Reproductive characteristics of two *Triturus* species (Amphibia: Caudata)

Tijana Vučić[1,2,*], Ana Ivanović[1], Sonja Nikolić[1], Jovana Jovanović[1] and Milena Cvijanović[2]

[1]*Institute of Zoology, Faculty of Biology, University of Belgrade*, Studentski trg 16, 11000 Belgrade, Serbia

[2]*Department of Evolutionary Biology, Institute for Biological Research "Siniša Stanković"- National Institute of the Republic of Serbia, University of Belgrade*, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

*Corresponding author: tijana.vucic@bio.bg.ac.rs

Abstract: During three consecutive years, we compared the reproductive characteristics and oviposition dynamics of two crested newt species, *Triturus ivanbureschi* and *T. macedonicus*. These two well-defined species are of special interest because of complex interactions at their contact zone, which include hybridization, species replacement and asymmetric mitochondrial DNA introgression. In common garden experiments, females were introduced to conspecific males, to males of other species and to hybrid males. We monitored the total number and size of the deposited eggs, as well as the dynamics and duration of oviposition during three consecutive years. The number of deposited eggs of *Triturus* species was much higher than previously reported. *Triturus macedonicus* lay more eggs that were of larger size in comparison to *T. ivanbureschi*. Also, the onset of *T. macedonicus* oviposition was considerably delayed compared to *T. ivanbureschi*. These are fundamental data, important for understanding complex species interactions in their contact zones.

Keywords: life history traits; hybrid zone; Salamandridae; *Triturus*

INTRODUCTION

Triturus newts, a monophyletic group of nine species of large-bodied newts, are strictly oviparous and all species have similar reproductive behavior and life history [1]. Females devote a significant amount of time and energy to producing and laying eggs. Complex courtship behavior occurs at the beginning of the aquatic period. After internal fertilization, oviposition lasts for several weeks. Eggs are protected with a jelly layer and are deposited individually, wrapped in leaves of underwater vegetation [1-6]. This specific oviposition behavior is thought to protect eggs from predation, mechanical damage and the negative effects of UV radiation [4,7,8], and it also increases embryonic survival rates [8,9]. The number and size of deposited eggs can vary significantly among amphibian populations within a single species and even among females of the same population. Variations in egg number and size can be interdependent and affected by different external and internal factors [10-13]. At the beginning of the breeding period, female newts have a certain number of formed yolked oocytes, which represent the maximum number of eggs that can be laid in a given season, but some eggs might not be deposited [3,14,15]. Egg size influences embryonic development and larval growth [16,17]. Previous studies on newt egg size established that this trait is species-specific [18] and that it differs between populations of the same species [19].

Triturus species belong to two distinct sister groups, the marbled (two species) and crested newts (seven species). On the Balkan Peninsula, particularly in its central part, in the territory of Serbia, four species of crested newts meet and hybridize [20,21]. In this study, we analyzed the reproductive characteristics of two morphologically and genetically distinct species, *T. ivanbureschi* and *T. macedonicus*. These two species have slightly different habitat preferences [22] and belong to different ecomorphotypes [23]. They show morphological differences at hatching, throughout larval development and after metamorphosis [24-26]. The larvae of the two species also differ in antioxidative response [27]. Regarding life-history traits, *T. ivanbureschi* has a shorter developmental period and

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metamorphoses earlier than *T. macedonicus*, while *T. macedonicus* has higher embryonic survival rates and a longer lifespan [18,19,28,29].

There is a wide zone of *T. ivanbureschi* and *T. macedonicus* overlap in Serbia where individuals possess nuclear DNA mostly of *T. macedonicus*, but mitochondrial DNA of *T. ivanbureschi*. The introgressed *T. ivanbureschi* DNA is considered a genomic footprint of the previous *T. ivanbureschi* range. According to this scenario, *T. macedonicus* expanded its range and transected that of *T. ivanbureschi*, dividing the latter into the northern enclave and the southern part of the species' range [30,31]. In eastern Serbia (Vlasi village, near Pirot), there is a confirmed viable hybrid population consisting of individuals derived from many generations of mutual hybrid crossing and backcrossing with parental species [32,33].

Given the hypothesized species displacement, one of the first important questions to address is whether differences in reproductive characteristics provide *T. macedonicus* an advantage over *T. ivanbureschi*. The number of gametes produced along with survival rate (i.e. reproductive success) are the main determinants of a species' biological potential. In species competing for the same resources, besides their survival rates and fitness, differences in reproductive characteristics could lead to the prevalence of the species that produces the highest number of gametes. Differences in oviposition dynamics could reflect a species' ecological preferences. To compare these characteristics of the two species, we analyzed their egg-laying performances and seasonal dynamics during their entire reproductive cycle. Common garden crossing experiments in three consecutive years (2016-2018) were performed to collect the reproductive data. We simulated the initial species' contact zone, and females were randomly introduced to conspecific males, males of the other species, and to F$_1$ hybrid males. Because the external environmental factors and experimental design (types of crossings) varied over the years, we indirectly assessed the effects of the environment and of intra- and interspecific female preferences. We tracked the following measures of reproductive success: the proportion of egg-laying females, the total number and size of deposited eggs, and we also followed the dynamic and duration of oviposition.

MATERIALS AND METHODS

Ethics statement

Collecting *T. ivanbureschi* adults from a natural population was approved by the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia (Permit No. 353-01-75/2014-08). Collecting *T. macedonicus* adults from a natural population was approved by the Environmental Protection Agency of Montenegro (Permit No. UPI-328/4). The experimental procedures were approved by the Ethics Committee of the Institute for Biological Research "Siniša Stanković", University of Belgrade (Decision No. 03-03/16). All animal procedures complied with the European Directive on the protection of animals used for experimental and other scientific purposes (2010/63/EU).

Experimental design

Adult females and males of *T. ivanbureschi* (Zli Do, Serbia; 42°25' N; 22°27' E) and *T. macedonicus* (Ceklin, Montenegro; 42°21' N; 18°59' E) were taken from natural populations well outside their hybrid zone, assumed to be unaffected by interspecific gene flow [34], and were transferred to the laboratory at the Institute for Biological Research "Siniša Stanković" in the spring of 2014 and 2015, respectively. We designed an experiment that focused on mating within and between the two species. In each of 3 experimental years (2016, 2017 and 2018) all crossings were performed after hibernation in a cold room chamber (4°C) at the beginning of March in semi-natural conditions. The semi-natural conditions during crossings and at the beginning of egg deposition allowed us to estimate the effects of external factors (e.g. temperature) at the start of the oviposition period. In 2016, females of *T. ivanbureschi* and *T. macedonicus* were allowed to mate only with males of the opposite species. In 2017, females were crossed both with conspecific males and with males of the other species. In 2018, females were crossed with conspecific males and males of the other species, as well as with F$_1$ hybrid males, as outlined in Supplementary Fig. S1 and Supplementary Table S1. The males also differed in age and could be divided into two age classes: unknown age (males from natural populations of the two species) and 2 years old (F$_1$ hybrid males obtained from 2016 crossings and raised in the laboratory).

When females started depositing eggs, they were transferred to separate 10-L aquaria, half-filled with dechlorinated tap water. Transparent plastic strips were provided as a substrate for deposition of eggs. During oviposition, females were kept in the laboratory under controlled conditions. The temperature was kept constant at 17-19°C and all females were fed every other day with earthworms or *Tubifex* sp. The same settings and conditions were maintained during all 3 years of the experiment.

Data collection

Each year at the beginning of March, after hibernation and before reproduction, the body mass of females was measured using an electronic device to the nearest of 0.01 g. In the last year of the experiment (2018), we photographed all females before reproduction. The body size (snout to vent length or SVL) of females was measured from photographs of the dorsal view of animals (from the tip of the snout to the level of the posterior edge of the hind legs). For all females that were laying eggs during the three-year experiment, the body condition index (BCI) was calculated in the last year of the experiment (2018). We used the residual index as the most recommended index for body condition in amphibians [35,36]. The BCI was calculated as the difference between the observed and predicted body mass of females. To obtain the BCI, we performed a regression of log body mass against log SVL of females, and for each female we extracted the residuals of the regression.

Eggs were collected, counted and photographed within 24 h of deposition. Eggs and females were photographed with a Nikon Coolpix 4500 digital camera or a Nikon Digital Sight Fi2 camera attached to a Nikon SMZ800 stereo zoom microscope. We recorded the start and end dates of deposition for both species in each experimental year. Egg dimensions (maximum length and width of jelly; maximum width of vitellus) were also measured from the photographs. Vitellus volume was calculated from the equation for the volume of a sphere or $V=4/3 \times r^3 \pi$, where r is the vitellus width/2. The volume of a jelly coating was calculated from the equation for the ellipsoid volume or $V=4/3 \times r_1 r_2^2 \pi$, where r_1 and r_2 are the radii (length/2 and width of jelly/2, respectively), as described [35]. All measurements were taken using ImageJ software (Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA; https://imagej.nih.gov/ij/, 1997-2018).

Statistical analyses

The body mass of females of both species in all years was normally distributed (Shapiro-Wilk test). The average body mass for egg-laying females of both species was calculated for each year. We used one-way ANOVA to test for the difference in the average body mass per year between species and to compare the body mass during three successive years within a species. An overall factorial ANOVA with species, year and their interaction was also performed. The body size (SVL) of females of both species was normally distributed and differences between species in SVL and BCI were checked by the two-tailed t-test. The total number of eggs deposited by females was recorded during the entire reproductive period (from the first egg deposited in common containers to the last egg deposited by each female) in 3 consecutive years for both species. Because the number of females included in the experiment and the percentage of reproducing females differed between years, we calculated the average number of eggs laid per female in each year for each species. The between-species differences in the proportion of reproducing females were tested using the test for difference in proportions, which compares two independent proportions taking into account sample size. The average number of eggs per species was calculated from the total number of eggs laid by a particular species and the number of reproducing females in each year. The number of laid eggs was not normally distributed for either species, so in further analyses we used their log-transformed values. To test for the difference in the average number of eggs per year between species, we used the two-tailed t-test. We also compared average numbers of laid eggs in three successive years for each species by one-way ANOVA. Two-tailed multiple comparisons of *p* values were used as a post-hoc test to establish which year contributed the most to the difference. An overall factorial ANOVA with species, year and their interaction was also performed.

Average values of egg dimensions (vitellus and jelly volumes) were compared with one-way ANOVA. To test for potential differences in egg size among females of each species laid in one year (2017), we used two separate one-way ANOVA tests on a subsample of 300 eggs for *T. ivanbureschi*, and 350 eggs for *T.*

macedonicus. To analyze the potential individual differences in egg sizes in 3 consecutive years, we measured 50 randomly-chosen eggs of three *T. macedonicus* females. We used the repeated ANOVA measures to assess the differences between years.

The total duration of the egg-laying period was expressed in days between the first and the last day of oviposition. We considered that the egg-laying period had ended when there were no eggs deposited for 7 consecutive days [38]. We calculated the percentage of overlap in egg-laying periods between the species in 3 years. To deduce in which part of the egg-laying period the species deposited the most eggs, the percentages of eggs laid during the early (the first third of the entire period), middle (the second third) and late (the last third) phase of oviposition periods were calculated for each species for each year. All statistical analyses were performed in Statistica 10 (StatSoft Inc. 2011).

RESULTS

Female body mass, size and BCI

The average body mass of egg-laying females (Table 1) did not differ between the two species in 2016 ($p > 0.1$), while there were significant differences in 2017 ($p = 0.013$) and 2018 ($p = 0.003$). In both species, the average body mass of females that were laying eggs did not change significantly during the 3 year period ($p > 0.05$ in both species). The factorial ANOVA showed a significant effect of species ($p < 0.001$) and year ($p = 0.027$), but a non-significant effect of species × year interaction ($p > 0.1$).

The values for SVL of *T. ivanbureschi* females (N=15) ranged between 6.96 and 9.10 cm, with a mean

Table 1. Average body masses of *T. ivanbureschi* and *T. macedonicus* egg-laying females during three experimental years.

		N	Mean (g)±SD	Range (g)	
				min	max
2016	*T. iva*	7	18.93±2.61	15.19	22.92
	T. mac	8	20.20±1.78	17.70	22.73
2017	*T. iva*	12	17.42±3.00	10.39	21.98
	T. mac	9	20.31±1.14	18.63	22.54
2018	*T. iva*	7	15.70±1.51	14.16	17.72
	T. mac	6	18.93±1.59	16.59	20.69

N – number of egg-laying females.

SVL±SD of 8.33±0.60 cm. The SVL for *T. macedonicus* females (N=9) ranged between 7.80 and 9.07 cm, with a mean SVL±SD of 8.70±0.37 cm. The two-tailed t-test showed no significant differences in female SVL between the two species.

The BCI did not differ between the females of the two species (t_{21}=0.543, p=0.592, *T. ivanbureschi* – N=15, mean BCI±SD: 0.015±0.075; *T. macedonicus* – N=9, mean BCI±SD: 0.000±0.039).

Differences in the number of laid eggs

An overview of the reproductive characteristics of the two species is given in Table 2. The number of reproducing females relative to the total number of females included in crossings did not differ between the species (p>0.5 in all three years). The two-tailed t-test showed that the average number of eggs laid per female differed between the species in 2017 (p=0.021) when *T. macedonicus* females laid considerably more eggs, while differences were not detected for 2016 and 2018 (p>0.05 in both comparisons). ANOVA showed no differences in the average number of eggs laid per female in three consecutive years in the case of *T. ivanbureschi* (p=0.675), while the average number of eggs laid by *T. macedonicus* females differed between years (p=0.026). The post-hoc test (two-tailed multiple comparisons of p values) showed that significant differences in the average numbers of deposited eggs in *T. macedonicus* existed between 2016 and 2017 (p=0.021), while other between-years comparisons were non-significant (p>0.05).

In total, the average number of eggs per egg-laying female was 96.36 for *T. ivanbureschi* and 338.33 for *T. macedonicus*, which is 3.5-fold higher. The factorial ANOVA showed a significant effect of species (p=0.039) and a non-significant effect of the year, and species × year (p>0.05 in both cases).

Inter- and intraspecific differences in egg size

Egg dimensions (vitellus and jelly volumes) were significantly larger in *T. macedonicus* in the same reproductive year (2017) (Table 3, p<0.001 for both dimensions). Egg size did not differ among the *T. ivanbureschi* females (p=0.070), while differences in egg size were found in *T. macedonicus* females (p<0.001).

Table 2. Egg-laying performance of *T. ivanbureschi* and *T. macedonicus* in three consecutive years.

Year	Species	N of females	% of egg-laying females	Total number of laid eggs	Range of the number of laid eggs per female		Average number of eggs laid per female
					min	max	
2016	*T. iva*	8	87.50	727	6	208	103.86
	T. mac	9	88.89	1381	6	371	172.63
2017	*T. iva*	16	75.00	2500	6	663	208.33
	T. mac	9	100.00	4631	73	759	514.56
2018	*T. iva*	12	58.33	1364	1	383	194.86
	T. mac	9	66.67	1967	47	619	327.83

Table 3. Egg dimensions of *T. ivanbureschi* and *T. macedonicus* during the same reproductive experimental year (2017).

Species	N	Vitellus volume mean±SD	Jelly volume mean±SD
T. ivanbureschi	300	5.09±0.96	14.87±1.53
T. macedonicus	350	5.48±0.89	21.18±2.26

Repeated ANOVA measures showed that the vitellus and jelly volumes of *T. macedonicus* varied between females (vitellus: F=50.72, *p*<0.001; jelly: F=77.23, *p*<0.001) and years (F=123.76, *p*<0.001; F=109.29, *p*<0.001), with significant interaction of these two factors (F=14.00, *p*<0.001; F=12.10, *p*<0.001).
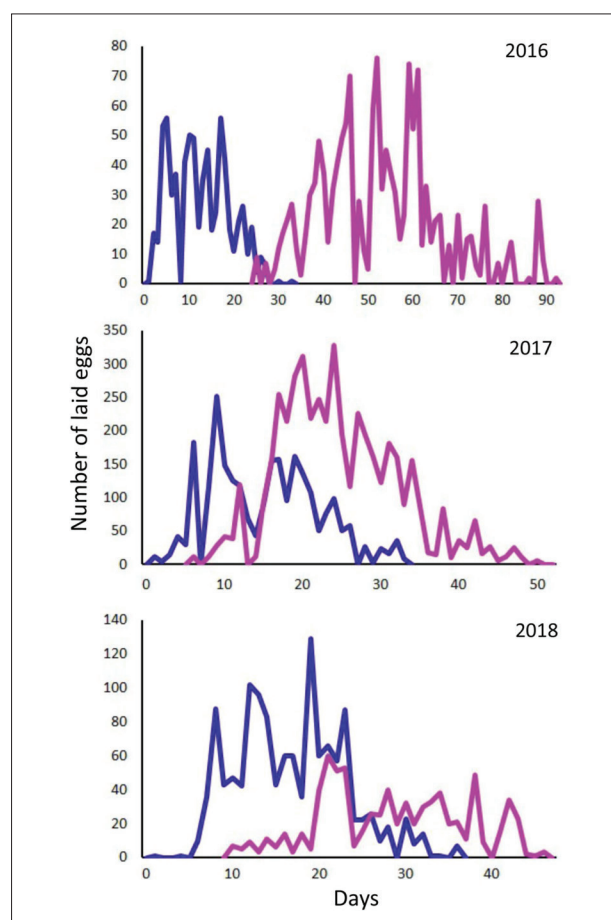
Egg-laying dynamics

In 2016, *T. macedonicus* females began laying eggs with almost a month delay compared to *T. ivanbureschi*, and their egg-laying periods overlapped by only 9.8%. In 2017, *T. ivanbureschi* and *T. macedonicus* started depositing eggs on similar dates and the entire period had a 56.0% overlap. The difference in the duration of oviposition periods between the species was not pronounced, but in *T. macedonicus* it lasted 12 days longer. In 2018, *T. ivanbureschi* started to lay eggs 10 days before *T. macedonicus*; oviposition periods had 58.7% overlap and their duration was almost equal (Fig. 1).

In 2016, *T. ivanbureschi* laid the largest number of eggs during the early phase of oviposition (48.9%). In the same year, there were two peak days with the same highest total number of deposited eggs (56 eggs on the 5th and on the 17th days, early and middle third of the entire oviposition period). In 2017 and 2018, most of the eggs were deposited during the middle phase (47.3% and 61.1%, respectively), while the main peaks were in the early phase (respectively, 9th day – 252 eggs and 19th day – 129 eggs) (Fig. 1). In all three years, *T. macedonicus* showed the same pattern, with the highest total numbers of laid eggs (2016: 50.9%, 2017: 58.2%, 2018: 47.6%), and the main peaks during the middle phase of the period of oviposition were as follows: 2016: 28th day – 76 eggs; 2017: 19th day – 328 eggs; 2018: 12th day – 60 eggs) (Fig. 1).

DISCUSSION

In three consecutive years of experimental crossings, we found that there were a significant differences in the number and size of eggs and a shift in the onset of

Fig. 1. Illustrative differences in egg-laying dynamics of *T. ivanbureschi* and *T. macedonicus* during three consecutive years. Color code: **blue** – *T. ivanbureschi*, **purple** – *T. macedonicus*.

oviposition between *T. ivanbureschi* and *T. macedonicus*. The maximum number of eggs laid by a single female, 759 in *T. macedonicus* and 663 in *T. ivanbureschi*, were 2- to 3-fold larger than the previous estimates for different *Triturus* species (189 to 489; [38,39]), showing that the possible number of laid eggs of *Triturus* species is much higher than previously thought. However, there is an indication that seasonal variations (variations between years) in the reproductive output exist, confirming that environmental factors have an impact on the number of eggs produced.

Estimations of the reproductive potential are affected by the methods of data collection. For example, the resulting number of deposited eggs per *T. macedonicus* female in one reproductive year (2016) is similar to results previously reported for this species [37], while this number is much larger for the other two years. In the same paper [37], pregnant females were collected from breeding ponds and transferred to lay eggs in the laboratory, while in our study females were laying eggs in the laboratory from the beginning of oviposition. It is very likely that in [37] some of the eggs had already been deposited in the wild, which could explain the observed differences in the number of eggs [38]. Another problem in considering the results from previous studies is the actual taxonomic status of the populations, since at the time when these studies were conducted, the phylogeny of *Triturus* newts had not been properly resolved. Some of the populations that were considered to contain one species were later confirmed as hybrid populations [32] or were separated as distinct species [40].

There is a considerable variation in the number of eggs laid by females within both species and high variation between years, which largely affects the statistical significance of between-species differences. However, on average, *T. macedonicus* females had a much higher reproductive output (a 3.5-fold larger number of eggs). Taking into account that *T. macedonicus* live longer on average [19,29], their lifetime reproductive success could be higher.

Vitellus volume, which reflects the maternal nutrient input [1], as well as jelly volume, varied significantly within both species (between females and among years). Vitellogenesis in all ectothermic vertebrates is sensitive to environmental conditions [11]. Different internal (e.g. individual age) as well as external biotic (e.g. presence of predators, food availability) and abiotic factors (e.g. temperature, desiccation) affect the size of eggs [11,41-46], which can explain the observed intraspecific variation. The significant variation in egg size among females surpasses intra-clutch variation [11]. Despite the fact that egg size is correlated with different environmental parameters, it was shown that in *Triturus* newts it is a species-specific trait [28,37]. *Triturus macedonicus* has significantly larger vitellus and jelly volumes than *T. ivanbureschi*. A larger jelly volume supports protection from harmful factors from the environment (e.g. UV rays), from mechanical injury, fungal infections and prevents desiccation [1,46,48]. Since the jelly coating has many functions, it is potentially adaptive, hence *T. macedonicus* could have a slight advantage over *T. ivanbureschi* regarding this trait.

Amphibian females can retain or reabsorb part of the oocytes and oviductal eggs if the conditions for insemination and/or oviposition are suboptimal, indicating that egg deposition can be environmentally constrained [38]. One of the external factors that affect the oviposition period is the permanency of ponds [43]. Removal of the vegetation in oviposition sites can also induce egg retention [49]. Reduced clutch size was also associated with amplexus with undesired males in frogs [50]. The accommodation time (the period that animals from natural populations spent in the laboratory before the experiment started), semi-natural conditions (crossings in artificial ponds in the common garden), and exposure to possibly undesired males, could affect reproductive output.

The beginning of oviposition and the oviposition dynamics pattern are the most consistent reproductive differences between the two *Triturus* species. Females of *T. ivanbureschi* began laying eggs earlier than females of *T. macedonicus* in all three seasons (experimental years). Both species were held under the same conditions (temperature, water, food, etc.). Therefore, the observed differences in the onset of oviposition may be explained by environmental temperature. Since *T. macedonicus* is more thermophilic than *T. ivanbureschi* [51], it is expected that in the former the reproduction and oviposition are triggered by higher temperatures. In addition, it was shown [52] that temperature changes can have species-specific effects on amphibian reproduction. The dynamic of egg laying differed between species but was largely

constant between seasons (experimental years). Since the onset of oviposition differed between species, this can lead to a different period of embryonic and larval development and metamorphosis of the two species in natural populations at the species' contact zone.

The differences in reproductive characteristics may be important in disclosing how the displacement of *T. ivanbureschi* by *T. macedonicus* occurred. Our results show that *T. macedonicus* females have higher reproductive performance (reflected by the higher average number of laid eggs per female during 3 years and the size of the deposited eggs), and different egg-laying dynamics compared to *T. ivanbureschi*. The obtained results represent basic information on species biology necessary for the explanation of complex species interactions, evolutionary processes of hybridization and species replacement. To better understand the spatial species displacement scenario in the case of *T. ivanbureschi* and *T. macedonicus*, the reproductive characteristics of reciprocal F_1 hybrids and the survival rates of species, F_1 and F_2 hybrid and backcrossed generations, as well as environmental factors, must be included.

Author contributions: MC and AI conceived and designed the study, all co-authors performed the experiments and collected data, TV, SN, AI and MC analyzed data and wrote the manuscript.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Vucic%20et%20al_5220_Supplementary%20Material.pdf