Larval pigmentation patterns of closely related newt species *(Triturus cristatus* and *T. dobrogicus*) in laboratory conditions

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Received: 13. November 2014 / Accepted: 05. May 2015 / Available online: 08. November 2015 / Printed: December 2015

*Triturus cristatus* (Laurenti, 1768) and *T. dobrogicus* (Kiritsescu, 1903) belong to a monophyletic closely related group of crested newts (Wielstra & Arntzen 2011). *Triturus cristatus* is widely distributed across Europe, while *T. dobrogicus* distribution is restricted to the valleys within the Danube River system. *Triturus dobrogicus* and, to a lesser extent, *T. cristatus*, inhabit permanent large water bodies - *T. dobrogicus* can be even found in slow running waters (e.g. Arntzen 2003). These two species come in contact and hybridize (Mikulíč et al. 2012) along the contact zone of their ranges, from Ukraine on the east to the Czech Republic on the west (Arntzen et al. 2014).

Identifying adults of *Triturus* species based on external morphology rely on their divergence in body size and shape (e.g., Arntzen & Wallis 1999, Jehle et al. 2011, Vukov et al. 2011), as well as on external qualitative traits (black dots on the ventral side of the head and body), which have been proven to be useful in discrimination of crested newt species (e.g., Arntzen & Wallis 1999). However, little is known about morphological differentiation of crested newt species at the larval stage (Schmidt & Van Buskirk 2005). The *Triturus* larvae can be easily recognized and distinguished from those of other European newts by having three pairs of external and protruding gills, which extend from either side of the neck like feathery plumes, and by having long toes and tapering tail fin with irregular black spots (Griffiths 1996, Arnold & Ovenden 2002). So far, larvae of crested newt species are considered as being morphologically very similar (Arntzen 2003). Study of variation in body shape among crested newt larvae (Ivanović et al. 2011) found that larvae, like adults and freshly metamorphosed juveniles (Vinsálková & Gvoždík 2007), differentiate in body shape (lateral head shape, interlimb distance).

Here, we focused on larval pigmentation patterns of two crested newt species, *Triturus cristatus* and *T. dobrogicus*. Such qualitative traits would be helpful in species identification at larval stage. All analysed larvae were obtained from eggs of females collected in populations which are out of the species contact zone. Females of *T. dobrogicus* (*n* = 4) originated from Kikinda town, Serbia (45º 06´ N, 21º 20´ E, 180 m a.s.l.), and females of *T. cristatus* (*n* = 5) were captured in Miroč, Serbia (44º 29´ N, 22º 20´ E, 440 m a.s.l.) and Vršački breg, Korkana, Serbia (45º 06´ N, 21º 27´ E, 180 m a.s.l.). In the laboratory, females were housed individually in 12-L aquaria containing six litres of dechlorinated tap water. The captive females were fed with fly larvae (*Musca* sp.) and *Tubifex* worms every other day. Aquaria were equipped with plastic strips for egg deposition and were daily checked for the presence of eggs. Eggs developed in Petri dishes (5 cm in diameter) containing a maximum of 10 eggs per dish and filled with enough dechlorinated tap water to cover the eggs under laboratory conditions (see below). The water was changed every second day. Upon hatching, the larvae were placed in 100ml plastic cups filled with 50ml of dechlorinated tap water. After 30 days, the larvae were transferred to 1-L plastic containers. The number of larvae per cup/container was kept constant during the experiment (maximum three larvae per container). Larvae were reared under controlled laboratory conditions. They were kept at 16-17ºC. Water was changed on a two-day basis. During the earlier stages, larvae were fed with *Artemia* sp. and during the later stages with *Tubifex* sp.

Pigmentation patterns data were obtained from 62 larvae of *T. dobrogicus*, and 24 larvae of *T. cristatus*. Larvae were examined at stage 62 (Glücksohn 1932), which is characterized by fully developed limbs and tail fin before the beginning of metamorphosis. Larvae were placed in a small water-filled glass chamber. The chamber was fitted with mirrors that project both a lateral and a ventral view of the larvae, and the individuals were photographed with a digital camera (Nikon Coolpix 4500). We used photographs of the lateral side to analyse larval pigmentation patterns.

We scored three traits to characterize larval pigmentation patterns: I. The number of dark blotches on the tail - (T1) sparse blotches (<5), (T2) moderate number of blotches (5-10), (T3) numerous blotches (>10); II. The position of the dark blotches on the tail - (P1) on the edges of...
The caudal fin, (P3) on the body of the tail, (P3) all over the tail; and III. The presence of visible lateral line – (L1) there is no lateral line, (L2) there is partially visible lateral line, (L3) there is whole lateral line. We counted only blotches, the clearly visible, larger pigmented areas. The smaller pigmented areas (dots) that are not clearly visible and distinguishable were ignored (see Fig. 1a). All traits were recorded by one person (MC).

We applied a Multiple Correspondence Analysis – MCA on scored traits to examine character contribution to species differences in pigmentation patterns. The interspecific variation in the pigmentation patterns was tested using a G-test (Sokal & Rohlf 1981). Statistical analyses were performed using Statistica 10.0 (StatSoft, Inc., Tulsa, OK).

The graphical presentation of variation in pigmentation patterns between species relative to the first two axes obtained by MCA on analysed traits are given in Figure 1b. The first two axes explained 55.17% of total inertia (Fig. 1b). Specifically, *T. cristatus* larvae were characterized by the presence of dark blotches on the edges of the caudal fin (p1) and the absence of lateral line (l1). In contrast, larvae of *T. dobrogicus* had few dark blotches restricted to the body of the tail (t1, p2) and the whole lateral line was clearly visible (l3) (Fig. 1b). We found significant differences in the position of dark blotches (G-test, $G_2 = 87.58, p < 0.001$), the visibility of the lateral line (G2 = 75.64, $p < 0.001$), and in the density of the dark blotches between *T. dobrogicus* and *T. cristatus* ($G_2 = 9.78, p < 0.01$).

Our results showed that *Triturus dobrogicus* larvae had a sparse number of dark blotches, usually on the surface of the tail, and the lateral line was visible. On the other hand, larvae of *T. cristatus* were clearly distinguished by having dark blotches on the caudal fin edges and no visible lateral line. The clear differences in larval pigmentation pattern suggest that larvae of both species are distinguishable using phenotypic traits at least under controlled laboratory conditions.

Generally, the pigmentation pattern in amphibian larvae show marked plasticity, which is induced by various biotic and abiotic cues, such as the presence of predators and canopy cover (Van Buskirk & Schmidt 2000, Van Buskirk et al. 2004, Van Buskirk 2011). Accordingly, the plasticity of larval pigmentation pattern in both *Triturus* species remains to be tested. Further field and lab studies will provide data on geographic variation and stability in pigmentation patterns and larval shape in natural populations of not only examined taxa but also other species of *T. cristatus* complex. Finally, the stability of pigmentation patterns through larval development and possible effect of hybridization on larval pigmentation provide promising areas for further research.

**Figure 1.** Pigmentation patterns and position of two crested newt species in MCA. The arrows show counted pigmented area (a) on *T. cristatus* (upper photo) and *T. dobrogicus* (bottom photo). b) The position of species (grey squares) relative to character states (circles). T – the number of dark blotches on the tail, P – the position of the dark blotches on the tail, L – the presence of visible lateral line.

**Acknowledgements.** We would like to thank Lumír Gvoždík and Dan Cogalniceanu for very constructive comments and discussions that improved the presentation of the data. This work was supported by the Serbian Ministry of Education, Science and Technological Development (project No. 173043). The experiments were approved by the Ethical Committee of the Institute for Biological Research “Siniša Stanković” (no. 6/06). All animals were collected under permits provided by the Ministry of Environmental Protection, Republic of Serbia (no. 353-01-309/2006-03).

**References**


Pigmentation pattern of Triturus larvae


