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RESEARCH ON CRESTED NEWTS IN SERBIA: INSIGHTS FROM A HYBRID POPULATION (*TRITURUS IVANBURESCHI* × *T. MACEDONICUS*) AS A BASELINE FOR BREEDING AND HYBRIDIZATION STUDIES

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The large-bodied newts (*Triturus* sp.) of the Balkan Peninsula have long posed a challenge for taxonomists due to their complex hybridization patterns and morphological variability. These newts, belonging to the Salamandridae family, are comprised of ten species divided into two main clades: the crested newts and the marbled newts. Over the years, advances in molecular research have significantly refined the taxonomy of *Triturus*, revealing cryptic species, clarifying species relationships, and resolving hybridization issues. The complex hybridization zones among the crested newts, particularly in the Balkan Peninsula, have led to significant phenotypic variation, complicating species delimitation. In Serbia, the presence of multiple subspecies and hybrid populations has been extensively studied, particularly in regions where high variability among individuals and sexes is observed. This study focuses on the Vlasi population, situated at the contact zone between two species, *T. macedonicus* and *T. ivanbureschi*. Our findings suggest that this hybrid population exhibits intermediate phenotypic traits and maintains a uniform sex ratio. Interestingly, significant differences in body size were observed between phenotypes. Additionally, significant body size differences were observed between males and females, and the seasonal phenology indicated a marked decrease in activity as the year progressed. These results contribute to our understanding of hybridization dynamics and the intricate taxonomic relationships within the *Triturus* species in the Balkan Peninsula. In conclusion, the Vlasi hybrid population of *T. macedonicus* and *T. ivanbureschi* exhibits a diverse range of phenotypic variations, providing a valuable model for studying hybridization, life-history traits, and evolutionary processes in newts. We dedicate this paper to Prof. Dr. Miloš Kalezić, whose pioneering work on newts in the territory of the former Yugoslavia laid the foundation for future research on amphibians, including the present study. His commitment as a professor and mentor has inspired many generations to continue work in this field.

Key words: Amphibians, Caudata, conservation, hybrids, phenology, Vlasi village

INTRODUCTION

For decades (even centuries) the large-bodied newts (*Triturus* sp.) distributed in the Balkan Peninsula represented an unsolved taxonomic puzzle. These newts from the Salamandridae family are comprised of ten species divided into two main clades – crested newts (seven species widely distributed throughout Europe and adjacent Asia except the Iberian Peninsula) and marbled newts (three species found in the Iberian Peninsula and France) (Arntzen 2003, Wielstra *et al.* 2014a, 2019, Rancilhac *et al.* 2021, Arntzen 2024a). The taxonomy of *Triturus* newts has undergone significant changes over the years, driven by advances in morphological and ecological studies, and especially molecular research. These studies have revealed new insights into species relationships, identified cryptic species, resolved issues caused by hybridization, and historical misclassification. For example, two species, alpine newt from the genus *Mesotriton* (*M. alpestris*) and smooth newt from the genus *Lissotriton* (*L. vulgaris*) were regarded as members of the genus *Triturus* until 2004 (García-París *et al.* 2004).

The morphological and genetic variation and differentiation among *Triturus* have been extensively studied during the past decades, from studies of allozyme polymorphism and morphology (Kalezić & Hedgecock 1980, Rafinski & Arntzen 1987, Giacoma & Balleto 1988, Arntzen & Sparreboom 1989, Kalezić *et al.* 1990, 1997), mitochondrial DNA (Arntzen & Wallis 1999, Zajc & Arntzen 1999), karyotype comparisons (Litvinchuk *et al.* 1999), to new molecular methods using both, mitochondrial and nuclear markers (Arntzen *et al.* 2007, Steinfartz *et al.* 2007, Themudo *et al.* 2009, Wiens *et al.* 2011, Wielstra & Arntzen 2011, Wielstra *et al.* 2012, 2013a, 2019).

One of the problems for resolving their phylogenetic relations is that all species can hybridize and produce fertile or infertile offspring depending on the phylogenetic relatedness of species (Arntzen & Hedlund 1990, Arntzen & Wallis 1991, Arntzen *et al.* 2009, 2014, 2018, 2021, 2024b, Cogălniceanu *et al.* 2020, Wielstra *et al.* 2017a). The two most studied hybridization events are between marbled and crested newts in Western France (e.g. Vallée 1959, Schoorl & Zuiderwijk 1981, Francillon-Viellot *et al*. 1990, Arntzen *et al*. 2009, 2021, Cogălniceanu *et al*. 2020, Arntzen, 2023a,b, 2024b,c) and between crested newts in the Balkan Peninsula, more precisely in eastern and south-eastern Serbia (Kalezić & Stevanović 1980, Stevanović & Kalezić 1980;Wallis & Arntzen 1989, Kalezić *et al.* 1990, Crnobrnja & Kalezić 1990, Wielstra & Arntzen 2012, Arntzen *et al.* 2014, 2018, Wielstra *et al*. 2014b, 2017a,b, Slijepčević *et al*. 2015, Vučić *et al*. 2018, 2019, 2020, 2022, Bugarčić *et al*. 2022).

Four species currently recognised in Serbia (*T. cristatus*, *T. dobrogicus*, *T. ivanbureschi* and *T. macedonicus*) were previously classified as subspecies of *T. cristatus* (for historical overview of the taxonomy of the genus *Triturus* see Vučić *et al.* 2020). In short, Bucci-Innocenti *et al.* (1983) distinguished four subspecies of *T. cristatus* as full species: *T. cristatus*, *T. dobrogicus*, *T. carnifex* and *T. karelinii* (Frost 1985, Macgregor *et al.* 1990), whose distributions can overlap in the Balkan Peninsula. Since then, *T. cristatus* and *T. dobrogicus* have retained their status, while *T. carnifex* was split into an additional two species: *T. carnifex* in the Apennine Peninsula and *T. macedonicus* in the Balkan Peninsula (Crnobrnja *et al*. 1989, Kalezić *et al*. 1997, Arntzen & Wallis1999, Arntzen *et al*. 2007). Additionally, after several decades of research, *T. karelinii* was divided into three species: *T. karelinii* (Caucasus, Crimea and the southern shores of the Caspian sea), *T. anatolicus* (northern Turkey and the southern shores of the Black sea), and *T. ivanbureschi* (western Turkey – Asian part and the Balkan Peninsula) (Wallis & Arntzen 1989, Litvinchuck *et al.* 1999, Themudo *et al.* 2009, Arntzen & Wielstra 2010, Wielstra & Arntzen 2012, 2014, 2016, Wielstra *et al*. 2010, 2012, 2013a,b, 2017a,b).

In Serbia, in the 1920s three subspecies were recognized within the *T. cristatus* species complex: *T. c. cristatus*, *T. c. dobogicus* and *T. c. carnifex* (Bolkay 1923, 1929, Karaman 1922, 1928, 1948, Pavletić 1964, Radovanović 1964, Pozzi 1966, Džukić 1972). More detailed studies of newts in former Yugoslavia started during the 1980s, with the pioneer work of Professor Miloš Kalezić, at a time when phylogeny and interspecific relations of newts were still largely unresolved and *I. alpestris* and *L. vulgaris* were still regarded as *Triturus* newts. During that period, Kalezić provided baseline studies for the research of newts, which can be sorted in two main directions – studies of genetic and morphological variability in newts (*L. vulgaris* – Kalezić 1982, 1983, 1984, Kalezić & Tucić 1984, Tucić & Kalezić 1984, 1986, Kalezić *et al.* 1987, *T. cristatus* – Kalezić 1978, Kalezić & Hedgecock 1980, Kalezić & Stevanović 1980, Stevanović & Kalezić 1980, Kalezić *et al.* 1989a), and together with Dr. Georg Džukić, studies of paedomorphosis (*L. vulgaris* – Džukić 1981, Džukić & Kalezić 1983, Tucić *et al*. 1985, Kalezić & Džukić 1985, 1986, *I. alpestris* – Džukić & Kalezić 1984, Kalezić *et al.* 1989b, *T. cristatus* – Kalezić & Džukić 1990, an overview for all three species – Džukić *et al*. 1990).

Considering species delimitation, phylogenetic relations of newts, and hybridization in the territory of former Yugoslavia, Kalezić (1978) published the first paper based on an analysis of allozyme polymorphism. The results showed that *T. cristatus* is more distantly related to *I. alpestris* and *L. vulgaris* (all classified into the same genus *Triturus* at that time) than these species are mutually. Additionally, the genetic distance between the subspecies *T. c. karelinii* and *T. c. dobrogicus* was large enough to support previous findings (e.g. Callan & Lloyd 1960, White 1973) that had already implied they are two different species. These results were further corroborated by a broader electrophoretic survey that assessed the level of genetic variation within and between populations of *L. vulgaris, I. alpestris* and *T. cristatus* (Kalezić & Hedgecock 1980). In 1980, Kalezić & Stevanović published two papers on the status of *T. cristatus* in central Serbia (Kruševačka župa and surroundings), considering its biogeography and morphology (Kalezić & Stevanović 1980, Stevanović & Kalezić 1980). They compared newts from this region to several other populations across the former Yugoslavia and Poland. They concluded that *T. c. carnifex* is present in central Serbia with high variability among individuals and sexes, while the Kruševačka župa population exhibited traits typical of three Serbian subspecies (*T. c. cristatus*, *T. c. dobogicus* and *T. c. karelinii*), likely due to hybridization in the zone where newts are expanding along large river valleys. This very important work was the first study of hybridization in natural populations of newts in Serbia and highlighted the importance of this region for further research. The allele distribution patterns of *Triturus* populations across the former Yugoslavia, based on electrophoretic data, showed that isozymes and mitochondrial DNA analyses are insufficient for resolving *Triturus* taxonomy. This limitation is due to the confirmed existence of hybrid zones among species of the *T. cristatus* complex, even when morphological analyses are considered (Crnobrnja *et al.* 1989, Kalezić & Crnobrnja 1989, Kalezić *et al.* 1989a). Indeed, the first phylogeographic study of newt populations in Serbia based on mitochondrial DNA analyses alone was somewhat misleading due to possible mitochondrial DNA introgression (Wallis & Arntzen 1989). Individuals from various populations across Serbia were recognized as different species based on morphology, yet their mitochondrial DNA suggested they belonged to the same species. However, this study confirmed the parapatric distribution of crested newts and thus, relatively narrow hybridization zones. It also revealed the geographical structuring within *T. carnifex* and *T. karelinii* populations and gene flow in transition zones or near them. Kalezić *et al.* (1990) conducted a comprehensive survey of 31 populations across the former Yugoslavia, examining various morphological characters to establish populations' affinities and their taxonomic implications. They also showed pronounced structuring within all species based on morphology, particularly within *T. carnifex*, where two separate groups (eastern and western) were recognized, suggesting that these two groups could represent different taxa at the subspecies level, at least. However, the morphological distinction between populations of *T. carnifex* and *T. karelinii* near or within the transition zone was not identified. Analyses of genetic structuring based on allozyme data from nine populations across the former Yugoslavia (Crnobrnja & Kalezić 1990) further confirmed the previous findings from mitochondrial DNA (Wallis & Arntzen, 1989) and morphology (Kalezić *et al.* 1990). In 1997, Kalezić *et al.* reassessed the affinities and taxonomical relations of the *T. cristatus* superspecies based on qualitative and quantitative morphological characters of 42 populations across the Balkan Peninsula to assess the position of hybridization zones. The results showed that, due to high variation in morphological characters within the crested newts, individuals exhibiting intermediate morphology between two species do not necessarily have to be hybrids, especially when backcrossing with parental species is present, and further genetic analyses were necessary to confirm hybridization. The authors concluded that based on morphology, *T. carnifex* and *T. karelinii* are the most closely related crested newt species, sharing similar phenotypes characterized by stout body, large heads and elongated limbs, resembling the presumed ancestral crested newts phenotype. The other two species, *T. cristatus* and *T. dobrogicus* are smaller and more elongated, suggesting that lowland and highland areas and their transition zones could be areas that generate morphological differentiation in crested newts. These morphological relationships did not correspond to any results of the genetic analyses conducted prior to 1997. The authors confirmed previously described structuring within *T. carnifex* and proposed that these should be two subspecies – eastern *T. c. macedonicus* (following Karaman (1922) who described morphologically distinct newts from Ohrid, North Macedonia as *Molge karelinii macedonica*) and western nomotypical *T. c. carnifex.* Based on the synthesis of previous findings, Crnobrnja *et al.* (1997)

highlighted the importance of the central Balkans (the area south of the Sava and Danube rivers), due to the remarkable level of phenotypic and genetic variation in crested newt populations. The authors suggested that the basal lineage of crested newts was restricted to the Balkans at least during the late Miocene, from which *T. carnifex*, *T. cristatus* and *T. karelinii* originated. Their findings suggest that morphologically *T. carnifex*-like populations mainly occupy areas south of the Sava and Danube rivers, while *T. karelinii*-like populations are more restricted to isolated regions in Peripannonian Serbia and eastern North Macedonia. Although Wallis and Arntzen (1989) confirmed the presence of *T. karelinii* mitochondrial DNA south of these rivers, this was based on limited sampling.

The aforementioned studies by Kalezić and colleagues served as a baseline for a series of molecular studies aimed to resolve the mystery of crested newts' taxonomy and distribution in the central part of the Balkan Peninsula, focusing on *T. carnifex* and *T. karelinii.* Arntzen & Wallis (1999) confirmed the findings of Kalezić *et al.* (1997) regarding the subspecies *T. c. macedonicus*, with a distribution in the Balkans, south and east of Bosnia and Herzegovina. The subspecies *T. c. macedonicus* was later elevated to the species level (*T. macedonicus*), based on a high level of genetic and morphological differentiation between subspecies and an estimated age of over 5 million years (Arntzen *et al.* 2007). The situation for *T. karelinii* was more complicated, especially in eastern and south-eastern Serbia. Litvinchuk *et al.* (1999) proposed that the *T. karelinii* populations in Serbia represent a distinct subspecies *T. k. arntzeni,* with a holotype description from the Vrtovac locality (between Knjaževac and Pirot) in eastern Serbia. Electrophoretic protein analysis indicated that samples from Serbia contained alleles identical to those of *T. cristatus* or *T. carnifex*, but without heterozygotes, suggestesting a lack of hybridization among the species, at least at Vrtovac. *Triturus k. arntzeni* was later elevated to species status (*T. arntzeni*) with a western distribution, while the nominotypical *T. karelinii* was restricted to the eastern distribution of the existing *T. karelinii* group (Themudo *et al.* 2009, Arntzen & Wielstra 2010). Further studies of mitochondrial and nuclear DNA revealed that the *T. karelinii* group consists of not two, but three clades – eastern, central and western which represent three cryptic species – *T. ivanbureschi, T. anatolicus* and *T. karelinii,* respectively (Wielstra *et al.* 2010, 2012, 2013a,b, Wielstra & Arntzen 2016). Wielstra *et al.* (2013a) first showed that the Vrtovac population belongs to *T. macedonicus* and recommended considering the species name *T. arntzeni* as a synonym of *T. macedonicus*. However, implementation of new nuclear markers and next-generation sequencing contradicted previous findings (Litvinchuck *et al.* 1999, Wielstra *et al.* 2013a) and proved that the Vrtovac population is a *T.*

macedonicus × *T. ivanbureschi* hybrid population consisting of Fn hybrid individuals resulting from long-term, multi-generational interbreeding and backcrossing with the parental species (Wielstra & Arntzen 2014, Wielstra *et al.* 2014a). The average genetic composition of hybrids' nuclear DNA is 72% *T. macedonicus*, 25% *T. ivanbureschi* and 3% *T. cristatus* (Wielstra *et al.* 2017a), which explains why resolving the taxonomic status of this population was problematic. Consequently, the name *T. arntzeni* should be used as a synonym for both *T. macedonicus* and *T. ivanbureschi* (Wielstra & Arntzen 2014).

The implementation of new molecular methods has also revealed the history of interactions between *T. ivanbureschi* and *T. macedonicus* in Serbia (Fig. 1). These two species clearly differ in life-history traits and morphology (Furtula *et al.* 2008, 2009, Cvijanović *et al.* 2009, Vučić *et al.* 2018, 2019, Vukov *et al.* 2011, 2014, Wielstra *et al.* 2019), but they reproduce in similar aquatic habitats, with their contact zone extending across the central, eastern and south-eastern parts of Serbia (Arntzen 2003, Džukić *et al.* 2016). *Triturus macedonicus*, while expanding its range

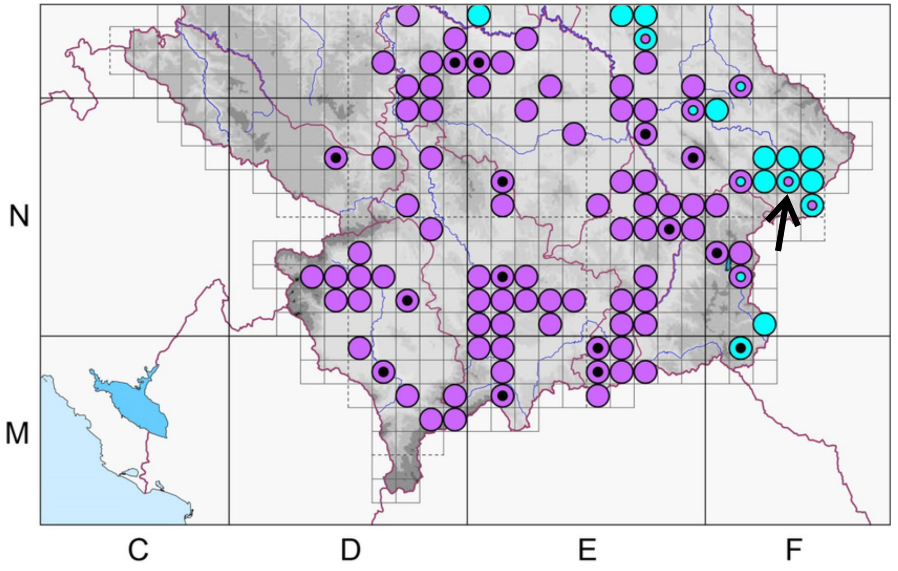


Fig 1. – Distribution (UTM grid 10 x 10 km) of *T. ivanbureschi* (blue) and *T. macedonicus* (purple). Solid-coloured round symbols represent species identified on the basis of morphology. Round symbols with a black interior dot represent species identified on the basis of genetic data. Concentric coloured circles indicate hybrids identified from genetic data, with the outer circle representing the species contributing most to the population's gene pool and the inner dot indicating the species with a smaller contribution. The Vlasi population is marked with an arrow. It is a modified map published in Vučić *et al.* (2020).

northward, has intersected the range of *T. ivanbureschi*, dividing it into a northern enclave in central Serbia and a larger southern portion of the range. In the contact zone, intensive hybridization has occurred, resulting in a broad zone of introgression (around 54,000 km^2). Due to asymmetric introgression of *T. ivanbureschi* mitochondrial DNA, individuals in this area possess *T. macedonicus* nuclear DNA and *T. ivanbureschi* mitochondrial DNA. The presence of *T. ivanbureschi* mitochondrial DNA is considered a genomic footprint of the previous range of this species (Wielstra & Arntzen 2012, Arntzen *et al.* 2014, Wielstra *et al.* 2017a).

In this study we report on morphological variability (coloration and size variation), sex ratio and phenology in the Vlasi population, which is geographically positioned at the contact zone between the two species (*T. macedonicus* and *T. ivanbureschi*), but closer to *T. ivanbureschi* (Wielstra *et al.* 2014a, Arntzen *et al.* 2018). We hypothesized that the hybrid population in the village Vlasi could be compared to an F1 hybrid generation. This is because the average nuclear genetic composition of the hybrids is nearly 50% from each species (60% *T. macedonicus*, 39% *T. ivanbureschi*, and 1% *T. cristatus*), with all individuals carrying mitochondrial DNA from *T. ivanbureschi* (Wielstra *et al.* 2017a). Additionally, the number of thoracic vertebrae of Vlasi individuals (13 to 14) covered the known range of the parental species (*T. ivanbureschi* 13 and *T. macedonicus* 14) (Slijepčević *et al.* 2015).

MATERIALS AND METHODS

The village Vlasi is situated in eastern Serbia (43.00 N, 22.64 E, altitude 468 m a.s.l.). It has a temperate and humid climate with warm summers (Cfb by Köppen classification) (Milovanović *et al.* 2017). The warmest month is August, and the coldest January, with May being the month with highest rainfall, and March and September have roughly 12 hours of daylight (Aladin.info, 2002–2024). In March, there is a significant increase in rainfall days compared to February, the average maximum temperature rises above 10°C, while the average minimum temperature does not fall below 0°C (Fig. 2).

The pond in the Vlasi village is situated 50 meters from the Jerma River, just beyond the river's exit from the gorge. It is surrounded by forest and overgrown with shrub vegetation. The pond measures 330 meters in perimeter, covers an area of 5,000 m^2, and has a maximum depth of 1.3 meters.

In September 2012, a fence was installed along one-quarter of the pond's circumference, positioned 2-3 meters away from the water. Pitfall

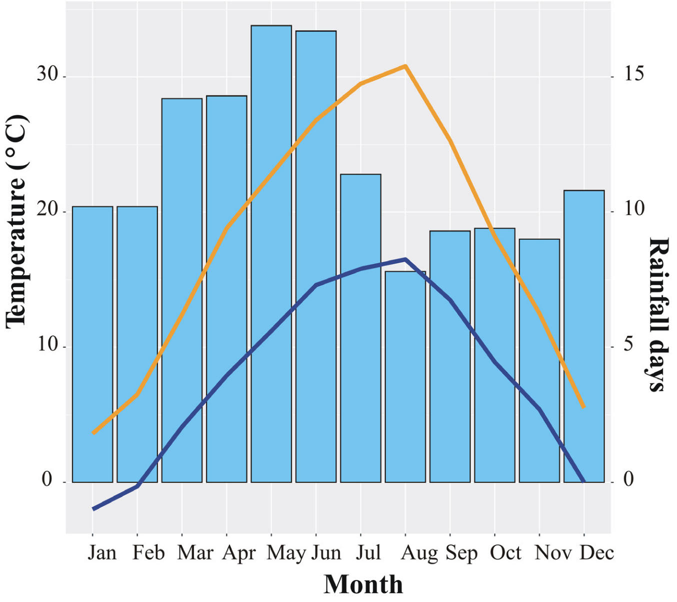


Fig 2. – Climate of the village Vlasi, according to the Aladin.info (2002–2024). Orange line – monthly average maximum temperature (in °C), dark blue line – monthly average minimum temperature (in °C), light blue bars – rainfall days per month.

traps were placed next to the fence on both sides and secured to prevent animals from falling into them during the winter (Fig. 3, top panel). At the end of February 2013, just before the migration season, five funnel traps were installed in the pond (Figure 3, bottom panel). The funnel traps are the most effective method for catching newts and have no adverse effects on the habitat or newt survival (Arntzen & Zuiderwijk 2022).

The migration season began in March (7. 03. 2013) and was monitored over the following five days. The traps and nets were checked twice daily during this period. The same arrangement of traps and five funnel nets were used again in April (1–4. 04. 2023). In May and July, only funnel traps were deployed to assess the presence of individuals in the water.

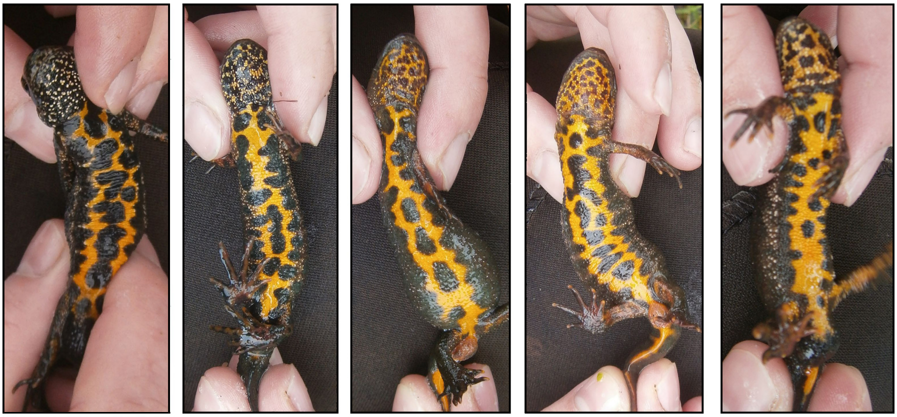
Each individual was photographed, measured snout-vent length (SVL) from the snout to the anterior edge of cloaca basis with digital caliper, and returned to the pond. Morphological classification, based on coloration characteristics (Arntzen & Wallis 1999), divided the specimens into three groups. The T. macedonicus-like individuals (mac group) had black, white-speckled throat and large, connected black spots on the abdomen. The T. ivanbureschi-like (iva group) had an orange throat with large dark spots and

Fig 3. – Fence with traps (top panels), and five funnel traps used for catching newts in Vlasi pond.

smaller, scattered black spots on the abromen. The The remaining individuals with intermediate characters (the hybrid group) could be further subdivided into a phenotype leaning towards *T. macedonicus* (hyb mac), a phenotype leaning towards *T. ivanburesch*i (hyb iva), and the remaining intermediates (hyb) (Fig. 4).

RESULTS

The entire sample of 326 adults and 13 metamorphosed juveniles (which were not analyzed for colour and body size variation) was collected from March to July 2013. Among captured adults, 5% displayed the *T. macedonicus* phenotype (mac), 15% showed the hyb mac phenotype, 45% displayed the intermediate hybrid phenotype, 25% exhibited hyb iva



mac hyb mac hyb hyb iva iva

Fig 4. – Recognized phenotypes in the hybrid population from Vlasi. *Abbreviations:* – T. macedonicus-like, *hyb mac* – phenotype leaning towards *T. macedonicus*, *hyb* – intermediate phenotypes, *hyb iva* – phenotype leaning towards *T. ivanbureschi*, and *iva* – *T. ivanbureschi*-like phenotype.

characteristics, and 20% had the *T. ivanbureschi* phenotype (iva). The distribution of colour morphs per sex is largely similar (Fig. 5). The sex ratio in adults is close to uniform but slightly female biased (149 males: 177 females, or 0.46 expressed as a proportion of males in population).

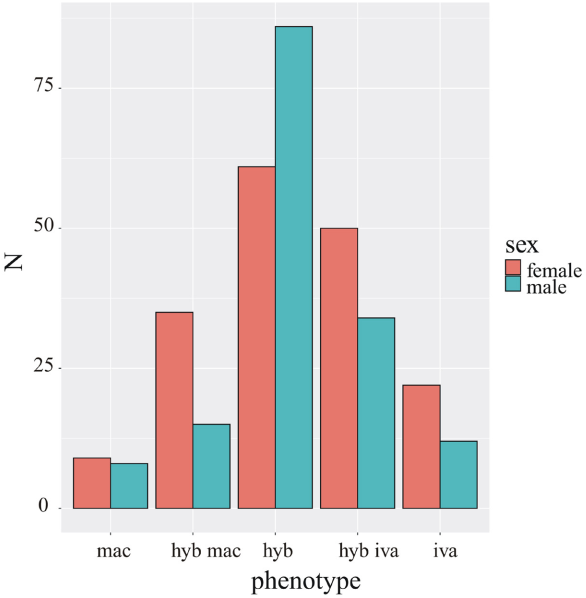


Fig 5. – The frequency distribution of colour phenotypes in the hybrid population Vlasi.

The size distribution showed that males were significantly smaller than females (SVL for males: 63.9 mm, SVL for females: 67.5 mm, Student's t-test, P<0.0001). We found statistically significant differences in SVL among five phenotypes (Table 1): for females (ANOVA, SS = 586.02, df = 4, F = 3.70, P < 0.01) and for males (ANOVA, SS = 446.0, df = 4, F = 3.03, P < 0.05).

Table 1. – Body size distribution in five phenotypes. Abbreviations: mac – *T. macedonicus*-like, hyb mac – phenotype leaning towards *T. macedonicus*, hyb – intermediate phenotypes, hyb iva – a phenotype leaning towards *T. ivanbureschi* and iva – *T. ivanbureschi*-like phenotype.

Females SVL (total N=177)	N	Mean	SD	Min	Max
Mac	8	71.3	6.8	59	81
hyb mac	33	70.2	6.3	57	87
Hyb	66	66.5	6.2	51	78
hyb iva	49	67.5	6.2	49	78
Iva	21	64.8	6.8	54	82
Males SVL (total N=149)					
Mac	8	70.4	3.3	64	74
hyb mac	15	63.7	6.0	52	72
Hyb	80	64.1	6.6	49	81
hyb iva	34	62.9	5.7	52	73
Iva	12	61.4	4.6	54	71

The activity in adults and metamorphosed juveniles was the most intensive in March (total of 218 individuals) and decreased in April (90 individuals). Juveniles were the least frequently documented class, with 10 records in March and 3 in April. For females and males, the dynamics of the water entry were similar, with fluctuations in the daily number of individuals. However, there was a decreasing trend in total number of animals detected from March to July (Fig. 6).

For the two months with the highest detected activity (March and April), there was a large reduction in the number of animals caught by the fence (during the migration towards the pond) from March to April, compared to the number of animals caught by the funnel nets in the pond (Fig. 7).

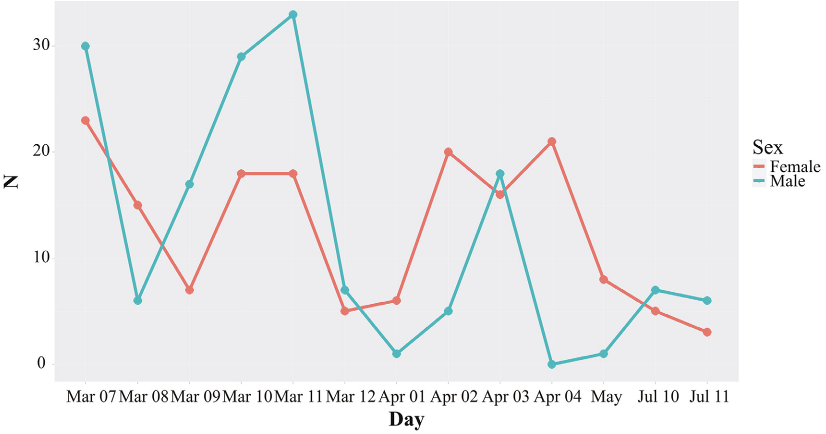


Fig 6. – Number of females and males (N) caught per study day between March and July.

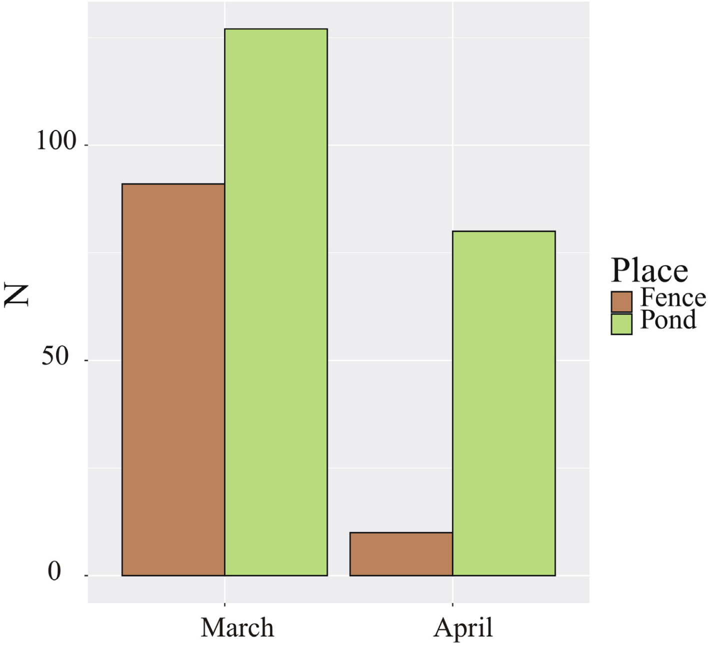


Fig 7. – Total number of individuals per month (N) caught by the fence or with traps/funnel nets.

The frequency of records for each phenotype in March and April was the highest in March for all three hybrid class phenotypes. However, for the mac and iva phenotypes, there was an increase of records in April (Fig. 8). During July, the nocturnal terrestrial activity was detected, probably related to adult individuals leaving the water (Fig. 9).

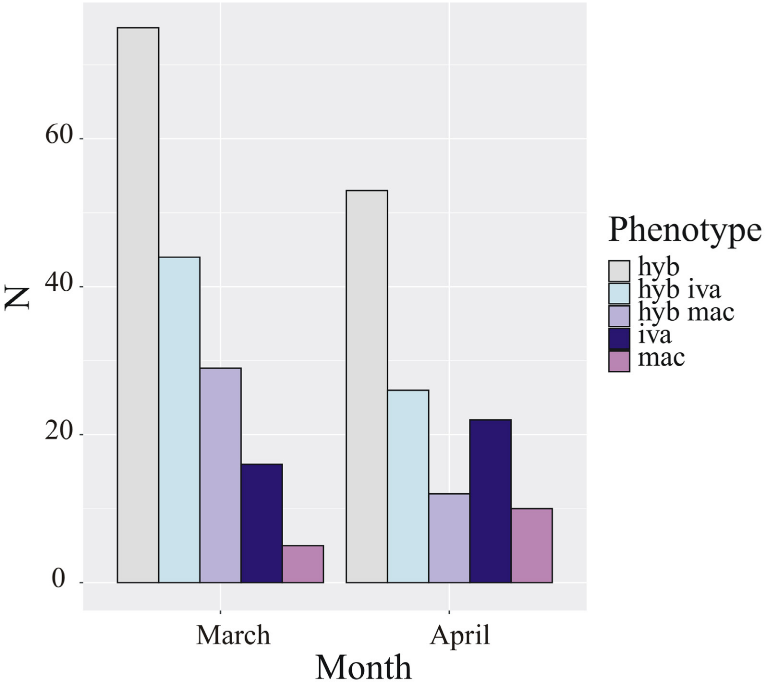


Fig 8. – Number of individuals of each phenotype (N) caught per month.

Fig 9. – Adult male individual photographed in situ near the pond during the night.

DISCUSSION

Phenotypic variation

All individuals collected from the Vlasi population are *Triturus macedonicus* × *T. ivanbureschi* hybrids, resulting from multiple generations of interbreeding with a3:2 share of nuclear DNA from the parental species (Wielstra *et al.* 2017a). Additionally, it has been shown that age, size and growth curves of hybrid individuals from the Vlasi population do not differ from those of their parental species (Arntzen *et al.* 2018). All individuals are Fn hybrids, however, the coloration pattern reflects all spectrum of morphologies,from individuals phenotypically recognized as *Triturus macedonicus*, over three classes of intermediate hybrid phenotypes, to individuals that look like *T. ivanbureschi* (Fig. 4). Among our sampled individuals two thirds have a hybrid morphology. The remaining third show either of the parent phenotypes (4:1 in favour of *T. ivanbureschi*) . Given the 3:2 (*T.m.*:*T.i.*) ratios of nuclear genome shares from their parental species (and *T.i.* predominant in mtDNA), such phenotypic distribution is not a surprise.

Sex ratio in Vlasi population is 0.46. In *Triturus* newts, sex ratio is usually 0.5, however some species with increased female fecundity such as *Triturus marmoratus* can have a male-biased sex ratio (Arntzen, 2002). On the other hand, the hybrid populations of *Triturus* newts can have a genetically-based female-biased sexual dimorphism (Valleé, 1959, Arntzen, 2002) which may also be the case here. Significant, female bias sexual dimorphism in body size is common for *Triturus* newts, females are known to have larger body sizes than males (Kalezić & Stevanović 1980, Kalezić *et al.* 1992, Cvetković *et al.* 1997, Kalezić & Đorović 1998, Malmgren & Thollesson 1999, Ivanović *et al.* 2024), also evident in our hybrid Vlasi population. We also detected significant differences in body size between colour morphotypes. Individuals of *T. macedonicus* colour phenotype have a greater mean size than individuals of *T. ivanbureschi* phenotype. We did not expect to find any differences in body size, as hybrid individuals at population level follow the same growth rate as the parental species (Arntzen *et al.* 2018), and no differences in body size between females of parental species have been found (Vučić *et al.* 2020). Given the robust sample sizes, the sampling error or genetic differentiation seem an unlikely explanation. Still, additional studies, possibly on other hybrid populations, should be conducted to completely rule the effects of genetic differentiation out.

Phenology

In terms of general seasonal dynamics, there is a general trend of decline in activity from spring towards late season, as previously observed for newts from the *Triturus cristatus* complex (Arntzen 2003, 2024a). The peak of the reproductive season in *Triturus* newts can greatly vary geographically, and can be prolonged from April to Mid-June in Poland (Kowalewski 1974) or from March to June with a peak in April in Romania (Covaciu-Marcov *et al.* 2023). In Vlasi village, peak activity was detected in March, and it somewhat reduced in April. This explosive breeding pattern could be related to a milder climate compared to Poland and Romania, with increased number of rainy days, temperature and end of night frosts in March, together with an increased duration of daylight hours. Daily fluctuations in number of animals caught, could be explained by variations in temperature and rainfall/humidity. The frequency of terrestrial migrations, i.e. the number of animals caught at the fence was also the most intense in March, likely because by April the majority of animals would have already migrated to the pond. During July, the number of animals caught in the water was reduced and the terrestrial migrations were observed during the night. Most adult crested newts usually leave the water from mid-July to October (Blab & Blab 1981), or sometimes even earlier by the end of June (Karaman 1948, von Bülow 2001). Most juveniles were detected in March, and they seemed to enter the pond together with the adults, as previously documented (Blab & Blab 1981, Verrell & Halliday 1985, Arntzen 2003). The second peak in the number of detected juveniles was in July and could be related to metamorphosis and increased activity prior to leaving the pond (Karaman 1948).

The significance of Vlasi population The data and results obtained from the hybrid Vlasi population formed the baseline for establishing an experimental colony of large bodied newts at the Institute for Biological Research "Siniša Stanković" in Belgrade. This colony was used for novel research of experimental hybridization, including the studies of viability, survival rates, morphological variability, and growth rates of hybrid individuals produced from experimental crossings between *T. ivanbureschi* and *T. macedonicus*. The research also included F1 hybrid interbreeding and backcrossing with parental species.

These breeding experiments revealed that hybrids differ from the parental species in life-history traits (Vučić *et al.* 2020, Bugarčić *et al.* 2022), morphology (Vučić *et al.* 2018, 2019), and physiological parameters related to oxidative stress (Prokić *et al.* 2018, Petrović *et al.* 2023). Furthermore, the research and the experimental colony established *T. ivanbureschi*, *T. macedonicus*, and their hybrids as a model system for evolutionary and developmental studies (Vučić *et al.* 2024).

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ИСТРАЖИВАЊЕ ВЕЛИКИХ МРМОЉАКА У СРБИЈИ: УВИД У ХИБРИДНЕ ПОПУЛАЦИЈЕ (*TRITURUS IVANBURESCHI* × *T. MACEDONICUS*) КАО ОСНОВА ЗА ПРОУЧАВАЊЕ ПРОЦЕСА РАЗМНОЖАВАЊА И ХИБРИДИЗАЦИЈЕ

МАЈА АЈДУКОВИЋ, ТИЈАНА ВУЧИЋ, ТАЊА ВУКОВ,
АЛЕКСАНДАР УРОШЕВИЋ, МИЛЕНА ЦВИЈАНОВИЋ,
ЉИЉАНА ТОМОВИЋ, АНА ИВАНОВИЋ

РЕЗИМЕ

Велики мрмољци (*Triturus* sp.) Балканског полуострва дуго су представљали изазов за таксономе због својих сложених образаца хибридизације и морфолошке варијабилности. Ови мрмољци, који припадају породици Salamandridae, обухватају 10 врста подељених у две главне кладе: крестасти мрмољци и мраморасти мрмољци. Током година, напредак у молекуларним истраживањима је значајно унапредио таксономију рода *Triturus*, откривајући криптичке врсте, разјашњавајући односе између врста и решавајући проблеме хибридизације. Сложене хибридне зоне између крестастих мрмољака, поготову на Балканском попуострву, довеле су до значајне фенотипске варијације, што отежава делимитацију врста. У Србији, присуство више подврста и хибридних популација је било екстензивно проучавано, нарочито у регионима у којима је висока варијабилност уочена међу јединкама и половима. Ова студија је усмерена на популацију у селу Власи, која се налази у контактној зони двеју врста, *T. macedonicus* и *T. ivanbureschi*. Наши резултати сугеришу да хибридна популација показује интермедијерне фенотипске карактеристике и одржава уједначен однос полова. Интересантно, уочене су значајне разлике у величини тела међу фенотиповима. Додатно, значајне разлике у величини су примећене између мужјака и женки, и сезонска фенологија указује на опадање

активности током одмицања године. Ови резултати доприносе нашем разумевању динамике хибридизације и сложених таксономских односа у оквиру рода *Triturus* на Балканском полуострву. Као закључак, хибридна популација *T. macedonicus* и *T. ivanbureschi* у селу Власи показује разноврстан опсег фенотипских варијација, што је чини вредним модел-системом за студије хибридизације, одлика животне историје и еволуционих процеса код мрмољака. Овај рад посвећујемо Проф. Др. Милошу Калезићу, чији је пионирски рад на мрмољцима поставило темеље за будућа истраживања на водоземцима на територији бивше Југославије, укључујући и овај рад. Његова посвећеност као професора и ментора инспирисала је многе генерације да наставе рад у овој области.