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RESEARCH ARTICLE

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A staging table of Balkan crested newt embryonic development to serve as a baseline in evolutionary developmental studies

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Abstract

There is an increased interest in the evolution and development of newts from the genus *Triturus* because: (1) morphological differentiation among the nine constituent species largely corresponds to different ecological preferences, (2) hybridization between different species pairs has various evolutionary outcomes in terms of life history traits and morphology, and (3) the genus expresses a balanced lethal system that causes arrested growth and death of half of the embryos. These features provide natural experimental settings for molecular, morphological, and life-history studies. Therefore, we produce a staging table for the Balkan crested newt (*T. ivanbureschi*). We provide detailed descriptions of 34 embryonic stages based on easily observable and interpretable external morphological characters, to ensure reproducibility. Compared with previous staging tables for *Triturus*, we include a vastly increased sample size and provide high-resolution photographs in lateral, ventral, and dorsal view, complemented by videos of specific developmental periods, and accompanied by detailed explanations on how to delineate the specific stages. Our staging table will serve as a baseline in comparative studies on *Triturus* newts: an emerging model system in evolutionary and developmental studies.

KEYWORDS
amphibia, external morphology, Salamandridae, *Triturus ivanbureschi*

1 | INTRODUCTION

A developmental staging table forms the foundation for research in the field of evolutionary and comparative biology (Muller & Newman, 2003; Wolpert et al., 2015). A staging table summarizes how an organism develops, by separating the continuous process of embryonic development into a certain number of stages, most often delineated based on the appearance of external morphological characteristics and pigmentation patterns (Duellman & Trueb, 1994; Gilbert, 2010; Richardson, 2021; Werneburg, 2009). By partitioning embryonic development into stages, it is possible to compare embryos of the same species at the same ontogenetic point, where they should share the same key traits, but may show intraspecific variation. Furthermore, the potential differences in development of homologous structures can be compared between species (Dünker et al., 2000; Richardson, 2021). Typically, staging tables are created

by comparing the ontogeny of one species to a related one for which a staging table already exists. As a consequence, the new staging table is presented as an ideal sequential series, while intraspecific variation is largely neglected (Werneburg et al., 2013).

Detailed staging tables have mostly been produced for widely used model organisms in embryological and evolutionary developmental (evo-devo) studies such as the nematode roundworm (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), the zebrafish (*Danio rerio*), and the mouse (*Mus musculus*) (Gilbert, 2010; Hopwood, 2007, 2011; Richardson, 2021; Stollewerk, 2016). Amphibians have a complex life cycle with larvae and adults generally inhabiting different environments (Moran, 1994), making them interesting models for evo-devo research. The most commonly used amphibian model organisms are *Xenopus* frogs (Keller, 1991; Nieuwkoop & Faber, 1975; Zahn et al., 2022). Yet, salamanders have been used in embryological studies from the 18th century (e.g., Harrison, 1918, 1925; Spallanzani, 1768; Spemann & Mangold, 1924). The advantage of salamanders for monitoring embryonic development is that eggs are protected with a transparent jelly layer that allows observation of external morphology during development, and are relatively large, containing relatively large embryos, which is conducive for experimental procedures. For the purpose of embryological work, the first widely adopted table of normal development was Harrison's classification system for *Ambystoma maculatum* (formerly *Amblystoma punctatum*), described at the beginning of the XX century and published later (Harrison, 1969).

Following Harrison's work, embryonic development in newts (family Salamandridae, subfamily Pleurodelinae) was first described by Glaesner (1925) for the smooth newt *Lissotriton vulgaris* (syn. *Triturus vulgaris*) and Knight (1938) for the alpine newt *Ichthyosaura alpestris* (syn. *Triturus alpestris*). The first staging tables for newts of the genus *Triturus* were produced in the 1980s (Horner & Macgregor, 1985; Sessions et al., 1988) and two more staging tables were produced this century (D'Amen et al., 2006; Lukanov & Tzankov, 2016). Although these staging tables were an important contribution to understanding embryonic development in *Triturus*, they are mostly focused on specific parts of embryonic development and ambiguous descriptions of the stages hamper reproducibility. For further evo-devo related studies in *Triturus* there is a need for a detailed staging table of embryonic development that can be readily interpreted by independent researchers.

Newts of the genus *Triturus* have proven themselves a suitable taxon for evolutionary studies on molecular (e.g., Arntzen et al., 2014; Wielstra et al., 2014; Wielstra, Burke, Butlin, et al., 2017; Wielstra, Burke, Butlin, Avcı, et al., 2017), morphological (Cvijanović et al., 2014; Ivanović & Arntzen, 2014; Vučić et al., 2019) and physiological level (Gvoždík, 2012; Petrović et al., 2023; Prokić et al., 2018), as well as for studies of life history traits (Bugarčić et al., 2022; Cogălniceanu et al., 2013; Cvijanović et al., 2009). Three aspects make embryonic development of *Triturus* particularly interesting from the perspective of evo-devo, namely the genus: (1) represents an eco-morphological radiation, (2) shows different outcomes of hybridization for different species pairs, and (3) expresses balanced lethal system.

The genus *Triturus* consists of three marbled (*T. marmoratus*, *T. pygmaeus*, and *T. rudolfi*) and seven crested newt species (Arntzen, 2024; Rancilhac et al., 2021; Wielstra & Arntzen, 2016; Wielstra et al., 2013, 2019) that can be divided into five distinct clades that differ in ecomorphology: (1) *T. marmoratus*, *T. pygmaeus*, and *T. rudolfi* (2) *T. ivanbureschi*, *T. anatolicus*, and *T. karelinii*; (3) *T. carnifex* and *T. macedonicus*; (4) *T. cristatus*; and (5) *T. dobrogicus*. These five clades differ in body shape (sturdiness) and ecology (in terms of the length of the aquatic period, that is, the number of months spent in water). Body shape and ecology are correlated: the five clades represent a morphocline from predominantly terrestrial species—*T. marmoratus*, *T. pygmaeus*, and *T. rudolfi* (shorter and stronger body; lower number of trunk vertebrae) to predominantly aquatic species—*T. dobrogicus* (elongated, slender body; higher number of trunk vertebrae) (Arntzen & Wallis, 1999; Arntzen, 2003; Slijepčević et al., 2015; Wielstra & Arntzen, 2011; Wielstra et al., 2019).

Triturus species are distributed across Europe and Western Asia (Wielstra & Arntzen, 2016; Wielstra et al., 2013, 2014) and in species contact zones hybridization occurs (Arntzen et al., 2014; Espregueira Themudo et al., 2012; Wielstra, Burke, Butlin, et al., 2017; Burke, Butlin, Avcı, et al., 2017). The outcome of interspecific hybridization is largely dependent on genetic divergence between taxa (Stelkens & Seehausen, 2009) and this also applies to *Triturus*. More closely related species produce morphologically similar and fertile offspring (Arntzen et al., 2014, 2018; Wielstra, Burke, Butlin, et al., 2017), while more distantly related species produce largely infertile and morphologically distinct F_1 hybrids (Arntzen & Hedlund, 1990; Arntzen & Wallis, 1991; Arntzen et al., 2009, 2021; Cogălniceanu et al., 2020). Hybridization is known to affect interspecific morphological variability (e.g., Arntzen et al., 2018; Slijepčević et al., 2015; Vučić et al., 2018) and hybrid larvae have been shown to already differ from both parental species at hatching (Vučić et al., 2019).

For over two centuries it has been known that only half of all *Triturus* eggs hatch (Rusconi, 1821). In the 1980s a balanced lethal system was discovered to be responsible. *Triturus* newts have two distinct versions of chromosome 1 and only heterozygotes are viable; homozygotes, 50% of the offspring given the rules of Mendelian inheritence, slow down their embryonic development and die during the mid-embryonic period at the tailbud phase (Grossen et al., 2012; Horner & Macgregor, 1985; Macgregor & Horner, 1980; Sessions et al., 1988; Wallace, 1987, 1994; Wielstra, 2020). When development comes to a halt, massive cell death occurs in most internal organs, leading to arrested development and, eventually, death (Sessions et al., 1988).

In this study, we document embryonic development of Balkan crested newt (*T. ivanbureschi*) based on almost 800 embryos to create a staging table. We provide precise descriptions of the key structures that characterize each stage, accompanied by high-resolution photos in lateral, ventral, and dorsal view and movies of specific developmental periods to visualize development. We also compare our staging table to all previous staging tables for *Triturus* and selected staging tables for other salamanders (Epperlein & Junginger, 1981; Harrison, 1969).

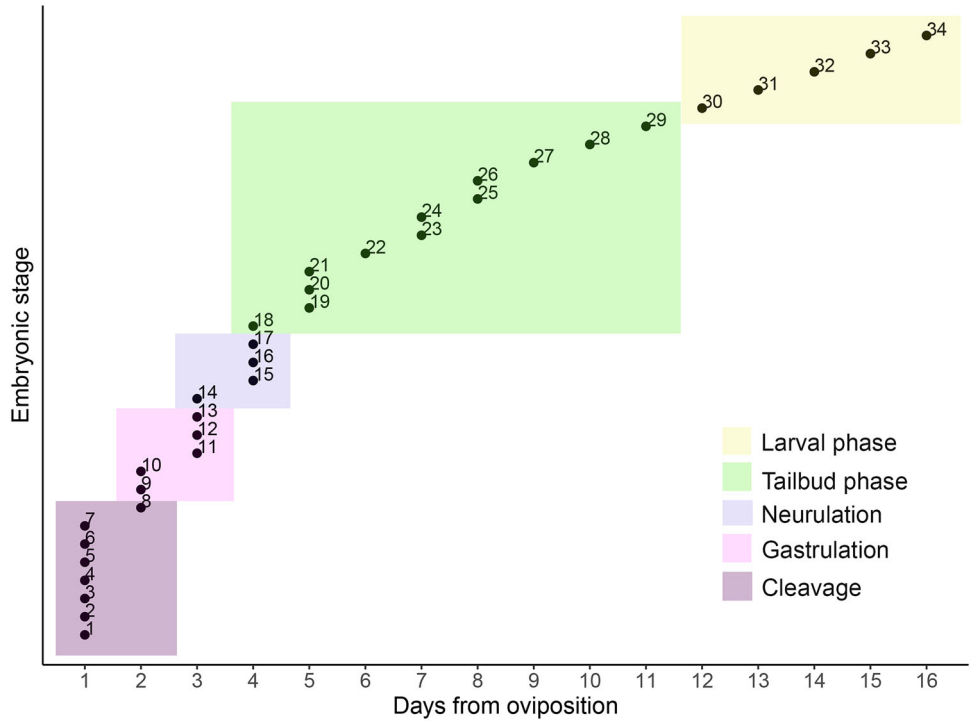

FIGURE 1 Approximate rate of *Triturus ivanbureschi* development (at 19°C) presented as days since oviposition for each embryonic stage. Day 1 marks the oviposition day. Colored rectangles mark different phases of embryonic development.

2 | RESULTS

We recognize and describe 34 embryonic stages of *T. ivanbureschi*, grouped into five main phases: cleavage (1–8), gastrulation (9–13), neurulation (14–17), tailbud phase (18–29), and prehatching larval phase (30–34). The 17 stages spanning the cleavage, gastrulation, and neurulation phases rapidly succeed each other during the first 4 days since oviposition. The tailbud phase is the longest (12 stages in 8 days), followed by the prehatching larval phase, where four stages proceed in 4 days (Figure 1). Thus, the time between stages is much shorter during the cleavage, gastrulation, and neurulation phases of embryonic development compared to the tailbud and prehatching larval phases.

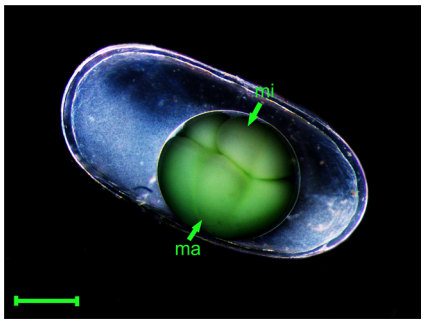

FIGURE 2 Diagnostic characters during *Triturus ivanbureschi* cleavage. Lateral view of an embryo at stage 4. Scale bar indicates 1 mm. ma, macromeres; mi, micromeres.

2.1 | Descriptions of embryonic stages

Cleavage (Stages 1–8, Days 1 and 2; Figures 2–4; Supporting Information: Video 1)

All stages of the cleavage phase are clearly observable (Supporting Information: Video 1). Cleavage is radially symmetrical and holoblastic. Macromeres (larger cells, filled with yolk) are formed at the vegetal pole, while micromeres (smaller cells) are formed at the animal pole (Gilbert, 2010).

Stage 1: Fertilized egg: Spherical egg protected with vitelline membrane and several jelly layers.

Stage 2: Two cells: The first cleavage furrow appeared on the animal pole and spread towards the vegetal pole. Two equally sized cells are visible.

Stage 3: Four cells: A second cleavage furrow appeared near the animal pole meridionally at an angle of 90° related to the first one, while the first is not yet complete on the vegetal pole.

Stage 4: Eight cells: A third, equatorial, cleavage furrow appeared closer to the animal pole, and divided the four cells from the previous stage into eight cells—four macromeres and four micromeres.

Stage 5: Early morula: There is a difference in size and number of cells between the two poles. The micromeres are smaller and more

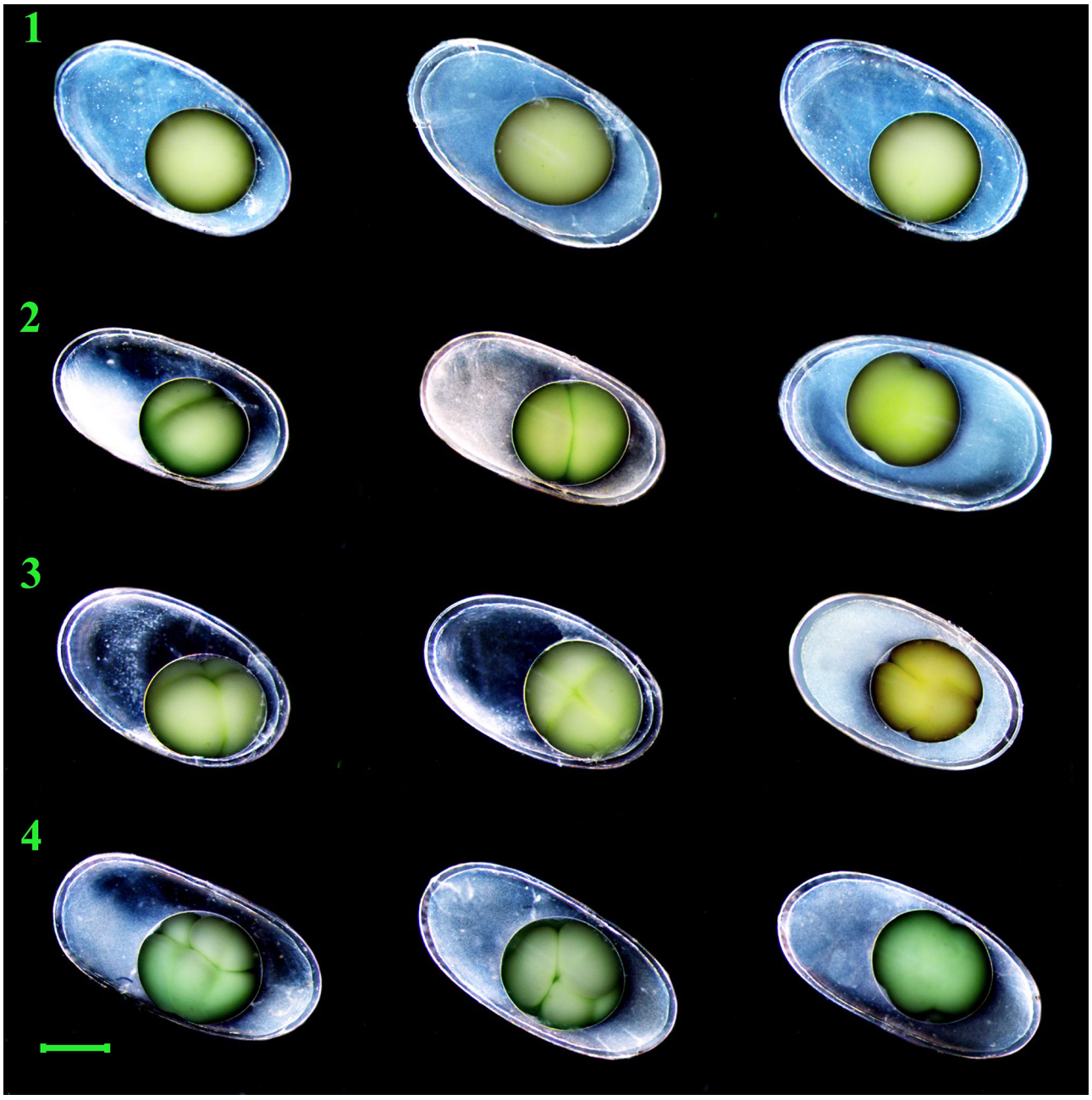

FIGURE 3 First part of cleavage during *Triturus ivanbureschi* development (the day of oviposition). Rows represent embryonic stages 1–4 and columns present a lateral view, animal and vegetal pole. Scale bar indicates 1 mm.

numerous than the macromeres. The cells can be counted and the number of cells is more than eight but less than 32.

Stage 6: Late morula: The difference in the size of the cells between the poles is prominent. There are numerous cells and it is difficult to count them.

Stage 7: Early blastula: The difference between the poles in cell size is not apparent, as cells on both poles are small. However, they can still be observed as small bumps.

Stage 8: Late blastula: Cells are so small that they can no longer be seen individually (at least using a stereo zoom microscope with 63X magnification). There is a slight difference in transparency between cell layers on the different poles due to blastocoel formation.

Gastrulation (Stages 9–13, Days 2 and 3; Figures 5 and 6; Supporting Information: Videos 1 and 2)

Gastrulation is initiated on the future dorsal side of the embryo, beneath the equator in the marginal zone, where the animal and vegetal hemispheres meet. During the initiation of gastrulation the cells change in shape ("bottle cells") and move over the dorsal blastopore lip (Gilbert, 2010; Supporting Information: Video 2). To observe all stages of blastopore formation, embryos need to be

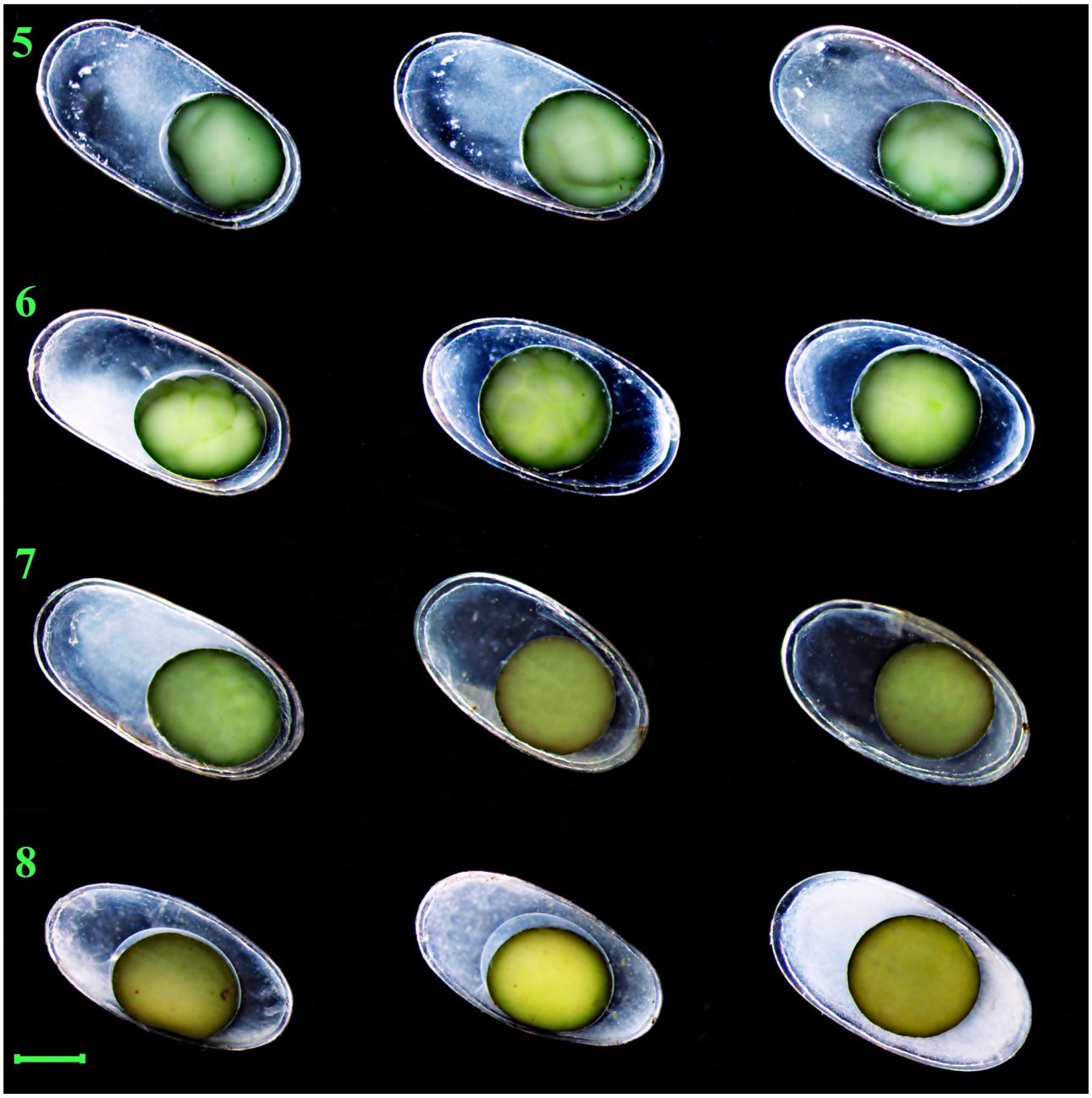

FIGURE 4 Second part of cleavage during *Triturus ivanbureschi* development (Days 1 and 2). Rows represent embryonic stages 5–8 and columns present a lateral view, animal and vegetal pole. Scale bar indicates 1 mm.

rotated (Supporting Information: Video 2). On the animal side of the embryo there are more transparent cell layers (Supporting Information: Videos 1 and 2).

Stage 9: Initial gastrula: The dorsal blastopore lip is formed as an invagination visible in the form of a small notch. The surrounding cells are elongated "bottle cells."

Stage 10: Early gastrula: The blastopore has a semicircular ("crescent") shape as the blastopore lip expanded laterally and formed lateral lips.

Stage 11: Middle gastrula: The lateral blastopore lips expanded ventrally and formed a ventral lip. The blastopore is fully formed in a ring shape with a clearly exposed yolk plug in the middle on the vegetal surface.

Stage 12: Middle gastrula: The blastopore lip extends all the way around the yolk plug, but does not expand over the yolk plug, which is round and concave.

Stage 13: Late gastrula: The blastopore is almost closed and expands over the yolk plug, which is small. The embryo is slightly elongated in the anterior-posterior direction, that is, has a more oval than round shape.

Neurulation (Stages 14–17, Days 3 and 4; Figures 7 and 8; Supporting Information: Video 1)

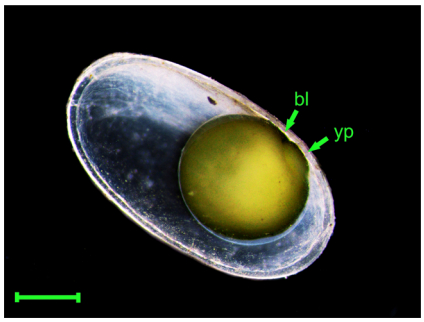

FIGURE 5 Diagnostic characters during *Triturus ivanbureschi* gastrulation. Lateral view of an embryo at stage 11. Scale bar indicates 1 mm. bl, blastopore; yp, yolk plug.

The formation of neural folds is clearly visible on the dorsal side of the embryo (Supporting Information: Video 1).

Stage 14: Beginning of neurulation: A neural plate with neural furrow is present in the form of a slight depression along the dorsomedial line of the embryo in an anterior-posterior direction.

Stage 15: Neurula: Two neural folds at the margins of the neural plate are formed and visible.

Stage 16: Neurula: The neural folds rise over the neural plate, which has the shape of a keyhole.

Stage 17: Neurula: The neural folds are close to the medial line in the trunk region, but they are still distant at the level of the emerging head and tail.

Tailbud phase (Stages 18–29, Days 4–10; Figures 9–13; Supporting Information: Video 1)

Embryos lay on their lateral side (Supporting Information: Video 1) and need to be rotated to see some of the main characters. Head and trunk regions can now be discerned. The blastopore remains visible at the base of the tailbud on the ventral side.

Stage 18: Beginning of the tailbud phase: The neural folds are touching, except in the head region, but not yet completely fused along the midline of the embryo. The tailbud is visible in lateral view.

Stage 19: Beginning of the tailbud phase: Neural folds are closed and they form a neural tube. The head is small, flattened, and "pinched." The eye vesicles are present, but not well-defined. The trunk is round.

Stage 20: Tailbud phase: The head and the rounded trunk are distinct. The eye vesicles are large and clearly visible. The tailbud does not exceed the trunk line when the embryo is in the lateral position.

Stage 21: Tailbud phase: The head is prominent, while the eye vesicles are positioned laterally. The trunk is bulging or slightly flattened and the tailbud slightly exceeds the trunk line. Somites are visible.

Stage 22: Tailbud phase: Head and trunk regions are well-defined. The head is bent ventrally so that it is not visible when the embryo is viewed dorsally. The eye vesicles are clearly defined. Gill buds are present. The abdomen is flattened. Somites are distinct. The tail surpasses the trunk line and curves toward the head region.

Stage 23: Tailbud phase: The head is straightened in relation to the trunk. The space between the head and trunk regions is slightly enlarged. Round gill buds can be seen in dorsal and ventral view. The width of embryo at the level of gill buds is smaller than the width at the level of the rounded trunk. The tail slightly overgrows the blastopore.

Stage 24: Tailbud stage: The head is still slightly bent. Dorsally and ventrally, gill buds have a triangular shape. The trunk is now more elongated and narrower. The tail extends over the blastopore. Melanophores are scattered dorsally.

Stage 25: Tailbud stage: The head is almost in the same plane as the trunk, but slightly bent. The gill buds are wider than the trunk. Balancer buds are not present yet. Melanophores are present dorsally, but not clearly organized in bands.

Stage 26: Tailbud stage: The head is in line with the trunk. Balancer buds are visible as swellings between the eyes and the gill buds. The tail is distinct from the trunk region and folds inside the capsule. Trunk pigmentation is apparent in the form of three bands—one dorsal and two laterals. The dorsal band is divided into two smaller ones in the head region. Muscular twitches can be observed, that is, the embryo begins to move.

Stage 27: Tailbud stage: The gill buds have three bumps. Balancer buds are present in the form of small nodes. Front limb buds are visible as small swellings behind the gill buds. Pigmentation is more pronounced, with more clearly defined lateral bands.

Stage 28: Tailbud stage: The gill buds are divided into three gill branches which are positioned at an angle of 90° to the trunk. Balancers are short and cylindrical. The forelimb buds are short, triangular and rounded at the top. The dorsal fin is visible. Blood circulation and heartbeats can be seen on the ventral side.

Stage 29: Tailbud stage: The three branches of the gills are elongated and positioned parallel to the trunk. Balancers are narrower at the base and wider at the end. A transparent cornea is visible in the eyes.

Prehatching larval phase (Stages 30–34, Days 11–15; Figures 14–15; Supporting Information: Video 1)

Embryos mostly lay on their lateral side, and frequently change their position (Supporting Information: Video 1). The stages during this phase can be discerned by the level of eye pigmentation.

Stage 30: The beginning of the prehatching larval period: The secondary gill branches can be observed as small nodes on one or more primary gill branches. Pigmentation in the eyes is present.

Stage 31: Prehatching larva: ⅓ of the eye is pigmented. The primary gills have clearly visible elongated secondary branches of different sizes.

Stage 32: Prehatching larva: ⅔ of the eye is pigmented.

Stage 33: Prehatching larva: The pigmentation of the eye is complete and the pupil is visible. The gills are well-developed and the balancers are long.

Stage 34: Prehatching larva: The condensations of digits I and II are visible as bifurcations at the distal end of the limb buds, indicating the formation of the first two digits. The individual is ready to hatch.

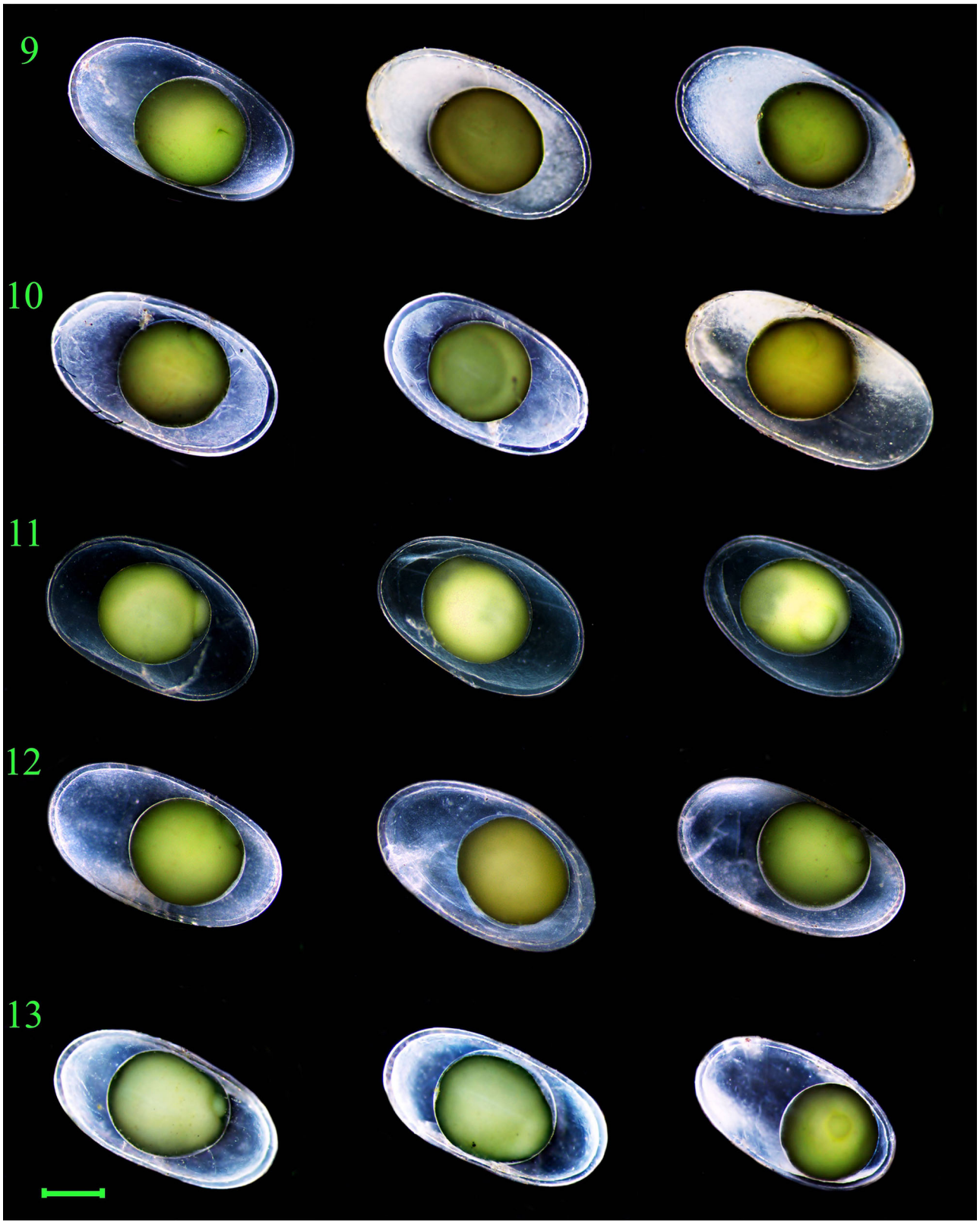

FIGURE 6 Gastrulation during *Triturus ivanbureschi* development (Days 2 and 3). Rows represent embryonic stages 9–13 and columns present a lateral view, animal and vegetal pole. Scale bar indicates 1 mm.

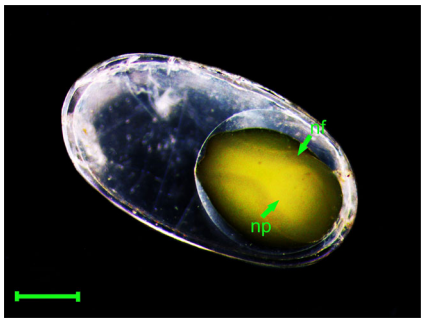

FIGURE 7 Diagnostic characters during *Triturus ivanbureschi* neurulation. Dorsal side of an embryo at stage 16. Scale bar indicates 1 mm. np, neural plate; nf, neural fold.

2.2 | Intraspecific comparisons

Out of 797 embryos followed, 135 were from the population Zli Dol and 662 from the population Brebevnica. We did not observe any differences in external morphological characters that determine embryonic stage or developmental rate between the two populations. We also did not notice any differences between the three breeding seasons (in 2021, 2022, and 2023 we observed 21, 334, and 442 embryos, respectively).

2.3 | Comparisons with published staging tables

The number of females and embryos that we included in this study is higher than in previous staging tables for *Triturus* (Table 1). Compared to previous *Triturus* staging tables, our new staging table for *T. ivanbureschi* has a different number of stages (Table 1). Stages during cleavage and gastrulation are similar. The differences concern the neurulation phase and, particularly, the tailbud phase.

3 | DISCUSSION

Studies on traditional amphibian model organisms, such as *Xenopus* (e.g., Keller, 1991; Nieuwkoop & Faber, 1975; Zahn et al., 2022) and *Ambystoma* (e.g., Adamson et al., 2022; Harrison, 1969), are crucial to deepen our knowledge of the molecular and morphological process of development. However, the need for developmental studies on nonmodel organisms is well recognized and is fundamental to understanding large-scale evolutionary events (Werneburg et al., 2013). Recently, an increased interest in developmental biology of the salamander family Plethodontidae (lungless salamanders) has led to the production of taxon-specific staging tables (Hurney et al., 2015; Kerney, 2011). These staging tables were then used to explore the developmental basis of lung loss (Lewis et al., 2022) and its effects on the circulatory system (Lewis & Hanken, 2017)—one of the major questions in salamander evolution.

To facilitate evolutionary developmental studies in *Triturus* newts, we provide a detailed and objective embryonic developmental table for *T. ivanbureschi*, based on a vast sample size. We document the development of 797 embryos, obtained from 25 different females from two populations in three breeding years, up to hatching. We present and explain 34 embryonic stages, based on external morphological characters commonly used to describe salamander embryonic development (e.g., Epperlein & Junginger, 1981; Glaesner, 1925; Harrison, 1969), which can be easily observed and recognized by independent observers. Werneburg (2009) developed a standard event system for vertebrate embryology to minimize diverging and subjective approaches to delimit stages. However, for newts, many of these specific marks would be hard to observe by stereomicroscope and/or would require histological analysis of embryos—which is not possible without sacrificing the embryo. For example, one of the most commonly used characters for describing stages in vertebrate staging tables is the number of somites. In *Triturus* newts, somites can be observed externally during early and mid tailbud phase, but not during the late tailbud and prelarval phases. Therefore, it is challenging to correctly estimate their exact number throughout embryonic development based only on external morphology. Our new staging table can be applied in laboratory experiments or field surveys during the entire period of embryonic development, without damaging the embryo.

It is crucial that a staging table is unambiguously interpretable. We encountered obstacles in determining certain stages when trying to apply previous salamander staging tables. For some stages, especially during the gastrulation and tailbud phases, the orientation of embryos and the availability of photographs are crucial to be able to recognize specific markers. The main challenge during gastrulation is to observe the formation of the blastopore lip and the yolk plug, because this process happens on the subequatorial, marginal zone where the animal and vegetal hemispheres meet (Gilbert, 2010) facing away from the observer's eye. During the tailbud phase, structures like balancers and gill buds appear, but are hard to observe from the lateral side, in which embryos are naturally positioned. To remedy this situation, we provide high-resolution photos of the lateral, ventral, and dorsal view for each stage, with additional figures to explain the main characters relevant to stage determination. The tailbud phase is the most difficult embryonic period in terms of delineating stages, because of the high variability in external morphological characters, especially in the head and tail regions, as a consequence of developmental arrest of half the embryos due to the balanced lethal system in *Triturus*. In previous staging tables, the development of healthy and diseased embryos was not properly disentangled (D'Amen et al., 2006; Horner & Macgregor, 1985; Sessions et al., 1988).

We extended the published staging table for *T. ivanbureschi* (Lukanov & Tzankov, 2016) by covering the entirety of embryonic development in a considerably larger number of stages. We identified fewer stages compared to previously published staging tables for other *Triturus* species (D'Amen et al., 2006; Horner & Macgregor, 1985; Sessions et al., 1988). This difference mainly

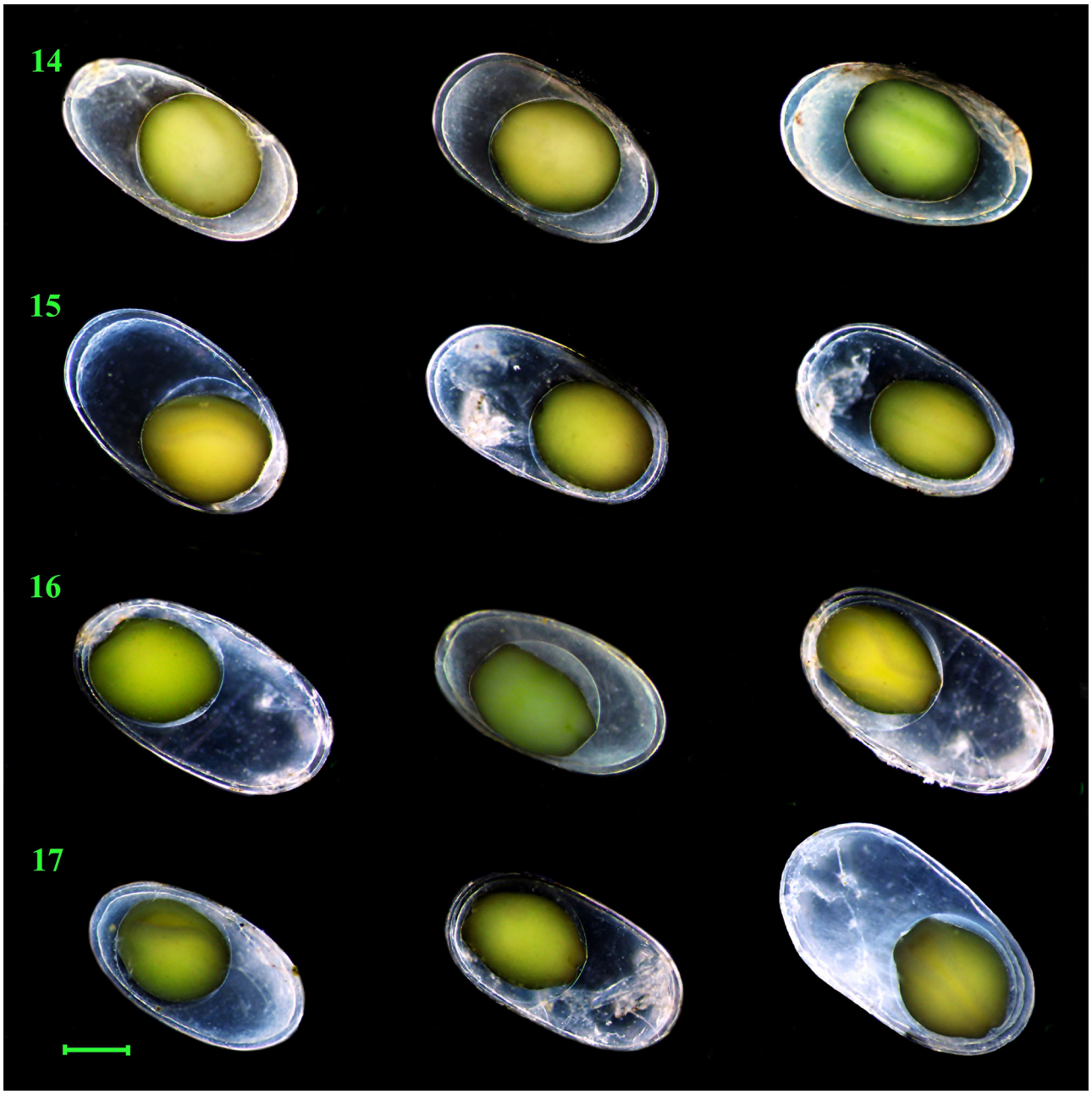

FIGURE 8 Neurulation during *Triturus ivanbureschi* development (Days 3 and 4). Rows represent embryonic stages 14–17 and columns present a lateral, ventral, and dorsal view. Scale bar indicates 1 mm.

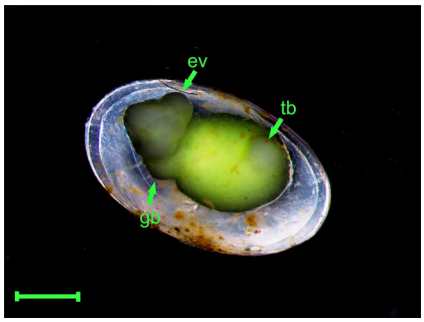

FIGURE 9 Diagnostic characters during early and middle parts of *Triturus ivanbureschi* tailbud phase. Ventral side of an embryo at stage 23. Scale bar indicates 1 mm. ev, eye vesicle; gb, gill bud; tb, tailbud.

reflects our effort to define stages in an objective and reproducible manner. Horner and Macgregor (1985) based their stages on a staging table for *Xenopus* (Nieuwkoop & Faber, 1975), with brief descriptions of 10 stages and drawings of 16 stages out of 40 proposed. Although the tailbud phase is well described and we could discern all stages Horner and Macgregor proposed, the cleavage, gastrulation, and neurulation are underrepresented and the pre-hatching larval phase is completely missing in their work. Sessions et al. (1988) compared Harrison's developmental stages for *Ambystoma* (Harrison, 1969) to three *Triturus* species (*T. marmoratus*, *T. carnifex*, and *T. cristatus*) up to the end of tailbud phase, but they did not document stages with drawings or photographs. Furthermore, these authors combined some of Harrison's stages without a clear

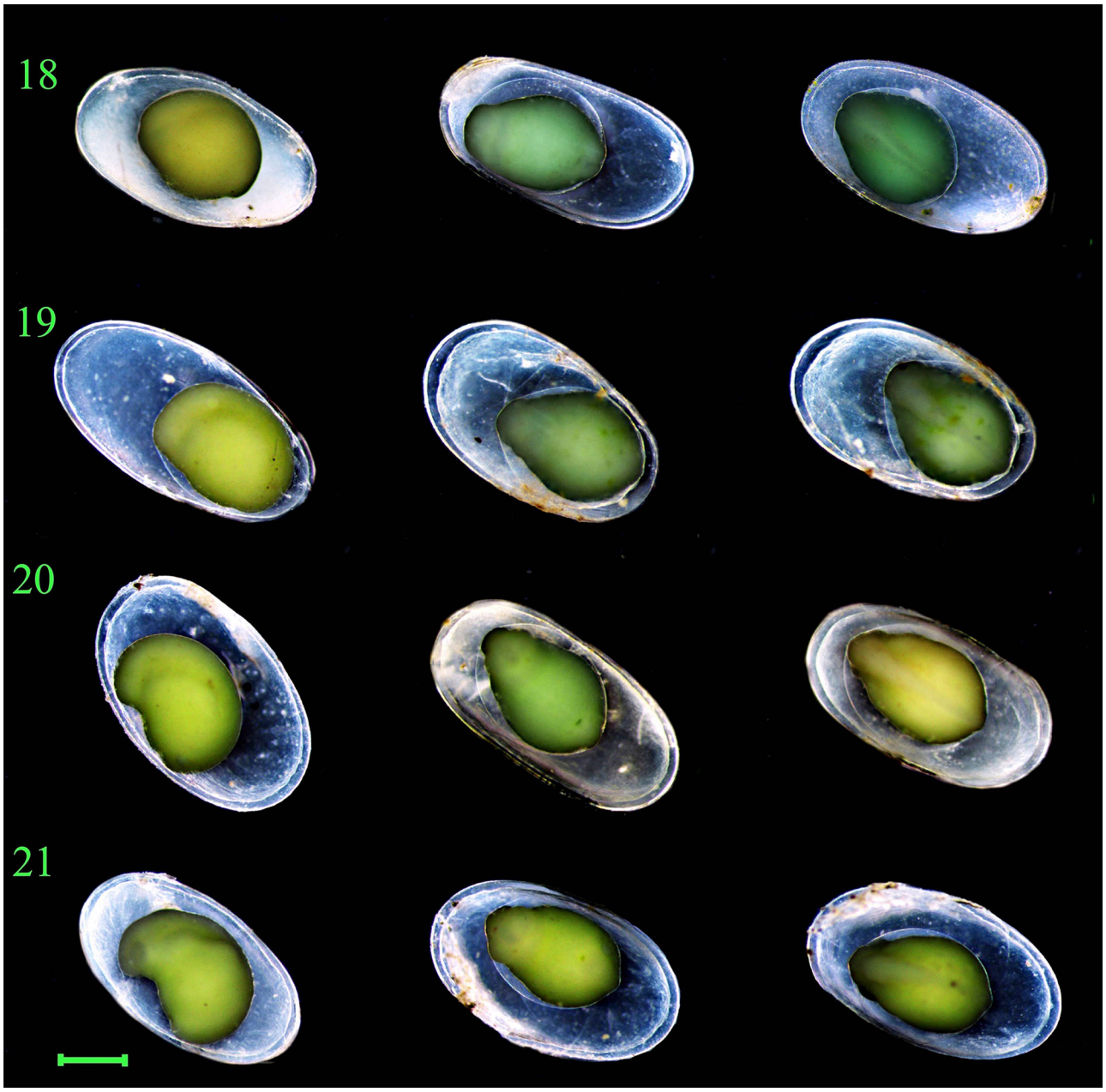

FIGURE 10 Early tailbud phase during *Triturus ivanbureschi* development (Days 4 and 5). Rows represent embryonic stages 18–21 and columns present a lateral, ventral, and dorsal view. Scale bar indicates 1 mm.

description, complicating the precise delineation of embryonic stages. Compared to the relatively comprehensive staging table by D'Amen et al. (2006), we provide a more detailed description of a reduced number but more ambiguously discernable stages during the gastrulation phase and we provide photographs in all three plains, with the relevant characters clearly visible, complemented by (time-lapse) movies of specific developmental periods.

Staging tables of the more distantly related *Ichthyosaura alpestris* (Epperlein & Junginger, 1981) and *Ambystoma maculatum* (Harrison, 1969) are as complete as our new *T. ivanbureschi* staging table and also represented by precise photographs or drawings. The main differences compared to our new *T. ivanbureschi* staging table most likely reflect actual differences during embryonic development between taxa as we could not clearly recognize some of the stages described in the *I. alpestris* staging table by Epperlein and Junginger (1981). For example, we did not recognize stage 16 (Epperlein & Junginger, 1981) during neurulation of *I. alpestris*, as the neural folds are always above the neural plate in *T. ivanbureschi* and approach at a more similar pace in the trunk and head regions. During the tailbud phase, the head of *I. alpestris* is more bent and almost leans on the trunk. This different position of the head in *I. alpestris* enables observation of some structures, such as the number of somites at stage 20 (Epperlein & Junginger, 1981), which cannot be easily observed in *T. ivanbureschi*.

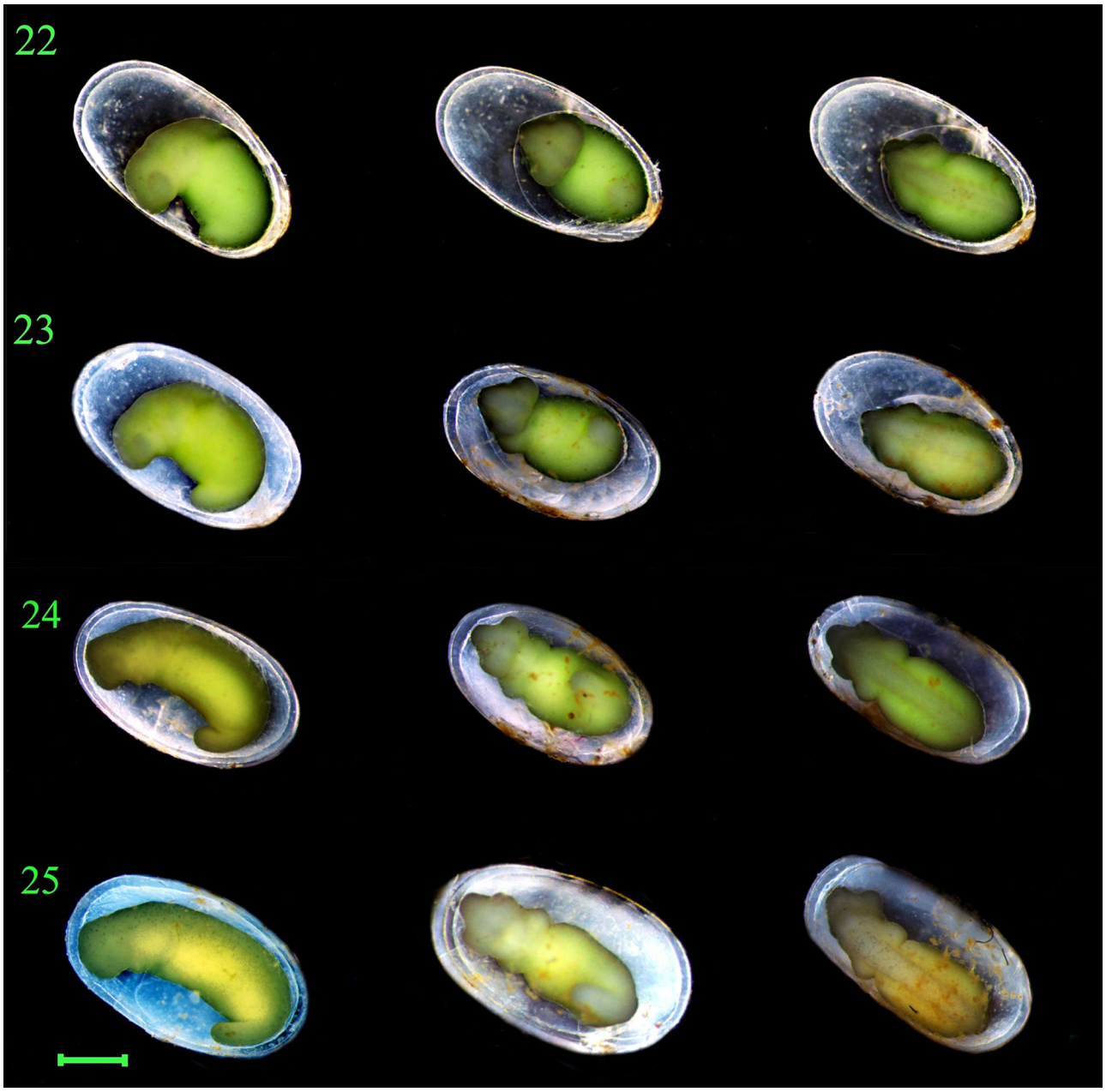

FIGURE 11 Middle part of tailbud phase during *Triturus ivanbureschi* development (Days 6–8). Rows represent embryonic stages 22–25 and columns present a lateral, ventral, and dorsal view. Scale bar indicates 1 mm.

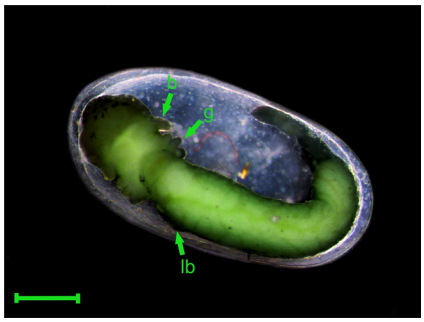

FIGURE 12 Diagnostic characters during *Triturus ivanbureschi* late tailbud phase. Ventral side of an embryo at stage 28. Scale bar indicates 1 mm. b, balancer; g, gills; lb, front limb bud.

There are two potential limitations to our study: (1) we lack a precise hourly rate of development and (2) we did not quantify potential intraspecific differences. The hourly rate of the development can be found in some traditional staging tables (e.g., Epperlein & Junginger, 1981; Sessions et al., 1988). As we tracked a large number of eggs, we provide an approximate timeline during embryonic development to facilitate precise determination of stages. Intraspecific variability among *T. ivanbureschi* populations in egg size (Lukanov & Tzankov, 2016) could affect embryonic development, but we only studied two Serbian populations, whereas the species range encompasses the southeastern Balkans and Western Turkey (Wielstra et al., 2014). Although the Serbian samples did not show discrepancies, either between populations or

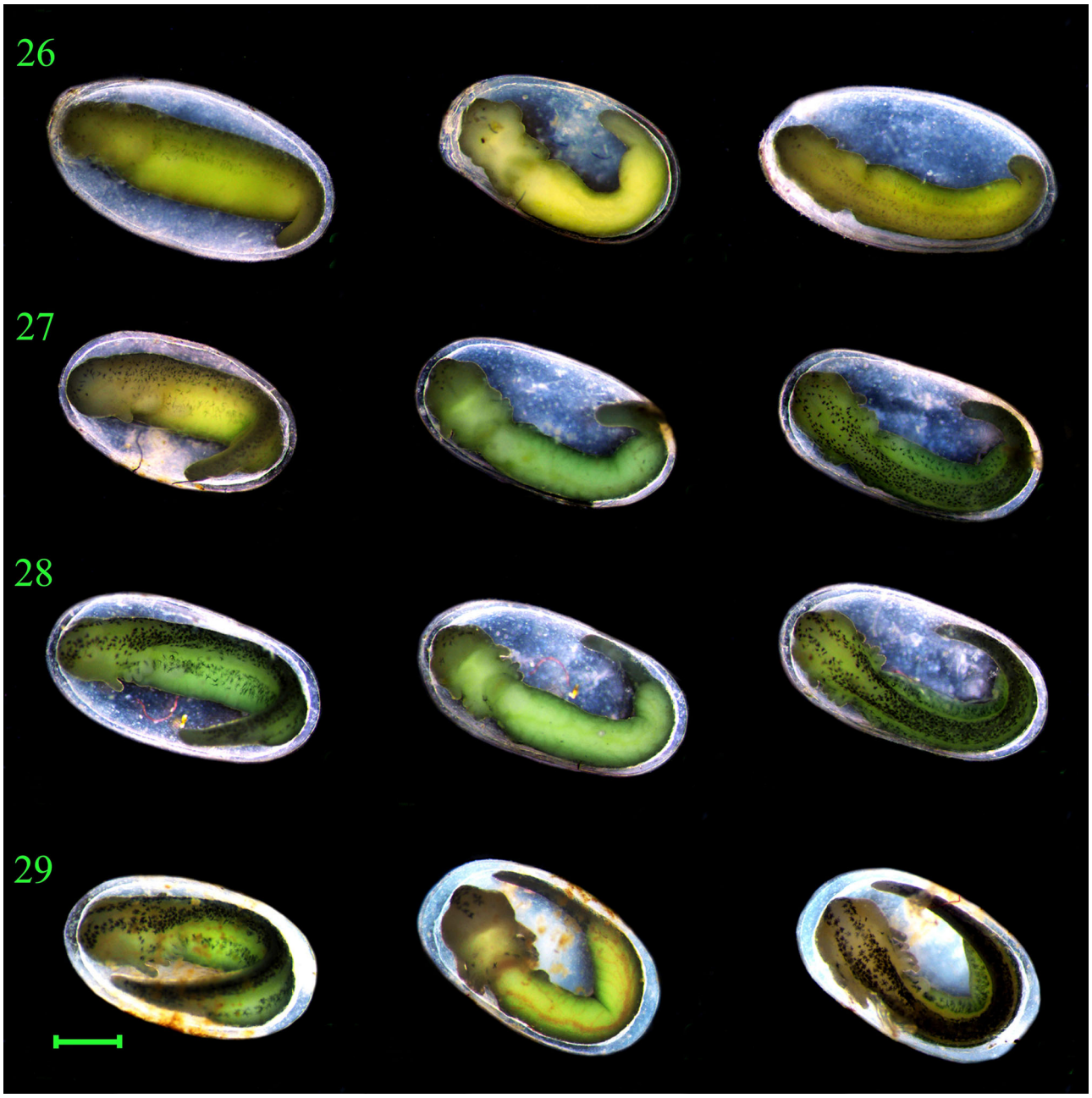

FIGURE 13 Late tailbud phase during *Triturus ivanbureschi* development (Days 8–11). Rows represent embryonic stages 26–29 and columns present a lateral, ventral, and dorsal view. Scale bar indicates 1 mm.

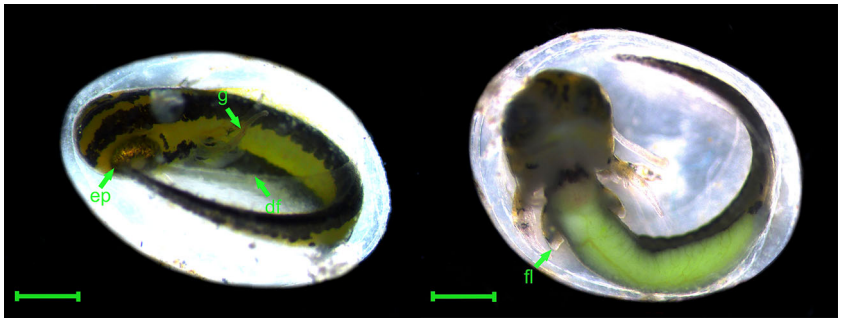

FIGURE 14 Diagnostic characters during *Triturus ivanbureschi* prehatching larval phase. Lateral view of an embryo at stage 32 (left) and ventral side of an embryo at stage 34 (right). Scale bar indicates 1 mm. ep, eye pigmentation; g, gills; df, dorsal fin; fl, front limb.

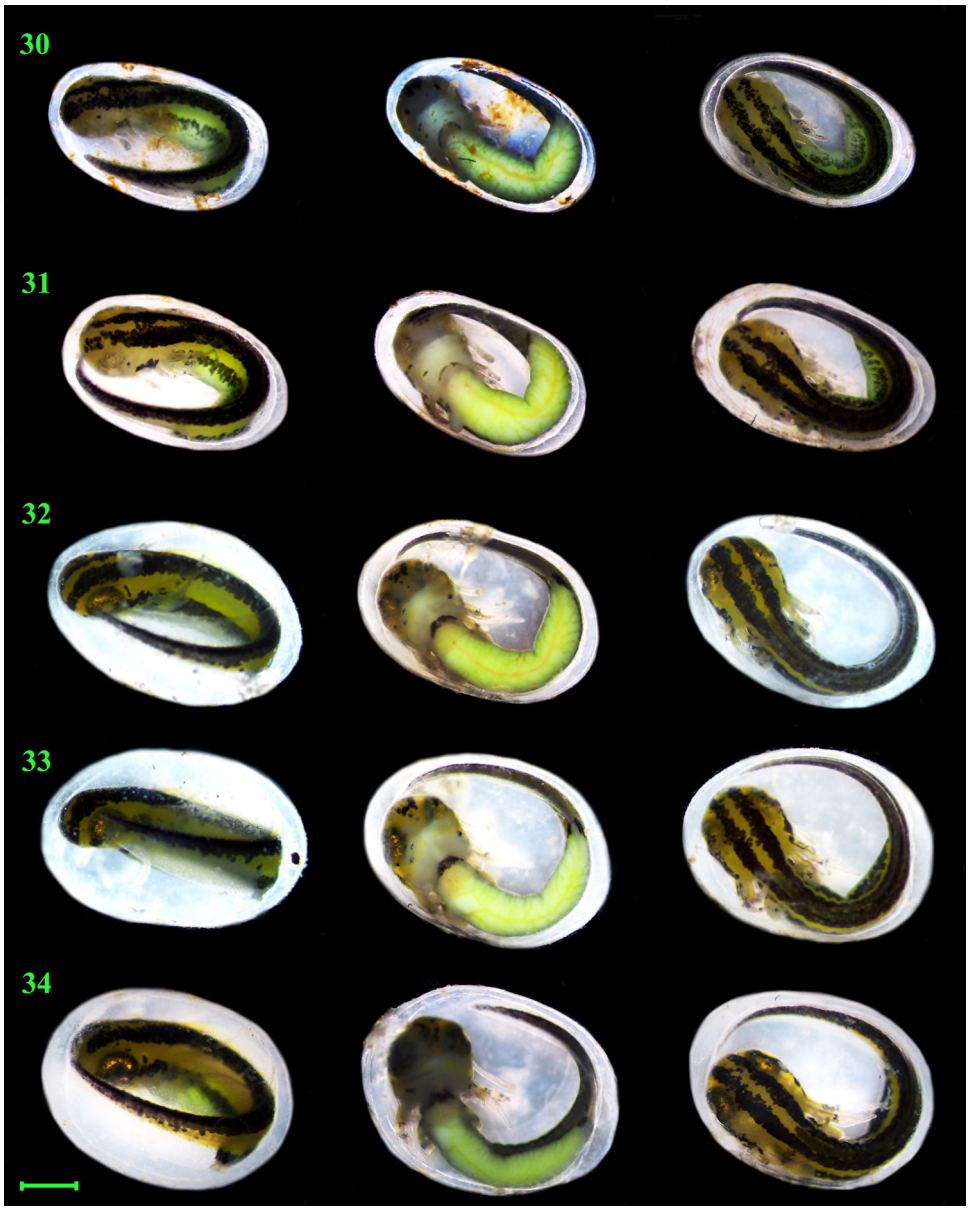

FIGURE 15 Prehatching larval phase of *Triturus ivanbureschi* development (days 12–15). Rows represent embryonic stages 30–34 and columns present a lateral, ventral, and dorsal view. Scale bar indicates 1 mm.

across seasons, future studies could include populations from distant parts of the range.

4 | CONCLUSION

Triturus newts are an emerging model system for evolutionary, developmental, ecological, morphological, and physiological studies. Our new staging table for *T. ivanbureschi* embryonic development is a valuable resource for future comparative evo-devo studies, including studies on the eco-morphological radiation, interspecific hybridization, and balanced lethal system expressed in *Triturus*.

5 | MATERIALS AND METHODS

To create the developmental staging table of *Triturus ivanbureschi*, we monitored embryos from two populations for three consecutive breeding seasons (2021–2023). We used individuals from two populations: Zli Dol, Serbia (permit no. 353-01-75/2014-08 of the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia) and Brebevnica, Serbia (permit no. 353-01-1506/2022-04 of the Ministry of Environmental Protection of the Republic of Serbia). The experimental procedures were approved by the Ethics Committee of the Institute for Biological Research "Siniša Stanković," University of Belgrade (decisions no. 03-03/16 and 01-1949). Experiments were performed in accordance with the Directive 2010/63/EU.

TABLE 1 Comparison of embryonic stages in the new *Triturus ivanbureschi* staging table with previously published staging tables for *Triturus* and *Ichthyosaura* newts, and *Ambystoma* salamanders.

T. ivanbureschi A Nf = 25 Ne/Nem = 1108/797	*T. ivanbureschi* B Nf = na Ne/Nem = na/215	*T. carnifex* C Nf=1 Ne/Nem = 311/178	*T. carnifex* D Nf = na Ne/Nem = na	*T. marmoratus*, *T. carnifex*, *T. cristatus* E Nf = na Ne/Nem Tm = 49/24; Ne/Nem Tca = 32/17; Ne/Nem Tcr = 49/24	*I. alpestris* F	*A. maculatum* G
1	1	1	1	1	1	1
2	-	-	2	2	2	2
3	-	-	3	3	3	3
4	-	-	4	4	4	4
5	-	-	5	5	5	5
6	-	-	6	6/7	6	6
7	-	8	7/8/8+	8	7/8	7/8
8	-	-	-	9	8+	9
9	-	-	9	10	9/10	10
10	-	10 1/2	10	11	10+/11	-
11	-	-	11/12	-	11	-
12	-	-	12	12	12	11
13	-	-	13/13+	13	13/13+	12
14	-	14	14	14	-	13
15	-	-	14+	15	14/14+	-
16	-	16	15	16	15	16
17	2	18	16/17/18	17/18	16/17	17
18	-	-	19/20	19	18/19	19
19	-	-	20	20	20/21/22	20
20	-	-	21	21-24	23	21/22
21	3	26	22	25	-	23/24
22	-	27	23/24	26/27/28	23/24	25/26
23	4	28	25	26/27/28	24	27/28
24	5	29	26	29/30	25	29/30/31
25	6	30	27	31/32/33	26	32/33
26	7	31	28	34/35	28	34
27	9	32	29	34/35	29	35
28	-	33	30/31/32	36	30	36
29	-	34	33/34	37	31	37
30	10	-	34/35	38/39	32	38
31	-	-	36	-	33	39
32	11	-	37	-	-	-
33	-	-	38	-	34	40
34	12	-	39	-	35	41

Note: A – this study; B – Lukanov and Tzankov (2016); C – Horner and Macgregor (1985); D – D'Amen et al. (2006); E – Sessions et al. (1988); F – Epperlein and Junginger (1981); G – Harrison (1969); na, not available; Nf: number of females; Ne, total number of eggs; Nem, number of normally developing embryos; Tm, *T. marmoratus*; Tca, *T. carnifex*, Tcr, *T. cristatus*.

Adult animals from Zli Dol were collected from a natural population in 2014 and brought to the Institute for Biological Research "Siniša Stanković" in Belgrade, Serbia, to establish a newt colony. In each breeding year, after hibernation at a constant temperature under refrigeration, females ($N = 7$) and males ($N = 7$) were transferred to outdoor tanks to mate, from the middle of February to the beginning of March, depending on weather conditions. Gravid females from Brebevnica were collected in April in 2022 ($N = 9$) and 2023 ($N = 9$), and transferred to the outdoor tanks in the courtyard of the Institute. Females from different populations were kept in separate tanks for mating and egg-laying. After the egg-laying period, females from Brebevnica were transferred back to the same pond where they were collected in both years. *Triturus ivanbureschi* females lay an average of 169 eggs during the breeding season (Vucic et al., 2020) and then wrap them inside leaves of underwater vegetation (Miaud, 1994). The color of the egg vitellus varies among females, ranging from yellowish to a slightly fluorescent green. Tanks (200-400 L) contained plastic strips as a substitute for underwater vegetation on which the females could lay their eggs, perforated bricks in which animals could hide and floating plastic platforms on which animals could rest. Tanks were protected with mosquito nets which allowed for a natural day/night regime. All animals were fed twice a week with *Lumbricus* sp.

Eggs were collected daily at 10 a.m. (24 h interval). The duration of embryonic development before the larva hatches is approximately 15 days (Lukanov & Tzankov, 2016), but various factors can affect the speed of development and temperature is one of the factors that has the greatest influence—higher temperatures lead to faster development (Brown, 1975). Therefore, after they were laid, we transferred eggs to the laboratory, where the temperature was kept constant at 19°C. Maximum of five embryos were placed in each Petri dish filled with dechlorinated tap water that was changed every second day.

To create a table of normal embryonic development, we only included embryos that successfully progressed to the hatching stage. Embryos that underwent developmental arrest (roughly halfway embryonic development, see introduction) were removed. This left us with 797 embryos for which development was followed and photographed with a Nikon DS-Fi2 camera attached to a Nikon SMZ800 stereomicroscope. Embryos were photographed on the lateral, ventral, and dorsal side at 20X magnification.

To visualize all phases of development as time-lapse movies, photographs of a subset of embryos were taken automatically at 20 min intervals over a 7–12 h period. For this purpose, photographs were taken at 10X magnification up to the prehatching larval phase, and at 5X magnification for the prehatching larval phase. For gastrulation, we additionally recorded a real-time movie of a single embryo at 25x magnification, to show how the embryo needs to be positioned to observe formation of the dorsal blastopore lip. Because time-lapse movies were recorded at room temperature (22–23°C), the development of these embryos is slightly faster than development of the embryos at a constant temperature of 19°C.

To depict the dynamics of embryonic development at a constant temperature of 19°C, we calculated the average day from egg deposition, where the first day marks the day of oviposition. During cleavage, we used the number of blastomeres to determine stages and during gastrulation, we used the shape of blastopore. To describe neurulation, we observed neural plate and neural folds. During organogenesis, we used body shape and the presence and shape of structures like gills, tailbud, balancers, limbs, and eyes. Finally, we cross-compared our results for the two *T. ivanbureschi* populations and the three breeding seasons and compared our new staging table with selected published staging tables listed above.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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