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Variation in thermal plasticity of larval morphology among crested newt species and their reciprocal hybrids (Salamandridae: *Triturus*)

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ABSTRACT

Phenotypic plasticity is assumed to play crucial role under climate change, as it enables organisms to cope with rapidly increasing environmental pressures. We investigated the effect of water temperature on head and tail morphology in larvae of two crested newt species (*Triturus ivanbureschi* and *T. macedonicus*) and their reciprocal F_1 hybrids. Larvae were raised under experimental conditions at 19 °C and, from the late larval phase (stage 62), were exposed to either 19 °C or 24 °C. Geometric morphometrics was applied to quantify and analyse size and shape variation and its relationship with the rate of metamorphosis. Our results revealed that the four analysed genotypes (two species and two reciprocal hybrids) displayed divergent responses to elevated temperature. *Triturus ivanbureschi* and hybrids with *T. macedonicus* mitochondrial DNA were the most thermally sensitive, showing the fastest metamorphosis and marked shape plasticity in both body regions. In contrast, *T. macedonicus* exhibited the slowest metamorphosis but increased growth. Temperature-induced allometric changes had genotypic-specific patterns. Plastic response was partially explained by a change in allometric trajectories only for head shape. Generally, changes in head shape were more pronounced than those in tail shape. Overall, our findings indicate that accelerated metamorphosis is the main factor contributing to a plastic response. Hybridization contributes to the level of plasticity, shedding light on potential mechanisms underlying the dynamic interaction between species within their natural hybrid zone, and highlighting the possible impact of rising temperatures on species distribution and interactions.

1. Introduction

The influence of environmental variation on phenotypic diversity has been widely acknowledged in evolutionary biology (Agrawal, 2001; Fusco and Minelli, 2010; West-Eberhard, 2003). Different genotypes typically exhibit divergent phenotypic responses, providing heritable variations for natural selection to act on and modify patterns of plasticity over the generations (Pigliucci, 2005; Suzuki and Nijhout, 2006). Recognizing that different individuals, populations, or species can respond distinctly to the same environmental factor due to differences in their genomes underscores the exceptional adaptive significance of phenotypic plasticity (Stearns, 1989).

Due to human-driven climate change (IPCC, 2021), rising temperatures are the factor with the most prominent effect on biodiversity, with the potential to influence the long-term ecological and evolutionary dynamics of many species (Parmesan, 2006; Pottier et al., 2022; Walther et al., 2002). In ectothermic organisms, such as amphibians, climate changes have profound evolutionary consequences since many developmental and metabolic processes in these animals depend on external temperature (Bickford et al., 2011; Ruthsatz et al., 2024; Seebacher et al., 2015; Wilbur, 1980). Limiting capacity of habitat selection and thermoregulation, together with a complex life cycle, make amphibians

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especially sensitive to thermal conditions (Navas et al., 2008; Pottier et al., 2025). Thus, understanding the effects of variable temperature on amphibian biology is of special interest, especially because they are among the animal groups most threatened by climate change (Li et al., 2013; Pottier et al., 2025; Schmidt et al., 2024; Wake and Vredenburg, 2008).

Amphibian larvae are a suitable model for studying phenotypic plasticity and climate effects. They inhabit highly variable environments (Newman, 1992), and are characterised by great sensitivity (Messina et al., 2023) and developmental plasticity across physiological (Burraco et al., 2022; Denver, 1997; Petrović et al., 2023), morphological (Hernández-Herrera et al., 2024; Van Buskirk and Schmidt, 2000), behavioural (Polo-Cavia and Gomez-Mestre, 2017; Schmidt and Van Buskirk, 2005), and life-history traits (Newman, 1998), with major effects on adult performance and survival (Richter-Boix et al., 2006; Tejedo et al., 2010; Stoks et al., 2022).

Variations in larval morphology and the timing of metamorphosis are important fitness components in amphibians (Wilbur and Collins, 1973). Metamorphosis involves profound morphological transformations, often including changes in body shape and overall form, to adapt from aquatic larval to more or less terrestrial juvenile and adult stages (Duellman and Trueb, 1994; Laudet, 2011; Wilbur, 1980). Age and size at the onset of metamorphosis can be decoupled to some extent, showing great sensitivity to thermal variations (Gomez-Mestre et al., 2010; Ruthsatz et al., 2018). However, trade-offs between growth and differentiation usually exist, where species with fast metamorphosis often reach smaller sizes at maturation (Newman, 1992). This suggests a complex relationship between the two processes, both of which are highly dependent on environmental conditions, while also emphasizing the potential cost and limitations of a plastic response (DeWitt et al., 1998).

Phenotypic plasticity has been extensively studied in amphibians, particularly in relation to warming conditions. However, there is a clear taxonomic bias, with more research focusing on anurans instead of salamanders (Sinai et al., 2022). For salamanders, it has been shown that an increase in temperature affects morphological, physiological, and life-history traits (Ficetola et al., 2016; Caruso et al., 2014; Gibbs and Karraker, 2006; Hantak et al., 2021; Petrović et al., 2023; Zhu et al., 2023). In the present study, we explored the effect of varying temperature on head and tail morphology (size and shape) in the larvae of two crested newt species (*Triturus ivanbureschi* and *T. macedonicus*) and their reciprocal F_1 hybrids (i.e., with mtDNA of either *T. ivanbureschi* or *T. macedonicus*), and estimated how variations in the larval morphology are linked to the rate of metamorphosis. The optimal developmental temperature for most *Triturus* newt species is around 18°C-19 °C, while the larvae can experience temperatures up to 25 °C, which is considered substantially above the optimum (Litvinchuk et al., 2007; Smolinský and Gvoždík, 2014; Winterová and Gvoždík, 2021).

Crested newts form a monophyletic clade within the genus *Triturus* (Wielstra et al., 2019). Two studied species, *T. ivanbureschi* and *T. macedonicus*, belong to two distinct lineages, which differ in morphology, life history, and ecological preferences (Vučić et al., 2018, 2019, 2020a; Wielstra et al., 2012). They meet in a contact zone in the central part of the Balkan Peninsula, where they hybridize. Only hybrids with *T. ivanbureschi* mitochondrial DNA persist in nature, indicating asymmetric introgression (Wielstra and Arntzen, 2012; Wielstra et al., 2014, 2017a). It has been shown that higher temperatures induce faster metamorphosis in *T. ivanbureschi*, while in *T. macedonicus*, they cause increased body growth (Petrović et al., 2023). Therefore, we expected that the two species would show different phenotypic responses to elevated temperature (higher than the optimal). Furthermore, we investigated how hybridization influences patterns of plasticity and whether the responses of hybrid genotypes differ from those of parental species. As no difference in head or tail shape was observed between reciprocal hybrids at the late larval stage in optimal experimental conditions (Vučić et al., 2018, 2019), and as hybrids show similar physiological response to increased temperature (Petrović et al., 2023), we expected that the two reciprocal hybrids may exhibit similar phenotypic responses. Furthermore, we tested whether the observed shape changes represent a direct morphological response to elevated temperature, which would mean that they are independent of the head and tail size and the rate of metamorphosis. First, we tested whether shape plasticity is size-dependent by estimating allometry, i.e., the relationship between size and shape (Pélabon et al., 2014; Klingenberg, 2016). Allometry has an important role in shaping morphological differences among *Triturus* species (Ivanović et al., 2008, 2025; Ivanović and Arntzen, 2014), and temperature can alter allometries (Nijhout and German, 2012; Rohner and Moczek, 2023). Therefore, we expect that allometry will have a role in shaping phenotypic response. Given that elevated temperatures increase developmental rate during larval development and accelerate metamorphosis in *T. ivanbureschi* and its hybrids, but not in *T. macedonicus* (Petrović et al., 2023), we expected that observed shape changes reflect normal metamorphic progression.

2. Methods

2.1. Experimental design

Triturus ivanbureschi and *T. macedonicus* adults were collected from natural populations outside the hybrid zone (Wielstra et al., 2014) in 2014 and 2015. *Triturus ivanbureschi* individuals originated from Zli Dol, Serbia (42°25′N, 22°27′E), while individuals of *T. macedonicus* originated from Ceklin, Montenegro (42°21′N, 18°59′E). Collected individuals were used as parental generations to obtain F_1 descendants for the subsequent experiment. Capturing animals for the experiment was approved by theMinistry of Energy, Development and Environmental Protection of the Republic of Serbia (Permit No. 353–01-75/2014–08), and the Environmental Protection Agency of Montenegro (Permit No. UPI-328/4). The experimental procedure was approved by the Animal Ethical Committee of the Institute for Biological Research "Siniša Stanković", University of Belgrade (Decision No. 03–03/16). The cross-breeding of the animals took place at the beginning of March, after hibernation in a cold chamber at a constant temperature of 4 °C. The detailed experimental procedure has already been published by Petrović et al. (2023), thus we only provided a summary and highlighted the most important points here.

Four types of crossings were made to acquire the larvae of the species and their reciprocal F_1 hybrids: 1) *T. ivanbureschi* (*T. ivanbureschi* ♀ × ♂), 2) *T. macedonicus* (*T. macedonicus* ♀ × ♂), 3) *T. ivanbureschi*-mothered (with *T. ivanbureschi* mtDNA) hybrids (*T. ivanbureschi* ♀ × *T. macedonicus* ♂), and 4) *T. macedonicus*-mothered (with *T. macedonicus* mtDNA) hybrids (*T. macedonicus* ♀ × *T. ivanbureschi* ♂). Upon hatching, all larvae were raised in 2L plastic containers (one larva per container), half-filled with dechlorinated tap water at the same controlled conditions (19 °C, natural day-night regime, water change every other day, fed *ad libitum* with *Tubifex* sp.). Upon reaching stage 62 (Glücksohn, 1931), the larvae were photographed and randomly separated into two thermal groups (19 °C and 24 °C). Thermal treatment of 19 °C is considered very close to the optimal developmental temperature in both studied species, whereas 24 °C substantially exceeds the optimum (Litvinchuk et al., 2007). The treatment lasted for 30 days. At the end of the experiment, individuals were sacrificed by immersion in liquid nitrogen to estimate parameters of oxidative stress (Petrović et al., 2023).

2.2. Geometric morphometrics and statistical data analyses

Each individual was photographed from a dorsal viewpoint to capture head shape and from a lateral view to capture tail shape, at the beginning and the end of the experiment. The total number of analysed photographs was 387 for the head and 380 for the tail, with sample sizes for each genotype given in Table 1. Photographs were taken using a

Table 1
Sample size per genotype for two morphological structures at two temperature treatments. Since some larvae died during the experiment, the appearance of "/" in the table divides sample sizes at the beginning (left value) and at the end (right value) of the experiment.

Genotype	Head		Tail	
	24 °C	19 °C	24 °C	19 °C
MAC	25/24	26/25	25	25
IVA	19	19	18/19	19
HMA	31	31/30	30/29	30/29
HIV	22	22	21/22	22

Abbreviations: MAC – *Triturus macedonicus*; IVA – *Triturus ivanbureschi*; HMA – Hybrid with *T. macedonicus* mtDNA; HIV – Hybrid with *T. ivanbureschi* mtDNA.

Nikon Digital Sight Fi2 Cameraattached to a Nikon SMZ800 stereo zoom microscope (Nikon Instruments Inc., NY, USA) and a Sony DSC-F828 digital camera (24-bit color and 3264 × 2448 pixel resolution, MP; Sony Corp., Tokyo, Japan).

During newt metamorphosis, the most notable externally visible changes are resorption of gills and a change in skin coloration and structure, which goes from thin and yellowish to thicker and darker (Ajduković et al., 2023; Duellman and Trueb, 1994). To estimate the rate of metamorphosis and how the progression of metamorphosis influenced shape variation, we defined four metamorphic categories at the end of the experiment and quantified the proportion of individuals belonging to each category. Metamorphic categories were defined as: 0 – individual did not enter the metamorphosis; criteria: presence of long branched gills and no signs of skin changes; 1 – beginning of the metamorphosis; criteria: visible reduction in the size of the gills, gills

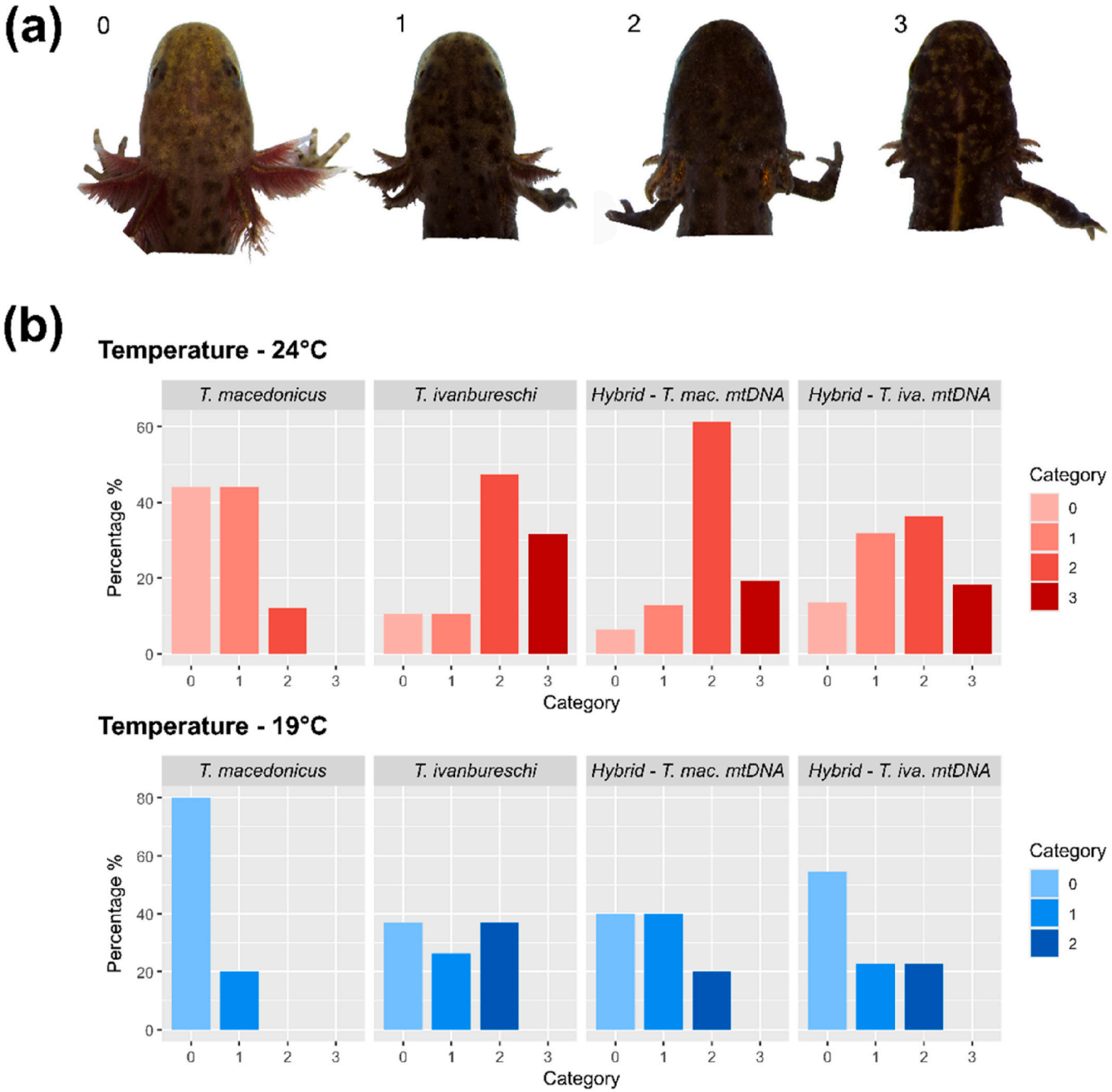

Fig. 1. Representation of each metamorphic category (a) and bar plots showing within-group proportions of metamorphosed individuals (b).

still branched, minor or no signs of skin changes; 2 – middle of the metamorphosis; criteria: significantly reduced and unbranched gills, beginning of changes in skin coloration and structure; 3 – end of the metamorphosis; criteria: only minor gill remains or no gills, juvenile skin coloration and structure (Fig. 1a and Table S1). To estimate the effect of temperature on metamorphosis, two treatment groups of the same genotype were compared, while also analysing the variation among species and hybrids, with Fisher's exact test for count data, using the *fisher.test*() function in R. A non-parametric approach using Monte Carlo simulations with 10.000 replicates was used to compute P values. Since we had four metamorphic categories, separation of the full dataset and the execution of multiple individual tests were necessary for the pairwise *post hoc* Fisher's test.

To quantify size and shape variation, we used the same photographs as for the analysis of metamorphosis. A landmark configuration of seven anatomical landmarks and 10 semilandmarks was used for the head, and three anatomical landmarks and eight semilandmarks for the tail, following Vučić et al. (2018, 2019) (Fig. S1; Tables S2 and S3). The MakeFan6 program from the Integrated Morphometrics Program (IMP) series (Sheets, 2007) was used to determine semilandmark positions and to ensure equal angular spacing between points along the curve. All landmarks and semilandmarks were digitized in tpsDig2 v. 2.32 (Rohlf, 2015).

Statistical analyses of morphometric data were done in R (v. 4.4.2) programming language (R Core Team, 2024), within *geomorph* (v. 4.0.10) and *RRPP* (v. 2.1.2) packages (Adams et al., 2025; Baken et al., 2021; Collyer and Adams, 2018, 2024) unless stated differently. Head and tail were analysed independently. The Generalized Procrustes Analysis (GPA) with the bending energy method for sliding semilandmarks was performed to remove the non-shape variation (scale, translation, and rotation) (Dryden and Mardia, 2016; Rohlf and Slice, 1990) using the *gpagen()* function. Centroid size (CS) was calculated as a size variable. For all further analyses involving size variation, a logarithm of CS (logCS) was used as the input variable (Tables S2 and S3). Using the *bilat.symmetry()* function, the symmetric component of shape variation was extracted from the data and was used as the shape variable in downstream analyses. The symmetric shape component was calculated as averages of the mirrored and the original landmark configurations for each specimen (Klingenberg et al., 2002). The effect of environmental variables on size and shape variation was statistically evaluated through Procrustes analysis of variance (Procrustes ANOVA) (Collyer et al., 2015). Models were described within *procD.lm()* function, with the ***shape/size ~ time * treatment * genotype*** formula. The "***time***" represents a categorical factor with values indicating the time when the individual was sampled, either at the beginning of the experiment or at the end. The "***treatment***" factor defines the affiliation to one of the two treatment groups, and the "***genotype***" factor indicates the species or hybrid group. Therefore, the statistical significance of *time* × *treatment* interaction would suggest divergent phenotypic responses between the two treatment groups, and the statistical significance of *time* × *treatment* × *genotype* interaction would suggest divergent phenotypic responses among treatment groups and genotypes.

The *post hoc* pairwise comparison of group means was performed to assess intra- and interspecific differences in size and shape using *pairwise ()* function. For the size comparison, distances were calculated as the absolute difference in the logCS values across group means. For the shape data, differences between groups were expressed as Procrustes distances between group means. Furthermore, the intraspecific pairwise comparison of shape variances was performed. A Bonferroni correction was applied to adjust P values for multiple comparisons.

The analysis of phenotypic change vectors (PCV) (Adams and Collyer, 2009) was used to assess the magnitude and direction of shape changes from the beginning to the end of the experiment, and to compare total shape change among experimental groups (Collyer and Adams, 2007, 2013). The magnitude of total shape change is estimated as the length of the phenotypic change vector defined by the distance between two points (shape means). Vector direction describes the orientation of the vector in the multivariate data space, where differences in directions between two vectors are expressed through their correlation coefficient and the angle they form. The analysis calculates differences in vector lengths and directions among desired groups, and those differences are used as test statistics to evaluate the hypothesis. For this analysis, we used the *trajectory.analysis()* function. To examine genotype-specific PCVs, we fitted a linear model with ***shape ~ genotype-treatment * time*** formula, where "genotype-treatment" was used as the "groups" argument and defined a factor containing information about genotype and its affiliation to either of the two treatments, while the "time" defined vector points. To visualize differences in vector attributes (magnitude and direction of phenotypic change), PCA was performed on fitted values from the linear model.

To account for the effect of allometry (covariation between size and shape) and to assess whether shape plasticity depends on size variation, a Procrustes analysis of covariance (ANCOVA) was performed using ***shape ~ logCS * genotype * treatment*** formula, including only individuals at the end of the experiment. To further evaluate allometric patterns, separate ANCOVAs were performed within each genotype (***shape ~ logCS * treatment***), and allometric slopes were estimated using the *emmeans* R package (Lenth and Piaskowski, 2025). Furthermore, to test whether shape differences between treatments were underlined by the different metamorphic categories, shape data (for the end of the experiment) were regressed on metamorphic categories using *procD.lm()* function with ***shape ~ metamorphosis * genotype*** formula for the full data set and within each genotype separately.

3. Results

3.1. Temperature-induced metamorphosis

Comparison of metamorphic categories revealed a general pattern of variation where each genotype exhibited significantly faster metamorphosis as a result of higher temperatures (P values from Fisher's exact tests: *T. macedonicus* – 0.013; *T. ivanbureschi* – 0.018; hybrids with *T. macedonicus* mtDNA – 0.0001; hybrids with *T. ivanbureschi* mtDNA – 0.012). This was indicated by consistently higher proportions of individuals in categories 2 and 3 at 24 °C relative to their corresponding proportions at 19 °C (Fig. 1b). Besides the general pattern of variation, there were significant differences in proportions of metamorphosed individuals among genotypes (P values from Fisher's exact tests: 19 °C – 0.00730; 24 °C – 0.00001). *Triturus macedonicus* larvae showed the slowest metamorphosis, reflected in the greatest proportion of individuals in categories 0 and 1 at 24 °C (88.00%, category 2 – 12.00%, category 3 – 0%). In contrast, *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA had the greatest proportion of individuals in categories 2 and 3 at 24 °C (*T. ivanbureschi*: 79.00%; hybrids with *T. macedonicus* mtDNA: 80.64%), indicating faster metamorphosis.

3.2. Size variation

At the beginning of the experiment, there were no significant differences in head or tail size among and within genotypes (Tables S4 and S5). Time, treatment, and genotype had statistically significant effects on both head and tail size during the experiment (Table 2). *Triturus macedonicus* exhibited a significant increase in size at 24 °C relative to 19 °C ($P < 0.001$ for head size and $P < 0.01$ for tail size), while in other genotypes, responses between two temperatures were similar ($P > 0.05$, in all comparisons; Fig. 2 and Table S4). At the end of the experiment, genotypes had similar head and tail sizes at both temperatures ($P > 0.05$, in all comparisons; Fig. 2 and Table S5).

3.3. Shape variation

All analysed factors significantly affected head and tail shape

Table 2
Analysis of variance for head and tail size (ANOVA) and shape (Procrustes ANOVA) data. Factor descriptions: "time" – differences between the beginning and the end of the experiment; "treatment" – differences between two thermal treatments (19 °C and 24 °C); genotype – differences between two species and two hybrid groups.

HEAD	Size ANOVA						Procrustes ANOVA				
	Df	MS	R^2 (%)	F	Z	P	MS	R^2 (%)	F	Z	P
time	1	9.343	88.19	4683.5	15.38	**0.0001**	0.112	23.70	201.7	9.46	**0.0001**
treatment	1	0.229	2.16	114.8	6.07	**0.0001**	0.020	4.25	36.1	6.21	**0.0001**
genotype	3	0.007	0.21	3.7	2.21	**0.0122**	0.027	16.76	47.6	11.61	**0.0001**
time × treatment	1	0.111	1.05	55.9	4.77	**0.0001**	0.015	3.17	27.0	6.17	**0.0001**
time × treatment × genotype	3	0.020	0.55	9.8	4.21	**0.0001**	0.003	1.65	4.7	4.24	**0.0001**
Residuals	371	0.002	6.99				0.001	43.59			
Total	386										
TAIL											
time	1	19.728	85.94	3508.5	15.26	**0.0001**	0.459	33.55	230.2	6.76	**0.0001**
treatment	1	0.541	2.36	96.3	5.68	**0.0001**	0.011	0.76	5.2	2.2	**0.0121**
genotype	3	0.026	0.34	4.7	2.62	**0.0037**	0.021	4.50	10.3	4.59	**0.0001**
time × treatment	1	0.334	1.46	59.4	4.92	**0.0001**	0.039	2.85	19.6	3.7	**0.0001**
time × treatment × genotype	3	0.018	0.24	3.2	1.98	**0.0207**	0.003	0.63	1.5	0.85	0.2045
Residuals	364	0.006	8.92				0.002	53.04			
Total	379										

Abbreviations: Df – degrees of freedom; MS – mean squares; R^2 – coefficient of determination; F – F value; Z – effect size; P – statistical significance.

(Table 2). At the end of the experiment, temperature had significant effects on head shape in all genotypes (Table S6, diagonal). In contrast, tail shape was significantly affected by temperature only in *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA (Table S6, diagonal).

At the beginning of the experiment, head shape differed between the parental species and between *T. ivanbureschi* and hybrids with *T. ivanbureschi* mtDNA (Table S7). At the end of the experiment, head shape differed among all genotypes at 19 °C, whereas tail shape did not (Table S6, below diagonal). At 24 °C, head and tail shape differed in most comparisons, except between the two hybrid groups and between *T. macedonicus* and hybrids with *T. ivanbureschi* mtDNA for the tail (Table S6, above diagonal).

At 24 °C, head shape variance increased significantly over time in *T. ivanbureschi* ($P < 0.01$) and both hybrids ($P < 0.001$ in both cases). Additionally, two treatments of both hybrids significantly differed in their variance at the end of the experiment ($P < 0.05$ in both cases; Table S8). A significant decrease in tail shape variance throughout the experiment was observed at 19 °C for *T. macedonicus* ($P < 0.01$) and at 24 °C for hybrids with *T. macedonicus* mtDNA ($P < 0.01$). All other comparisons of shape variance were statistically non-significant (Table S8).

3.3.1. The magnitude and direction of head and tail shape changes

The increase in magnitude of head shape changes at 24 °C compared to 19 °C was observed in both species and hybrids with *T. ivanbureschi* mtDNA (Table 3, Fig. 3a). *Triturus macedonicus* and hybrids with *T. ivanbureschi* mtDNA showed the same vector lengths at both 19 °C and 24 °C, with approximately 50% increase in magnitude at 24 °C compared to 19 °C. Direction of shape changes differed between 19 °C and 24 °C in *T. ivanbureschi* and both hybrids (Table 3). Overall, *T. ivanbureschi* is characterized by a unique pattern of shape change within the morphospace, with more than twice the magnitude of head shape change in the 24 °C group (compared to the 19 °C group), reflecting the strongest response to the higher temperature (Fig. 3a). For tail shape (Fig. 3b), increased magnitude of shape change was observed in *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA at 24 °C (Table 3). There were no differences in the direction of tail shape change across genotypes (Table 3; Fig. 3b).

Both species and hybrids experienced widening of the posterior part of the head at the end of the experiment at 19 °C (Fig. S2a). At 24 °C, widening was more pronounced and coupled with shortening of the posterior part of the head. The most notable difference between species was recorded in the eye region. In *T. macedonicus* this region was narrower at both temperatures, while in *T. ivanbureschi* it was wider at 24 °C compared to the beginning of the experiment. At the end of the experiment, the overall shape difference between the 19 °C and 24 °C groups was the most prominent in *T. ivanbureschi*. Considering tail shape, *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA showed a notable decrease in relative tail width throughout the experiment, which is associated with caudal fin resorption (Fig. S2b).

3.3.2. Contribution of allometry and metamorphosis in the shape plasticity

Head shape exhibited clear genotype-specific size-dependent plasticity, as indicated by a significant *size* × *treatment* × *genotype* interaction (Table 4). *Triturus ivanbureschi* exhibited strong treatment effects with temperature-dependent allometry but retained positive slopes across both temperatures. *Triturus macedonicus* displayed pronounced allometry with a shift along a common allometric trajectory, but no size-dependent plasticity. Both hybrids showed significant *size* × *treatment* interactions and a temperature-dependent reversal in allometric direction (Tables S9 and S10, Fig. 4). In contrast, the three-way interaction was not significant for tail shape (Table 4). Although *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA showed apparent shifts in slope direction, these changes were small and inconsistent across groups (Tables S9 and S10, Fig. 4). Thus, tail plasticity was not size-dependent.

The effect of the rate of metamorphosis on head and tail shape was significant (head: $R^2 = 22.41\%$, $F = 24.8$, $P < 0.0001$; tail: $R^2 = 25.52\%$, $F = 23.98$, $P < 0.0001$), whereas the *metamorphosis* × *genotype* interaction was non-significant (both $P > 0.05$). These numbers should be interpreted with caution, considering the unbalanced design of the model (small number of individuals in category 3). The rate of metamorphosis significantly affected head shape ($P < 0,05$; Table S11) across all genotypes, but it explained a noticeably smaller amount of shape variation in *T. macedonicus*. Tail shape was affected by the rate of metamorphosis in *T. ivanbureschi* and hybrids ($P < 0.01$; Table S11). *Triturus ivanbureschi* showed the strongest association to metamorphic categories for both traits (Fig. 5).

4. Discussion

Our results indicate that the shape changes observed under elevated temperature do not represent a direct plastic response of morphology to temperature. Instead, they reflect developmental plasticity expressed primarily through an acceleration of metamorphosis, with the observed shape changes corresponding to normal metamorphic transformations occurring at a faster rate. The two species exhibited divergent responses to elevated temperature. *Triturus ivanbureschi* showed high sensitivity, with accelerated metamorphosis driving pronounced shape changes by changing the elevation of allometric trajectories. In *T. macedonicus*,

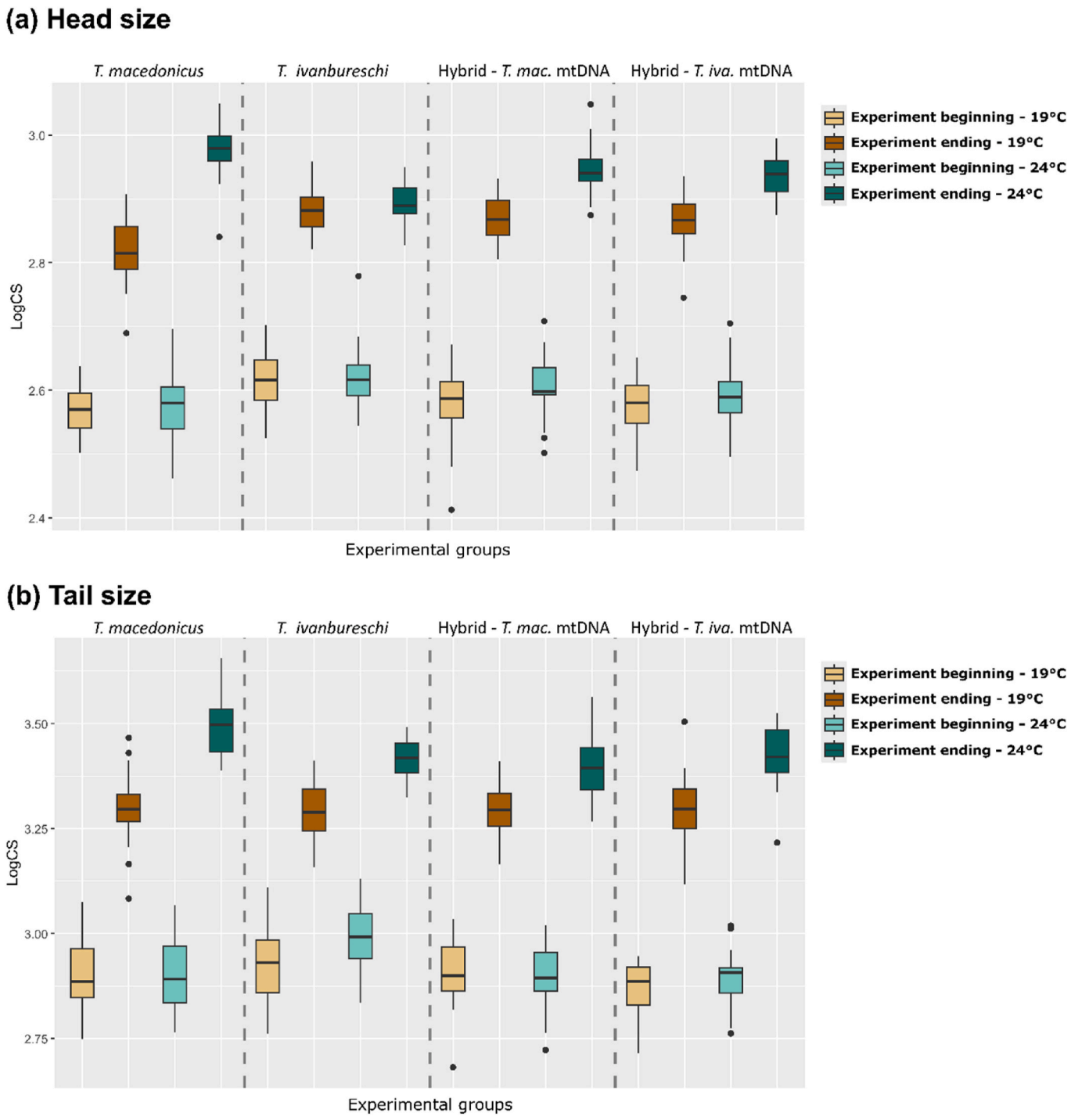

Fig. 2. Differences in mean head (a) and tail (b) size (logarithm of centroid size – LogCS). Each box plot is defined by the median (horizontal bold line), the interquartile range (colored box), the upper and lower whiskers (vertical lines), the minimum and maximum values (whisker tips), and outliers (black points).

metamorphosis acceleration was observed, but not as prominent as in *T. ivanbureschi.* This acceleration was coupled with increased growth, resulting in relatively minor changes in head shape that largely reflected shifts along a common allometric trajectory for both temperatures. Tail shape differed between temperature treatments in *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA, regardless of size differences, indicating limited size-dependent variation in tail morphology. Reciprocal hybrids, with different mtDNA genotypes, also showed genotype-specific responses, including pronounced changes in the allometric trajectories of head shape. In addition, plastic responses differed between body regions: head shape exhibited a greater plasticity with patterns that were both genotype-specific and size-dependent, whereas tail shape was less affected by elevated temperature. Overall, elevated temperature enhanced morphological divergence among genotypes over time through its effect on the rate of metamorphosis, particularly influencing head shape.

4.1. *Patterns of genotype-specific size plasticity*

In ectotherms, the effect of temperature on variation in size and age at metamorphosis is often explained by the temperature–size rule, which predicts that individuals reared at higher temperatures undergo shorter

Table 3
Comparisons between groups reared at 19 °C and 24 °C in the magnitude (vector length) and direction (vector angle) of shape change vectors from the beginning to the end of the experiment within each genotype. P values in bold were significant after the Bonferroni correction (P < 0.0125).

HEAD	Vector length			Vector angle			
Compared groups	PD	Z	P	r	degrees	Z	P
MAC t_{19}–t_{24}	0.0147	2.39	**0.0032**	0.91	25.16	1.79	0.0387
IVA t_{19}–t_{24}	0.0370	4.40	**0.0001**	0.76	40.88	2.90	**0.0011**
HMA t_{19}–t_{24}	0.0028	−0.09	0.5490	0.80	36.50	3.34	**0.0001**
HIV t_{19}–t_{24}	0.0148	2.26	**0.0068**	0.75	41.48	3.25	**0.0004**
TAIL	Vector length			Vector angle			
Compared groups	PD	Z	P	r	degrees	Z	P
MAC t_{19}–t_{24}	0.0166	0.65	0.2727	0.99	7.69	0.25	0.3976
IVA t_{19}–t_{24}	0.0567	2.56	**0.0010**	0.99	6.01	−0.47	0.6758
HMA t_{19}–t_{24}	0.0575	3.06	**0.0002**	0.98	11.48	1.18	0.1233
HIV t_{19}–t_{24}	0.0272	1.30	0.0981	0.99	8.56	0.33	0.3667

Abbreviations: MAC – *Triturus macedonicus*; IVA – *Triturus ivanbureschi*; HMA – Hybrid with *T. macedonicus* mtDNA; HIV – Hybrid with *T. ivanbureschi* mtDNA; PD – Procrustes distance; r – correlation coefficient; Z – effect size; P – statistical significance; t_{19} – 19 °C treatment group; t_{24} – 24 °C treatment group.

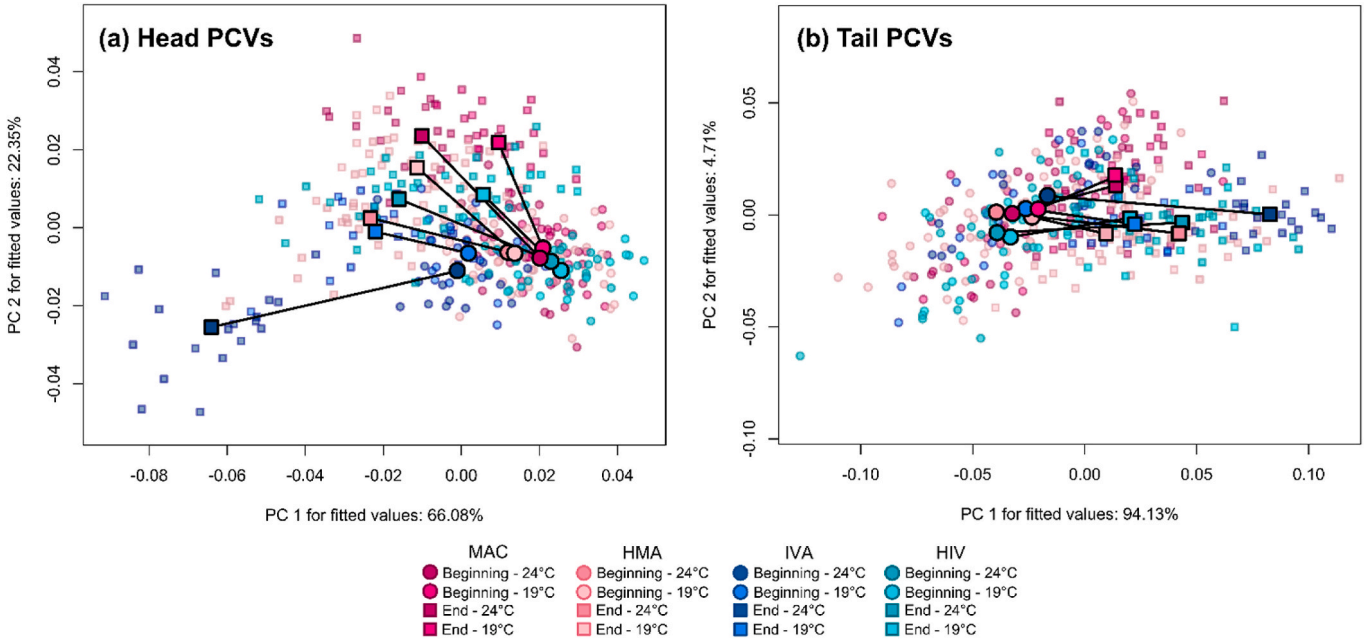

Fig. 3. Genotype-specific phenotypic trajectories (phenotypic change vectors – PCVs) describing the magnitude (vector length) and direction (vector angle) of shape changes from the start (mean shape per genotype represented as circles) to the end (squares) of the experiment in the head (a) and tail (b), plotted in morphospace defined by the first two principal components. Lines connecting the circles and squares represent the vectors of shape change. Vector length corresponds to the magnitude of change, and angle corresponds to the direction of shape change. Differences in magnitude and direction illustrate genotype-specific responses to different temperatures, with *Triturus ivanbureschi* (IVA) showing the strongest response in both head and tail. Abbreviations: MAC – *Triturus macedonicus*; IVA – *Triturus ivanbureschi*; HMA – Hybrid with *T. macedonicus* mtDNA; HIV – Hybrid with *T. ivanbureschi* mtDNA.

larval periods and reach smaller sizes at metamorphosis, compared to conspecifics reared at lower temperatures (Atkinson, 1994). Recent studies indicate that overall developmental rate in amphibian larvae is more sensitive to thermal variation than growth rate, which leads to generally faster metamorphosis in species or populations at higher temperatures, whereas size at the onset of metamorphosis remains largely species- or population-specific (Ruthsatz et al., 2018; Sinai et al., 2022). Our results support these findings, as the plastic response in head and tail size (centroid size) did not follow the temperature-size rule. Instead, size changes were genotype-specific. *Triturus macedonicus* exhibited increased growth in head and tail regions, while growth in *T. ivanbureschi* was largely unaffected by temperature, and hybrids showed distinct responses compared with both parental species.

4.2. Patterns of genotype-specific shape plasticity

The phenotypic trajectories of shape changes showed that under elevated temperature, head shape vectors diverged in magnitude (*T. macedonicus*), direction (hybrids with *T. macedonicus* mtDNA), or both (*T. ivanbureschi* and hybrids with *T. ivanbureschi* mtDNA). For tail shape, only *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA exhibited a greater magnitude of shape change. These patterns of shape changes indicate higher thermal responsiveness of *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA in both body regions. The observed patterns of head shape plasticity may be at least partially explained by the potential of variable external factors, including temperature, to modify the relationship between head size and shape (West-Eberhard, 1989; Nijhout and German, 2012; Viscosi, 2015) and produce genotype-specific static allometric patterns. Interestingly, in *T. macedonicus*, which was the only genotype to exhibit increased

Table 4
Procrustes analysis of covariance (ANCOVA) testing allometric effects (shape–size covariation) and whether phenotypic plasticity in shape is size-dependent across treatments and genotypes. Factor descriptions: "logCS" – log-transformed centroid size as a size proxy; "treatment" – differences between two thermal treatments (19 °C and 24 °C); genotype – differences between two species and two hybrid groups. Data used for the analysis includes only individuals from the end of the experiment.

HEAD	Df	MS	R^2 (%)	F	Z	P
logCS	1	0.004	14.52	60.2	31.00	**0.0005**
treatment	1	0.003	9.84	40.8	24.27	**0.0062**
genotype	3	0.001	14.65	20.2	19.20	**0.0274**
logCS × treatment	1	0.002	0.92	38.3	23.26	**0.0088**
logCS × genotype	3	0.001	14.61	20.2	18.95	**0.0271**
treatment × genotype	3	0.001	1.53	20.2	20.75	**0.0185**
logCS × treatment × genotype	3	0.001	1.52	46.04	20.52	**0.0196**
Residuals	176	0.001	42.46			
Total	191					
TAIL						
logCS	1	0.026	6.89	21.2	39.71	**0.0001**
treatment	1	0.023	6.07	18.7	39.44	**0.0001**
genotype	3	0.024	19.34	19.9	64.23	**0.0001**
logCS × treatment	1	0.005	1.22	3.8	18.93	**0.0279**
logCS × genotype	3	0.005	4.20	4.3	29.56	**0.0008**
treatment × genotype	3	0.006	4.81	4.9	33.44	**0.0002**
logCS × treatment × genotype	3	0.001	0.98	1.0	0.25	0.3985
Residuals	174	0.001	56.49			
Total	189					

Abbreviations: logCS – logarithm of centroid size; Df – degrees of freedom; MS – mean squares; R^2 – coefficient of determination; F – F value; Z – effect size; P – statistical significance.

growth under elevated temperature, there was a negligible contribution of allometry in head shape plasticity. This suggests a pattern of ontogenetic scaling, indicating a more stable and canalized developmental pathway (Parsons and Albertson, 2013; Klingenberg, 2016) compared to *T. ivanbureschi* and hybrids. On the other hand, tail shape plasticity was not affected by the allometry. In general, the observed contrast between head and tail shape responses to elevated temperature might suggest functional constraints. The larval head and tail have distinct functional roles, both essential for survival and overall fitness. Head is primarily involved in feeding and sensory input (Duellman and Trueb, 1994; Fabre et al., 2020), while the tail is the primary locomotive organ in larvae (Schmidt and Van Buskirk, 2005; Van Buskirk, 2009; Wassersug, 1989).

4.3. The rate of metamorphosis and its effects

Metamorphosis is closely linked to environmental cues, and although it is a process tightly regulated by genetic and hormonal cascades (Laudet, 2011), its timing and progression can be largely affected by thermal variability (Hickerson et al., 2005; Lowe et al., 2021). At a constant temperature, the larval period of *T. macedonicus* is longer and they start metamorphosis at a larger size compared to *T. ivanbureschi* and hybrids (Furtula et al., 2009; Vučić et al., 2019). We found that elevated temperature consistently accelerated metamorphosis, with genotype-specific responses placing *T. ivanbureschi* and *T. macedonicus* at opposite ends of the spectrum. The rate of metamorphosis was a strong predictor of changes in head and tail shape, explaining 22%–33% of the variation among faster-developing genotypes. Such shape changes are consistent with the typical metamorphic transformations of these body regions (Duellman and Trueb, 1994; Vučić et al., 2019). This finding highlights the importance of accounting for the rate of metamorphosis in studies of larval morphology. It is particularly crucial in comparative analyses of shape change, where differences in the progression of metamorphosis may obscure stage-specific variation and lead to incomplete or misleading interpretations.

4.4. Hybridization and developmental plasticity

The evolution of phenotypic plasticity has enhanced organisms' success in novel habitats and plays a key role in genetic differentiation and speciation. Hybridization influences this process by modifying gene flow, recombination, and adaptive divergence, either accelerating or slowing speciation through mechanisms like introgression. The phenotypic novelties stemming from hybridization and plasticity are shaped by adaptive responses, not random variations, and can lead to ecological changes, expand evolutionary potential, and alter dynamics in contact zones (Abbott et al., 2013; Agrawal, 2001; West-Eberhard, 1989, 2005). The interplay of developmental plasticity and hybridization may be of special importance for crested newts, as hybridization plays an important role in shaping their evolution (Arntzen et al., 2014, 2021; Patton et al., 2020; Wielstra et al., 2017a, 2017b).

As noted before, two reciprocal *T. ivanbureschi* × *T. macedonicus* F_1 hybrids express similar patterns of morphology and life-history, which are divergent from both parental species under optimal experimental conditions (Vučić et al., 2018, 2019, 2022; Bugarčić et al., 2022). Physiological traits of hybrids also differ substantially from those of parental species (Prokić et al., 2018), with hybrids experiencing greater oxidative stress as a physiological response to thermal elevation (Petrović et al., 2023). Vinšálková and Gvoždík (2007) found that temperature preferences of *T. carnifex* × *T. dobrogicus* F_1 hybrids resembled the maternal species (*T. carnifex*), while body length was intermediate between the parental species during both the larval and juvenile periods. In contrast, reciprocal *T. ivanbureschi* × *T. macedonicus* F_1 hybrids exhibited increased body growth under elevated temperature, similar to *T. macedonicus* (Petrović et al., 2023), whereas head and tail sizes remained largely unchanged, resembling *T. ivanbureschi*. The patterns of shape changes were also divergent among both hybrids and parental species, as well as between the two hybrids. The divergence in allometric direction observed in hybrids suggests that the synergistic effects of hybridization and thermal stress may lead to a breakdown of more canalized allometric patterns in both species, where temperature did not induce a change in the allometric direction.

The observed temperature-induced differences in plastic response between the two hybrids suggest that mito-nuclear interactions may influence the direction and magnitude of morphological change. A similar pattern was observed in oxidative status and responses to oxidative stress in these individuals (Petrović et al., 2023). Also, temperature-induced increase in head shape variance in hybrids (compared to the lower temperature group) highlights that both hybridization and maternal inheritance largely contribute to the plastic developmental landscape, which may be crucial in unpredictable and

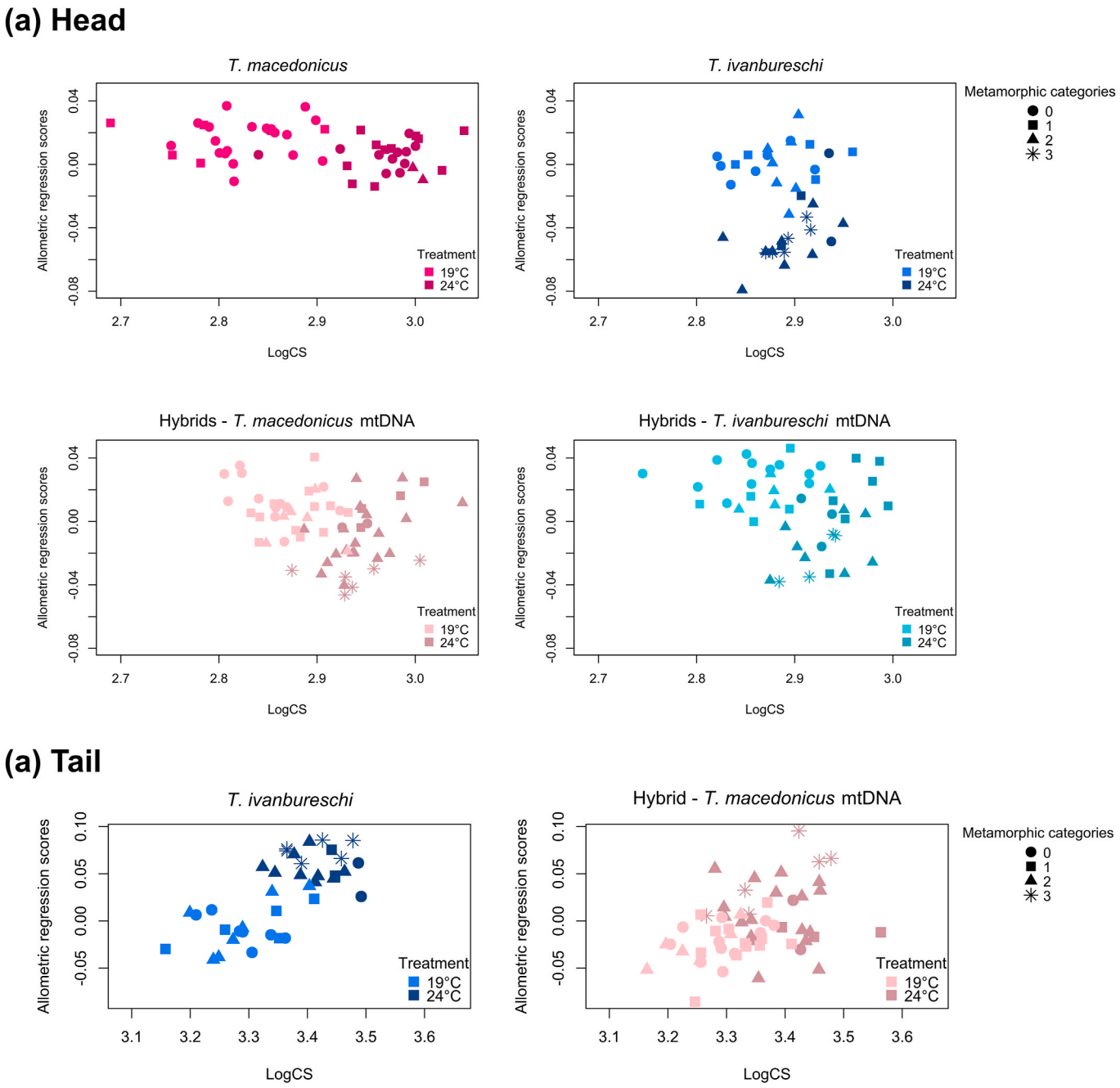

Fig. 4. Relationship between size (x-axis) and shape (y-axis), including information about metamorphic categories. Divergent allometric trajectories between two treatments are shown for head shape in *T. ivanbureschi* (change in elevation) and both hybrid genotypes (change in direction). Statistically similar allometric trajectories, with shifts along the common trajectory for two temperatures, are shown for head shape in *T. macedonicus* and for tail shape. The figure shows no correlation between allometric patterns and metamorphic categories.

fast environmental changes.

4.5. Possible implications for species interactions

In nature, the geographical distribution of the two examined crested newt species is characterized by dynamic interactions at their contact zone, where *T. macedonicus* has been gradually displacing *T. ivanbureschi* over generations. The hybrid zone is relatively narrow and consists of hybrid crosses and backcrosses, all with *T. ivanbureschi* mtDNA (Wielstra and Arntzen, 2012; Wielstra et al., 2017a). It has been previously shown that, in optimal laboratory conditions, *T. macedonicus* can outcompete *T. ivanbureschi* in several life-history traits (Cvijanović et al., 2009; Vučić et al., 2020a). Our results, together with those of (Petrović et al., 2023), provide important evidence that the two species and their hybrids exhibit distinct patterns of phenotypic change under elevated temperatures. *Triturus macedonicus* may display a preference for, or higher tolerance to, elevated temperatures compared to other genotypes, but also has the slowest metamorphosis.

Slower metamorphosis coupled with larger body size, as found in *T. macedonicus*, could be favored by natural selection under specific environmental conditions (Kingsolver and Huey, 2008) and may influence a species' ecological niche and competitive interactions. In contrast, the high thermal sensitivity observed in *T. ivanbureschi* and hybrids with *T. macedonicus* mtDNA may have reduced fitness in past environments, potentially contributing to their replacement or historical extinction. This aligns with Price et al. (2003), who emphasized that

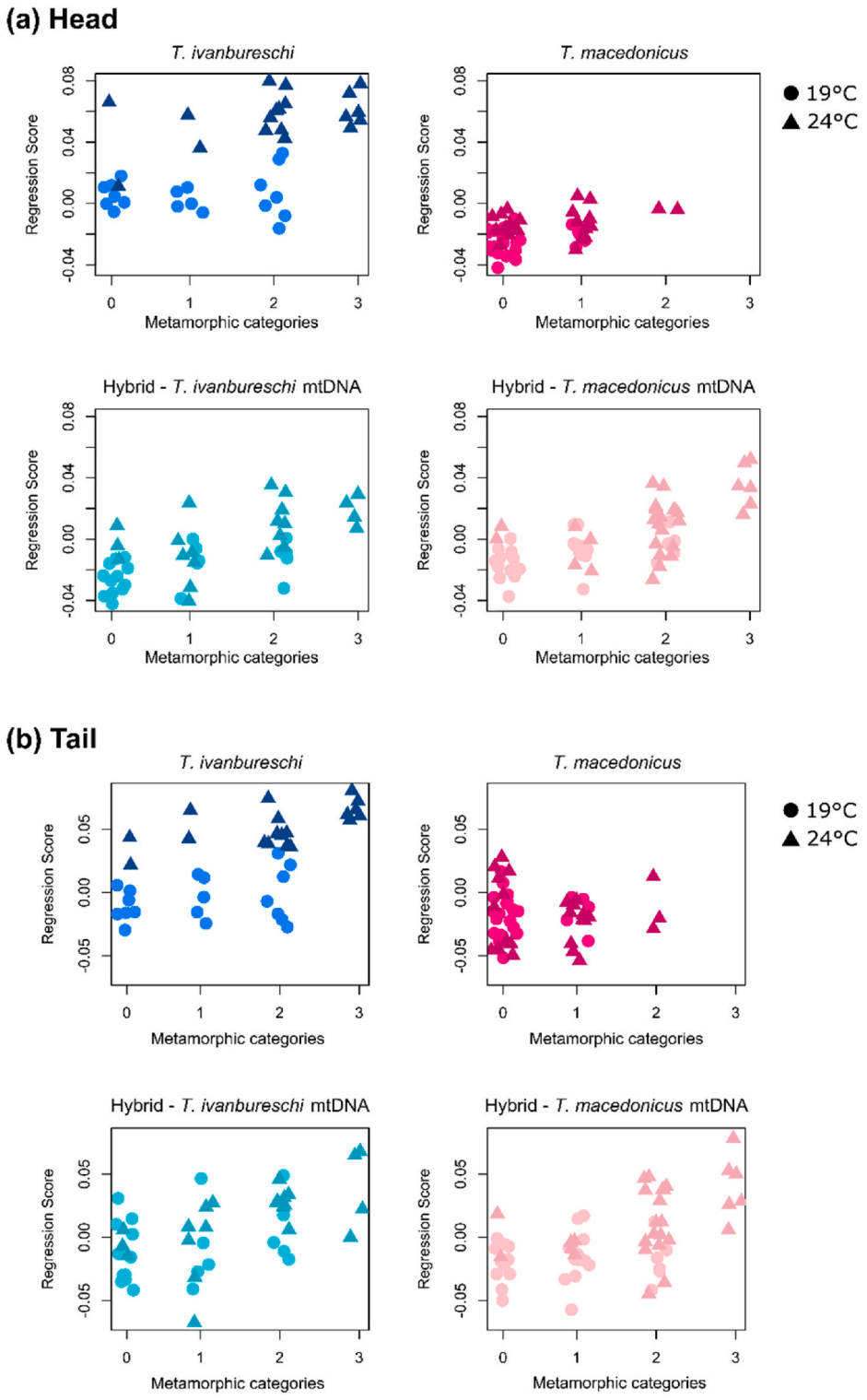

Fig. 5. Relationship between shape and metamorphic stage for head (a) and tail (b) across temperature treatments. Points and triangles represent individual regression scores obtained from the multivariate regression of shape on metamorphic category (0 – individual did not enter the metamorphosis; 1 – beginning of the metamorphosis; 2 – middle of the metamorphosis; 3 – end of the metamorphosis).

moderate levels of plasticity permit population survival in novel environments, whereas excessively high plasticity can constrain adaptive evolution. As historical climate changes likely shaped the replacement of species and the formation of the *T. ivanbureschi* × *T. macedonicus* hybrid zone (Wielstra and Arntzen, 2012), temperature-dependent differences in life-history traits, together with specific developmental, morphological, and physiological plastic responses, could have provided a competitive advantage to *T. macedonicus* in natural populations.

The Central Balkan Peninsula, a region encompassing the contact zone of the two species (Wielstra et al., 2017a; Vučić et al., 2020b), is significantly affected by climate change, including rising average temperatures and increased precipitation fluctuations (Vuković et al., 2018). Under such conditions, *T. macedonicus* may continue to expand into the range of *T. ivanbureschi*, exposing the latter to potential local

extinction risks mediated by human-driven climate change. Conversely, if future conditions bring decreased precipitation and increased drought during the larval development season, these circumstances may favor *T. ivanbureschi* and hybrids exhibiting faster metamorphosis, which rely less on aquatic habitats (Newman, 1992). Increased shape variance in hybrids under warming conditions may indicate both potential for adaptive evolution and signs of developmental instability (DeWitt et al., 1998; Ghalambor et al., 2007; Gibert et al., 2019), depending on genotype-specific responses.

It should be noted that our experimental design was based on two constant temperature regimes, which do not fully capture natural thermal conditions, particularly under climate change scenarios predicting increasing temperature variations. However, the diversity of plastic responses among genotypes suggests that future environmental conditions could lead to a variety of evolutionary outcomes associated with the specific circumstances. Natural temperature variation with high-temperature peaks should further accelerate metamorphosis. Such acceleration is likely to increase divergence among genotypes in developmental trajectories and associated shape changes. Future work should assess how thermal fluctuations and climate-driven niche shifts affect the fitness and evolutionary trajectories of *Triturus* species and their hybrids.

5. Conclusion

This study reveals species- and hybrid-specific patterns of temperature-induced plasticity in *Triturus* newts, which mostly affect the rate of metamorphosis and related changes in head and tail shape. Not only the two different species involved, but also their reciprocal hybrids, exhibit divergent patterns of phenotypic plasticity, suggesting that mito-nuclear interactions may play a role in shaping the plastic responses. Our findings contribute valuable insights into the composition of the natural hybrid zone, as well as the potential adaptability of *T. macedonicus* and *T. ivanbureschi* to ongoing climate change.

Data accessibility statement

Data is available within the supplementary file. Coding scripts, along with input files, will be available on the following link: https://github.com/amph-evodevo-lab-ub/variation-in-thermal-plasticity-larval-morphology.

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CRediT authorship contribution statement

Mihajlo Milić: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Ana Ivanović:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing. **Sonja Nikolić:** Investigation, Writing – review & editing. **Antonija Avdalović:** Formal analysis, Writing – review & editing. **Tamara Petrović:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Marko Prokić:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Tijana Vučić:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2026.104479.

Data availability

Data is available within the supplementary file. The link to coding scripts and input files is given at the Attach Files steps.

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