, clutch size and larval period length differences. *Biol. J. Linn. Soc.* **43**: 197-210.
- Seymour RS. 1999. Respiration of aquatic and terrestrial amphibian embryos. *Am. Zool.* **39**: 261-270.
- Warton DL, IJ Wright, DS Falster, M Westoby. 2006. Bivariate line-fitting methods for allometry. *Biol. Rev.* **81**: 259-291.