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Dynamics of Size and Shape Variation During Embryonic Development in the Balkan Crested Newt

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ABSTRACT

Despite its relevance for developmental and evolutionary studies, intraspecific variation, particularly among-individual variation in size and shape during embryogenesis, remains poorly understood. Here, we apply geometric morphometrics to quantify external morphology and to examine variation in embryo size and shape in *Triturus* across the tailbud and prehatching phases. *Triturus* newts, an emerging model system in developmental and evolutionary biology, possess a balanced lethal system that delays development, alters morphology, and induces embryonic arrest in approximately half of the embryos. We hypothesize that this arrest increases morphological variance during the tailbud phase, a period that should be highly constrained due to organogenesis. In contrast, we expect more uniform variance during the prehatching phase, which follows arrest, when only viable embryos remain and approach hatching, a major developmental milestone. Our results reveal gradual changes in size and shape across both developmental phases. Morphological variance increased at tailbud stage 23, driven in part by allometric effects, and coincided with the onset of developmental arrest. During the prehatching phase, variance was uniform but higher than that reported in a previous study of the posthatching period. Overall, our findings provide new insights into morphological variability during early development and establish a foundation for comparative studies aimed at testing developmental hypotheses, including the effects of the balanced lethal system.

1 | Introduction

Embryonic development is a dynamic process that begins with a single cell, the zygote, and progresses through a series of cell divisions, cell differentiation, and morphogenesis, to ultimately form an organism as a whole (Gilbert 2003). During the early development, basic cell types and tissues differentiate and give rise to organs (Gilbert 2003; Richardson 2021). Embryonic development is considered to be a highly constrained period, exhibiting a low level of variation, as even small deviations can produce major changes in phenotype and impair further development (Galis and Metz 2001; West-Eberhard 2003). However, variation during embryonic development exists, and it is the raw material upon which natural selection acts (Kirkwood et al. 2005; Sears 2014; Hallgrimsson et al. 2015). The amount of variation during embryonic development depends on inherent limitations or restrictions imposed by an organism's developmental processes (Gilbert 2003; Galis et al. 2018), which is empirically documented (Badyaev and

Martin 2000; Zelditch et al. 2012; Richardson 2021). Changes in the amount of variation during development produce a particular developmental landscape (Waddington 1942; Siegal and Bergman 2002), with periods that show more and less variation. These less variable periods are regarded as a result of the ability of development to follow a predetermined pathway and produce the same phenotype even in the face of perturbations (canalization) (Waddington 1942; Wagner and Misof 1993; Zelditch et al. 2004).

Quantifying morphological variation across different ontogenetic stages, including different phases of embryonic development, is integral to our understanding of the evolution of developmental processes (West-Eberhard 2003; Cooper and Albertson 2008; Hallgrimsson and Hall 2011; Von Dassow and Davidson 2011). Quantitative analyses aid in identifying conserved developmental stages (Richardson 1999), exploring the genetic basis of trait inheritance (Klingenberg 2002; Zelditch et al. 2012), and play a crucial role in detecting evolutionary patterns, adaptive processes and the mechanisms driving life's diversity (Adams and Collyer 2009; Zelditch et al. 2012). However, studies that quantify morphological variation during the embryonic period of development are methodologically challenging and have largely been neglected until the last two decades. Previously, embryological studies were mostly focused on qualitative descriptions, while the quantification of phenotypic variation was rarely performed and the variation within groups was not calculated (Cooper and Albertson 2008). With the use of morphometry in developmental studies, it became possible to quantify and compare size and shape variation during embryonic development (Cooper and Albertson 2008; Hallgrimsson et al. 2015). Such studies can be divided into two groups. The first group quantifies the differences in craniofacial development between different strains or experimentally modified embryonic development of well-established model organisms such as zebrafish (Albertson and Yelick 2007), chicken (Young et al. 2010), and mouse (Parsons et al. 2008; Green et al. 2017). The second group explores dynamics of within- or between-group variation during the embryonic development of fish (Schmidt and Starck 2004; Mayer et al. 2014) and birds (Kim et al. 2011; Smith et al. 2015).

We explore patterns of size and shape variation and ontogenetic dynamics of variance during embryonic development in a tailed amphibian, the Balkan crested newt (*Triturus ivanbureschi*). The Balkan crested newt is an emerging model organism in evolutionary and developmental biology (Wielstra et al. 2012, 2017; Vučić et al. 2019, 2024; de Visser et al. 2025; France et al. 2025). The morphological variation during postembryonic development of *Triturus* newts has been extensively studied (Ivanović et al. 2011; Cvijanović et al. 2014; Ivanović and Arntzen 2014; Vučić et al. 2018, 2019). The patterns of size and shape changes and dynamics of variance are largely similar across *Triturus* species (Ivanović et al. 2011; Cvijanović et al. 2014; Vučić et al. 2019), with a high level of variance at hatching and right after metamorphosis, whereas the mid-larval stages are highly constrained (Ivanović et al. 2011; Vučić et al. 2019). Information on variation during embryonic development is scarce, even though *Triturus* newts are suitable for non-invasive approaches in monitoring embryonic development: they have relatively large eggs with a transparent protective jelly layer that are not laid in clutches but separately. Due to these characteristics, they have been used as a model system in classical embryological studies more than a century ago (e.g., Spemann and Mangold 1924). Furthermore, *Triturus* newts have a particularly remarkable feature where 50% of individuals slow down their development and die during the tailbud phase as a consequence of a genetic disorder known as a balanced lethal system (Horner and Macgregor 1985; Wielstra 2020; de Visser et al. 2025; France et al. 2025).

Here, we focus on two important phases of embryonic development – tailbud and prehatching. The tailbud phase is a crucial period in vertebrate embryonic development, characterized by the ongoing process of organogenesis (Gilbert 2003). The beginning of the tailbud phase is regarded as (part of) the phylotypic period. This period occurs during the middle stages of vertebrate embryonic development, and it is considered to cover the period when the foundations for the vertebrate body plan are laid down (Slack et al. 1993). It is a time of rapid cellular and molecular events that set the stage for subsequent organ development and the overall morphogenesis of the organism. Therefore, we investigated changes in size and shape of the entire embryo during the tailbud phase. The tailbud phase is a highly conserved phase of ontogeny, influenced by pleiotropic constraints (Galis et al. 2018). This conservation suggests a lower level of variance compared to other developmental periods. However, the timing and duration of the tailbud phase can vary across species, and differences in variance levels within this phase are also possible (Richardson et al. 1997, 2009; Beck 2015). Since the balanced lethal system in *Triturus* newts affects both internal and external morphology during the tailbud phase (Horner and Macgregor 1985; Sessions et al. 1988; D'Amen et al. 2006), we hypothesize that it will increase size and shape variance at certain stages of developmental arrest, leading to fluctuating levels of variance throughout this phase.

The prehatching period in salamanders, such as those belonging to the genus *Triturus*, encompasses the final stages of embryonic development leading up to hatching. During this phase, the embryos undergo significant morphological and physiological changes in preparation for independent life outside the egg (Duellman and Trueb 1994). Hatching is similar to other amphibian taxa and can be considered as a developmental milestone (e.g., Strauss and Altig 1992; Duellman and Trueb 1994). Since the influence of the balanced lethal system ends before the prehatching phase and the embryos remain protected by the egg membranes, we hypothesize that this phase represents a canalized phase of development with a consistent level of morphological variance.

To assess shape variation during embryonic development of the Balkan crested newt, we first tested whether differences in embryo shape at the tailbud phase and head shape at the prehatching phase, quantified using landmark-based geometric morphometrics, correspond to the developmental stages previously defined for this species (Vučić et al. 2024). Additionally, we analyzed allometric changes in shape within each developmental stage and assessed their contribution to overall variance, as size-related changes in shape (allometry) are a significant component of morphological variance (Klingenberg 2016), particularly during periods of rapid growth and development.

2 | Materials and Methods

Triturus ivanbureschi adults used in experimental breeding to obtain embryos were collected from two populations in Serbia: Zli Dol (42°25′ N, 22°27′ E) and Brebevnica (43°02′ N, 22°53′ E). In total, 7 females from Zli Dol and 18 females from Brebevnica were used for breeding (for details, see Vučić et al. 2024). The collection of animals from natural populations was approved by the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia and the Ministry of Environmental Protection of the Republic of Serbia (permits no. 353-01-75/2014-08 and 353-01-1506/2022-04). The animals were transported to the laboratory of the Department of Evolutionary Biology at the Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade. The maintenance, breeding, and housing of these individuals were approved by the Ethics Committee of the Institute for Biological Research "Siniša Stanković" (decisions no. 03-03/16 and 01-1949). All experiments were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

Fertilized eggs and embryos were raised in the same controlled experimental conditions (for the experimental settings, see Vučić et al. 2024). Eggs were placed in plastic Petri dishes in groups of five. During embryonic development, temperature was kept constant at 19°C in a well-lit room with natural day/night dynamics. Water in Petri dishes was changed every other day. Embryos that stopped developing during any phase of development were discarded daily.

2.1 | Data Collection

The stage of the individual embryo was determined based on a staging table defined specifically for *T. ivanbureschi* (Vučić et al. 2024). Embryos were continuously examined and photographed at particular ontogenetic stages using a Nikon Digital Sight Fi2 camera attached to a Nikon SMZ800 stereo zoom microscope (Nikon Instruments Inc., NY, USA). In total, we randomly selected 20 embryos from a larger pool of individuals at each developmental stage independently (Table 1).

To quantify the size and shape of embryos, we divided the analyzed embryonic stages into two groups. The first group covered the early and middle part of the tailbud phase – stages 21–25 (5–8 days old). At these stages, embryos are positioned laterally (i.e., they lie sideways on the surface, see Vučić et al. 2024). The second group covered the prehatching larval phase – stages 30, 31 and 32 (12–14 days old). At these stages, embryos are elongated and folded inside the vitelline membrane and are positioned on their ventral side. During the late tailbud phase (from stage 26 to 29), embryos are not aligned with the substrate, either laterally (like at earlier stages) or ventrally (at later stages). To take a photo of the head of the embryo in these stages, we had to hold it with tweezers and lift it from the substrate, which increases measuring error and affects analyses of size and shape. Therefore, we excluded these stages from the analyses.

The shape of the embryo during the tailbud phase was described by 7 landmarks and 14 semi-landmarks (Figure 1A; Table S1). The shape of the dorsal side of the head during the prehatching phase was described by 11 landmarks and 16 semi-landmarks following Vučić and colleagues (2019) (Figure 1B; Table S1). Landmark coordinates were scored with the tpsDig program (Rohlf 2005), using the *Resample curve* function to obtain equidistant spacing of semi-landmarks that compose the curves.

All data preparation and analyses were done in R (R Core Team 2023). The package *xlsx* (Dragulescu and Arendt 2020) was used to load factor data (developmental stage, age, population) and *abind* (Plate and Heiberger 2016) was used for combining 3-dimensional arrays of coordinates.

As a preliminary analysis, measurement error induced by landmarking was calculated using two replicates for the total number of embryos with the *measurement.error* function in the *RRPP* package (Collyer and Adams 2018, 2021, 2024). For the tailbud phase, the analysis indicated significant shape variation among individuals (Rsq = 0.333, $p < 0.001$). Systematic measurement error was not statistically significant (Rsq < 0.001, $p = 0.438$) nor was the systematic measurement error × stage interaction (Rsq < 0.001, $p = 0.173$). There was no statistically significant measurement error within each of the analyzed tailbud stages (Table S2). For the prehatching phase, differences between individuals were highly significant (Rsq = 0.849,

TABLE 1 | Description of analyzed stages and sample size per stage.

Stage	Age	*N*	Description of embryonic stages (Vučić et al. 2024)
Tailbud phase			
21	5	20	Head formed, belly round, eye vesicles moving laterally, somites visible
22	6	20	Eye vesicles fully formed, belly flat, gill buds present
23	6–7	20	Head aligning with body, gill buds round, tailbud elongating
24	7–8	20	Head continues to straighten, gill buds triangular, tail grows over the blastopore
25	7–8	20	Head almost in line with the body, wide gill buds
Prehatching phase			
30	12–13	20	Secondary gill branches, start of eye pigmentation
31	12–13	20	33% of eye pigmented
32	13–14	20	66% of eye pigmented

Note: Stage - embryonic stage; Age - number of days since oviposition; N - number of embryos.

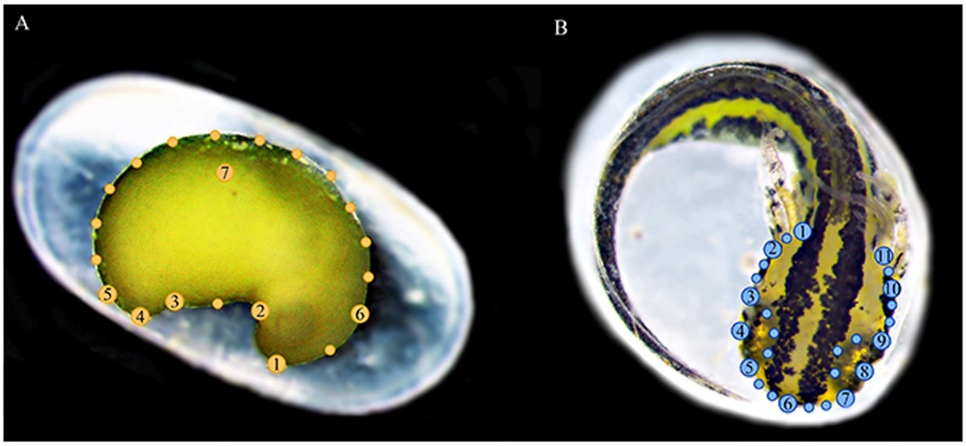

FIGURE 1 | Landmark configurations for the tailbud stages (A) and the prehatching stages (B). Landmarks are denoted with numbers, while semi-landmarks are unmarked circles.

$p < 0.001$). Systematic measurement error was not statistically significant (Rsq < 0.001, $p = 0.098$) and systematic measurement error × stage interaction was also not statistically significant (Rsq < 0.001, $p = 0.410$). There was no statistically significant measurement error within each of the analyzed prehatching stages (Table S2). As there were no differences between replicates, we decided to use the average coordinates from the first and second replicates as the data for subsequent analysis for both phases.

The variables for size and shape were obtained by the *geomorph* package (Adams et al. 2016; Baken et al. 2021) using the *gpagen* function. As a size variable, we calculated centroid size (CS) as the square root of the summed squared distances of the landmarks from the centroid (Tables S3 and S4). We obtained Procrustes coordinates as shape variables for tailbud stages. For the prehatching stages, because the head is a symmetric structure, we used the *bilat.symmetry()* function to obtain a symmetrical component. In both developmental series, semi-landmarks were slid using minimized bending energy between the target and reference individual in tangent directions.

For analyzing the tailbud stages, we used individuals from a single population (Brebevnica; Table S3), whereas prehatching stages (30 and 31) included two populations (Brebevnica and Zli Dol; Table S4). Pairwise permutation tests (1000 permutations) detected no significant population differences in mean head shape at any stage (Table S5).

2.2 | Data Analysis

Data analyses were done using the *geomorph* (Adams et al. 2016; Baken et al. 2021), *RRPP* (Collyer and Adams 2018, 2021), *car* (Fox and Weisberg 2018) and *Hdclassif* (Bergé et al. 2012) packages, while the *ggplot2* (Wickham 2016) package was used for plotting data. Due to differences in analyzed morphological structures and landmark configurations, all statistical analyses were performed separately for the tailbud and prehatching stages.

A common approach in studies of embryonic development is defining developmental stages based on one or more easily recognizable traits described in standardized staging tables. These traits are commonly qualitative in nature and they enable comparisons between developmental stages (Hopwood 2007; Love 2010; Richardson 2021). To quantify variance in size and shape, we first examined whether changes in embryo or head shape (defined by landmark configuration) correspond to the developmental stages defined by the staging table of the Balkan crested newt (Vučić et al. 2024), which were used to classify the stages of embryos we studied. In the other words, we assessed whether these stages are suitable candidates for studying morphological variation. We performed high-dimensional cluster analysis of Procrustes coordinates, pooled for the tailbud and prehatching series separately. We applied unsupervised pattern recognition analyses with the *hddc* function under model evaluation with the Bayesian information criterion. The number of clusters was not *a priori* defined – it was selected from 1 to 10 based on the Bayesian information criterion. This analysis clusters embryos into the optimal number of clusters solely by their shape. By comparing clusters obtained by pattern recognition and positions of embryos of particular stages within these clusters, we will have an estimate of how well we can infer embryonic stage based on shape data.

We used One-way Analysis of Variance (ANOVA) to test for differences in mean size (CS) between consecutive stages, and the TukeyHSD test using the *tukeyHSD* function as a post hoc test. We also calculated the variance of CS for each stage and compared the variance in a pairwise manner using the *leveneTest* function with Bonferroni correction for multiple comparisons.

Principal component analysis was performed using the *gm.prcomp* function to explore the pattern of variation within and between analyzed stages. We further analyzed the differences in shape among developmental stages by calculating Procrustes distances. To test whether differences were statistically significant, we used Procrustes pairwise permutation tests with the *pairwise* function and applied Bonferroni correction to adjust for multiple comparisons, ensuring more robust results.

To visually interpret the progression of shape changes across subsequent stages, we performed a multivariate regression of shape coordinates on developmental stages. This analysis

generated regression scores, which were then plotted against developmental stages to highlight trends in shape changes over time (Hallgrimsson et al. 2015). Additionally, to better understand the direction and magnitude of these changes, we calculated vectors representing shape transformations between the mean shapes of consecutive stages using the *plotRefToTarget* function.

To visualize variance within developmental stages, we calculated Procrustes distances of each individual from the mean shape of the corresponding stage, along with the mean value of Procrustes distances and standard error. The within-group variance in shape for each developmental stage and the statistical significance of pairwise differences between stages were calculated using the *morphol.disparity* function. The statistical significance of differences in shape variance was then adjusted for pairwise comparisons using Bonferroni correction.

We explored size-related shape changes (allometry) within stages. Using multivariate regression of shape variables on logCS with the *procD.lm* function and adjusted statistical significance using Bonferroni correction. To more closely inspect the effect of allometry on variance in shape and shape changes between stages, we calculated these parameters after correction for allometry.

3 | Results

3.1 | Unsupervised Cluster Analysis of Embryo Shape

The unsupervised cluster analysis found five shape clusters as an optimal number of clusters based on embryo shape for tailbud stages. 100% of embryos belonging to developmental stage 21 grouped together in cluster A, and 75% of embryos at stage 22 grouped in cluster B. In cluster C, there was a similar number of embryos at stages 23, 24, and 25. Cluster D contained 65% of embryos at stage 23% and 60% of embryos at stage 24. In Cluster E, 60% of embryos were at stage 25. Overall, the average percentage of embryos at the same stage that clustered together was 72% (Table 2 and S6). Clustering of the tailbud stages suggests that early tailbud stages could be strong candidates for comparative research, given that we were able to distinguish stage 21 based solely on shape data.

For the prehatching stages, the unsupervised cluster analyses found three clusters as an optimal number of clusters based on head shape. 85% of individuals at stage 32 were grouped together, while for embryos at stages 30 and 31 that proportion was much lower (55% and 45%). Overall, the average percentage of embryos at the same stage clustered together was 62% (Tables 2 and S6).

3.2 | Pattern of Change in Size

We mainly see a gradual increase in mean size (CS) as development unfolds (for both phases $p < 0.001$) except between the first two analyzed stages 21 and 22 ($p > 0.05$, Table S7; Figure 2). The variance of CS was not significantly different across stages (for both phases $p > 0.05$, Table 3).

TABLE 2 | Summary of posterior probabilities of each embryo belonging to the given cluster obtained by the unsupervised cluster analysis.

Tailbud stages						
Stage	**A**	**B**	**C**	**D**	**E**	**%**
21	20					100
22	2	15		3		75
23		3	3	13	1	65
24			5	12	3	60
25			5	3	12	60
					Average:	72

Prehatching stages				
Stage	**F**	**G**	**H**	**%**
30	11	6	3	55
31	9	5	6	45
32	2	1	17	85
			Average:	62

Note: Stage – embryonic stage assigned using the staging table. A–H: arbitrary names for clusters. Column "%" displays the percentage of embryos at the same embryonic stage clustered together.

3.3 | Patterns of Shape Change

For the tailbud stages, the first two principal components (PCs) accounted for 87.49% of the total variation in shape (Figure 3A). The stages were distributed in the horseshoe shape, with stages 21 and 22 separated from each other as well as from stages 24 and 25, which grouped together on the opposite side of PC1. Embryos belonging to stage 23 connected the aforementioned groups. The first two PCs accounted for 59.71% of the total shape variation for the prehatching stages (Figure 3B), with a large overlap between individuals at consecutive stages.

There is a statistically significant difference in mean embryo shape among tailbud stages except for stage 24, which did not differ from stages 23 and 25 (Table 4). During the tailbud phase, as development unfolds, head and tail gradually start to align with the central part of the body as the embryo is elongating (Figure 4A). The first analyzed stage (stage 21) is the last in the early tailbud phase, when the head and the tail are not yet fully defined. During the mid-tailbud phase (stages 22–24), the embryo has an overall arched shape. At stage 22, the head is well-defined, but the tail is only visible as a small bump in lateral view. In other mid-tailbud stages (23–25), both the head and the tail are clearly distinct in lateral view and pointed towards each other. Stages 23 and 25 differ from each other as the embryos gradually straighten out due to the growth of the entire body. At the last observed mid-tailbud stage (stage 25), the embryo is almost fully straightened. Mean head shape differed among prehatching stages, with stages 30 and 31 characterized by a more slender and elongated head relative to stage 32 (Table 4; Figure 4B).

Shape variance was higher at stage 23 than at other tailbud stages ($p < 0.01$), except for stage 25, which did not differ from the remaining stages ($p > 0.05$). No significant differences in shape variance were detected among prehatching stages (Table 3; Figure 5).

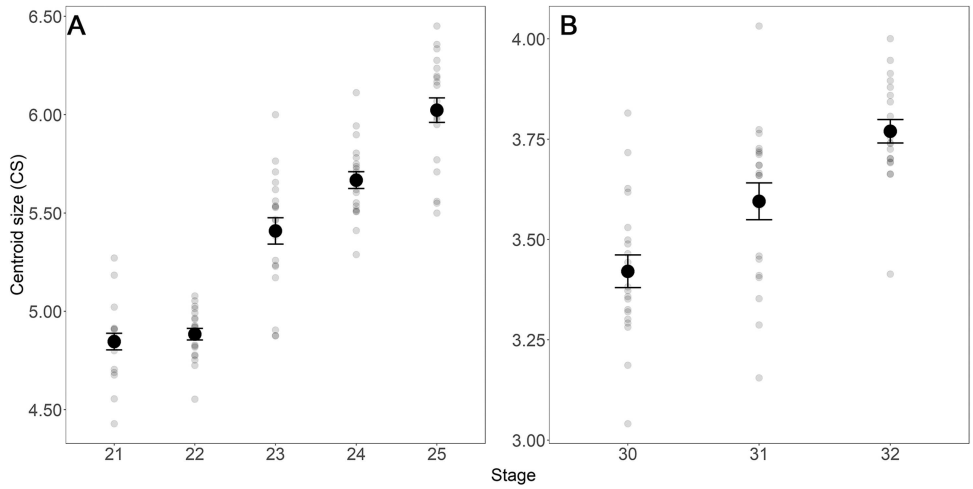

FIGURE 2 | Ontogenetic dynamics of embryo size (centroid size – CS) at the tailbud stages (A) and head size (CS) at the prehatching stages (B). Black dots represent the mean value, gray points represent individual observations, and whiskers display standard errors.

TABLE 3 | The stage-specific variance in size (centroid size – CS) and shape (Procrustes distance from the mean shape of the given stage).

Tailbud stages		
Stage	**Variance of CS**	**Variance of shape**
21	0.035	0.004
22	0.017	0.004
23	0.090	0.009
24	0.037	0.004
25	0.078	0.006
Prehatching stages		
Stage	**Variance of CS**	**Variance of shape**
30	0.033	0.001
31	0.042	0.001
32	0.017	0.001

3.4 | Allometry and Changes in Embryo Shape During Embryonic Development

Within stages, allometry was found to be statistically significant for embryo shape at tailbud stage 23 (allometry explained 44% of shape variance, $p = 0.001$), but not for other tailbud stages (Table S8). When we removed the effect of allometry and calculated the variance of shape for stage 23 again, it was comparable to the variance of shape of other tailbud stages (reduced from 0.009 to 0.005). However, this drop in variance was not statistically significant ($p = 0.064$). Allometry within stages was not present in head shape at the prehatching stages ($p > 0.05$, Table S8).

4 | Discussion

By examining the tailbud and prehatching phases of embryonic development in *Triturus* newts, we aimed to assess the dynamics of size and shape variance in relation to two hypotheses. First, we expected that variance would fluctuate during the tailbud phase due to the influence of the balanced lethal system, as it affects external morphology. The second hypothesis concerns the prehatching phase, where we expected a uniform level of variance, as only healthy embryos (not affected by the balanced lethal system) remain and, although ready to hatch, are still protected by the egg membranes.

The tailbud phase, a critical and highly constrained period of organogenesis in vertebrates (Galis et al. 2018; Richardson 2021), is also particularly vulnerable to developmental disruptions that result in higher mortality than during other developmental phases (Galis and Metz 2001). It is a period of rapid axis elongation, somite formation, and neural development (Gilbert 2003), which are processes that are considered highly sensitive to genetic imbalance (Pourquié 2003). Indeed, we found that shape variance fluctuates across tailbud stages in *T. ivanbureschi*, influenced by the balanced lethal system. The balanced lethal system leads to death of all *Triturus* embryos homozygous for chromosome 1 (which occurs in an A and a B form), but first slows down their development, affecting morphology (Horner and Macgregor 1985; Sessions et al. 1988; D'Amen et al. 2006). The effects of the balanced lethal system can be observed externally, especially in the head and the tail regions during the mid-tailbud phase (Horner and Macgregor 1985; Sessions et al. 1988), which was fully covered by our study (stages 22–25). Our results showed that although size variance remains consistent across stages, shape variance is higher at stage 23 compared to other tested tailbud stages (except stage 25), with significant allometry. The observed fluctuation in variance may reflect developmental instability induced by lethal genetic interactions, as the increased level of variance occurred at the beginning of the arresting period. Stage 23 of *T. ivanbureschi* (Vučić et al. 2024) corresponds to stage 25 described for *T. carnifex* (D'Amen et al. 2006), the stage where D'Amen and colleagues first observed differences in the developmental progression between heterozygous and homozygous embryos. They described an evident slowdown in developmental rate across stages 25–27 (which corresponds to stages 23 to 25 in *T. ivanbureschi*), unaffected by different pH or temperature, which represents the onset of embryonic arrest that

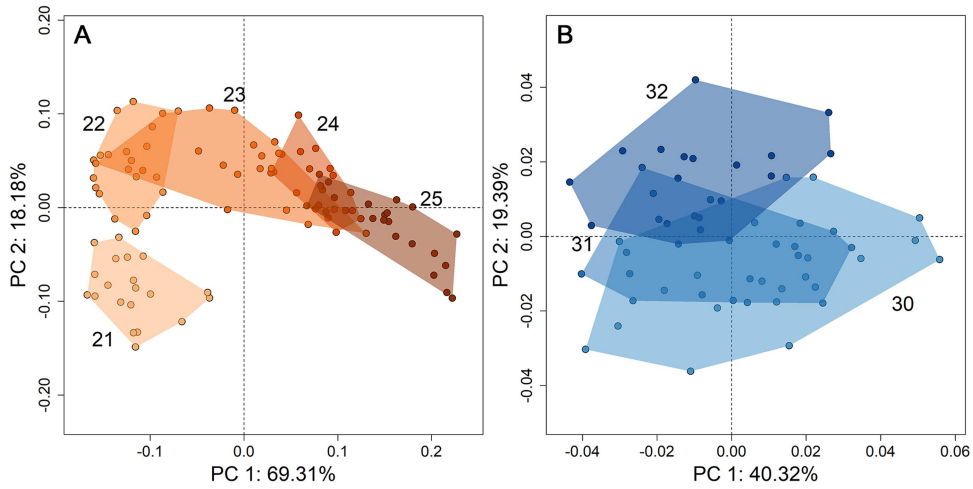

FIGURE 3 | Principal component analysis of Procrustes coordinates. The position of embryos at the tailbud stages (A) and the prehatching stages (B) in the morphospace defined by the first two principal component (PC) axes.

TABLE 4 | Procrustes distances (PD) and statistical significances of mean shape differences (p) between embryonic stages during the tailbud and prehatching larval phases.

Tailbud stages		
Pair	**PD**	***p***
21:22	0.1340	**0.001**
21:23	0.1788	**0.001**
21:24	0.2287	**0.001**
21:25	0.2860	**0.001**
22:23	0.1299	**0.004**
22:24	0.2086	**0.001**
22:25	0.2908	**0.001**
23:24	0.0869	0.028
23:25	0.1757	**0.001**
24:25	0.0926	0.018
Prehatching stages		
Pair	**PD**	***p***
30:31	0.0160	0.103
30:32	0.0319	**0.001**
31:32	0.0229	**0.004**

Note: Statistically significant results for the Bonferroni-adjusted statistical significance ($p < 0.005$ for the tailbud stages and $p < 0.017$ for the prehatching stages) are given in bold.

occurs between stages 26 and 30 (stages 24–28) (D'Amen et al. 2006). We also found that this period (covering stages 23–25 in our study) showed interesting results, not only for variance, but also because stage 24 does not differ in shape from stages 23 to 25, even though there are significant differences among other stages. Moreover, after correcting for allometry, shape variance at stage 23 becomes comparable to other stages, suggesting that the balanced lethal system likely affects growth.

To better understand how the balanced lethal system influences developmental variance, it would be valuable to genotype each embryo to determine whether it is heterozygous or homozygous for the specific region on chromosome 1 associated with arrested growth and embryonic lethality (de Visser et al. 2025; France et al. 2025) and to incorporate a wider range of developmental stages (from late neurulation through to the late tailbud phase). This approach could help identify whether certain morphological features appear early on that reliably predict developmental failure, offering insight into how lethal genetic combinations disrupt normal development. Due to morphogenetic processes, particularly in early development, identifying homologous landmarks, defined as the biological correspondence of structures across ontogeny (Hallgrimsson et al. 2015), requires the use of different methodological approaches and the production of 3D models of embryos (e.g., Metscher 2009; Hallgrimsson et al. 2015; Smith et al. 2015), as well as optimizing the protocol for genotyping (Meilink et al. 2025) after scanning (Green et al. 2017). As newt embryos are tiny and very fragile, this would be extremely time-consuming work.

In line with our second hypothesis, head shape variance showed limited change throughout the prehatching stages. Vučić et al. (2019) used the same landmark configuration to assess head shape variance during *Triturus* larval development and reported that variance in hatchlings was higher than in the middle and late larval stages, but comparable to that of metamorphosed individuals. Before hatching, embryos remain enclosed within their protective egg membranes, which may buffer them from external environmental fluctuations. Following hatching, larvae enter a free-living phase characterized by increased environmental heterogeneity and potentially stronger selective pressures, where variation in head and body shape may affect locomotor performance, feeding efficiency, and ultimately survival (Duellman and Trueb 1994; Vučić et al. 2019). However, when considered in relation to the pattern reported by Vučić et al. (2019), our results suggest that head shape variance during the prehatching period may actually exceed the levels observed immediately after hatching. This pattern is consistent with the view that early ontogenetic stages exhibit greater developmental flexibility and weaker functional constraints (Zelditch et al. 2003, 2012). Overall, these findings suggest that head shape variance follows a dynamic trajectory

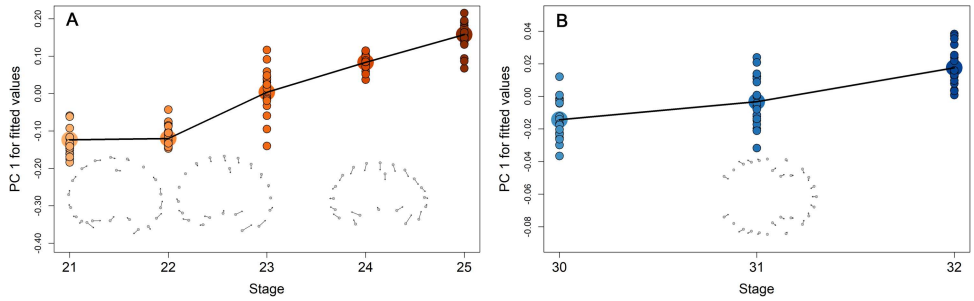

FIGURE 4 | Fitted values from the regression of shape on developmental stage for embryos at the tailbud stages (A) and the prehatching stages (B). For tailbud stages, illustrations represent vectors of shape change in mean shape in a pairwise manner between stages, with the last illustration representing shape change from stage 23 to stage 25. For prehatching stages, illustrations represent vectors of shape change in mean shape between stages 30 and 32, with three times magnification.

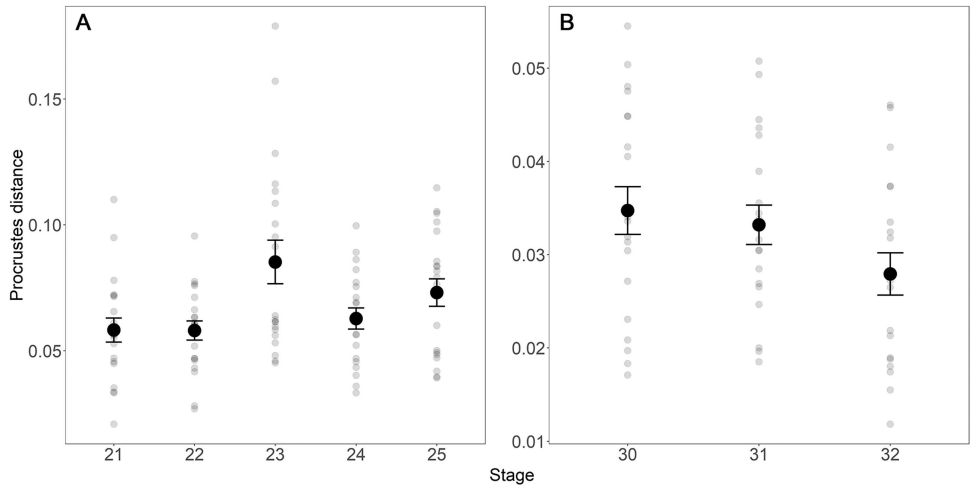

FIGURE 5 | The ontogenetic dynamics of Procrustes distance during the tailbud stages (A) and the prehatching stages (B). Black dots represent the mean value of Procrustes distance for a specific stage, gray points represent individual distances calculated from its stage mean shape, and whiskers display standard errors.

across key developmental transitions, at least under controlled laboratory conditions (Vučić et al. 2019; this study).

5 | Conclusions

The quantitative analyses of the dynamics of size and shape changes during the two studied embryonic phases of *T. ivanbureschi* development covered by our study provide a cornerstone for solving the puzzle of the "developmental landscape". Although the variance in size and shape observed across analyzed tailbud stages cannot be regarded as high or low without data from preceding and subsequent stages, our results underscore that the dynamics of size and shape variation during the tailbud phase are crucial to understand how the balanced lethal system in *Triturus* drives morphological differentiation between heterozygous and homozygous embryos. Furthermore, we propose that embryo shape can be used to compare embryonic development among *Triturus* species during the tailbud phase and may serve as a tool to test the conservativeness of the phylotypic period. Based on the stages during the prehatching phase and results from previous studies, variance in newts declines after hatching when environmental conditions are constant. However, future experiments that include various external conditions would give insights into whether the same trend of change in variance is present across natural populations.

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Data Availability Statement

The data supporting the findings of this study are provided in the Supporting Information. The complete code used for all analyses, together with all input files, is publicly available on GitHub at https://github.com/amph-evodevo-lab-ub/dynamics_of_size_and_shape_variation.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1002/jez.b.70041.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File S1: jezb70041-rev-1-editor-decision-letter.pdf.
Supporting File S2: jezb70041-rev-1-reviewer-1-report.txt.
Supporting File S3: jezb70041-rev-2-editor-1-recommendation.pdf.
Supporting File S4: jezb70041-rev-2-editor-decision-letter.pdf.
Supporting File S5: jezb70041-rev-2-reviewer-1-report.txt.
Supporting File S6: jezb70041-sup-0001-Supplementary_material.xlsx.