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PAGES: 192-207

ORIGINAL PDF URL: <https://dergipark.org.tr/tr/download/article-file/3362933>

EMBRYONIC DEVELOPMENT OF THE LEMON-YELLOW TREE FROG, *Hyla savignyi* AUDOUIN, 1827

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ABSTRACT. Amphibians are widely used in temperature adaptation studies due to their compatibility in laboratory experiments. We investigated the embryonic development stages (from fertilization to 25th) of *Hyla savignyi* following Gosner's generalized table. Three pairs of *H. savignyi* were collected during the breeding season (February 2015) from Northern Cyprus, Kalkanlı Region and maintained at 21±1 °C in the laboratory. The samples were set in 3 groups and examinations of embryos and photographs taken every 10 minutes were carried out during the 9-days embryonic period. Embryos hatched at stage 20 or 21 come up to 3rd – 4th days after fertilization. Embryonic development of *H. savignyi* is about 157 hours (7 days). Cleavage is unequal holoblastic. The embryonic developmental stages of *H. savignyi* were compared with the result of a similar study of two other *Hyla* species (*H. orientalis* and *H. annectans*) at various temperatures, and the possible temporal effect of the temperature and ovum size on the growth rate of these species was discussed.

1. INTRODUCTION

Amphibians have been considered model organisms for developmental studies for a long time. Although the South African clawed frog, *Xenopus laevis* (Daudin, 1802) is currently the most popular amphibian model, others had already been spotlighted for the issue [1, 2, 3]. *X. laevis* was raised through its use in pregnancy testing, and it was established by Nieuwkoop and Fischberg as a model for development [2]. Among the frogs, several *Rana* species have been used in developmental studies [4, 5, 6, 7].

Different salamander species were used in classic embryological studies [8, 9, 10, 11, 12, 13, 14, 15, 16]. While the Spanish ribbed newt, *Pleurodeles waltl* Michahelles, 1830 was popular in French labs, the lowland newt, *Cynops pyrrhogaster* (Boie, 1826) was in Japanese laboratories. The Mexican axolotl, *Ambystoma mexicanum* (Shaw and Nodder, 1798)

Keywords. Fertilization, cleavage, gastrula, tadpole, growth rate, ovum size, gosner stages, hylidae

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became the most popular one because it could be easily bred and kept in captive conditions and laboratory colonies [17, 18].

There are fundamental differences in development within different groups of amphibians. Fertilization is monospermic in most anurans, frogs, as in mammals, but fertilization is polyspermous in most urodeles; newts and salamanders [19, 20]. Primordial germ cells form a cytoplasmic localization in anurans via germ plasm, but by induction in urodeles [21, 22]. The shape of the body changes completely and abruptly at anurans metamorphosis, but the body form undergoes minimal and gradual changes in urodeles. Also, urodeles possess remarkable regeneration abilities, not found in anurans [23].

In 1960 Gosner proposed a table that is now used as a standard for development in anurans. The table contains 46 stages but just the first 25 stages are embryonic or prefeeding and the other 21 are larval stages and investigates metamorphosis in the frog as stages [24]. However, the sequence of changes in the early embryo from fertilization to cleavage, the blastula and the gastrula is essentially similar in most species.

Published data of the development of hylid species referred only to *Hyla regilla* Cocroft, 1994, *Hyla avivoca* Viosca, 1928 and *Hyla japonica* Günther, 1859 [25, 26, 27, 28]. Recently, the developmental stages of *Hyla orientalis* Bedriaga, 1890 and *Hyla annectans* Jerdon, 1870 have been described in detail [29, 30]. However not much is known about the embryonic development of *H. savignyi* Audouin, 1827.

This is the first study on the embryonic development of *H. savignyi* that provides a staging temporal table and a complement to previous studies in the literature. This laboratory work also reveals the timing and characterization of similar species' external development to understand the embryonic process's importance and define the hylid life history.

2. MATERIALS AND METHODS

The fertilized eggs of *H. savignyi* were obtained from two mating pairs that were collected during the breeding season (09 Feb. 2015) from Northern Cyprus (Kalkanlı Region, 35° 15' 59.48" N, 33° 02' 34.22" E). These pairs were separately transferred to the laboratory and placed in a glass container (26 × 38 × 16 cm) with de-chlorinated tap water. Spawns were observed three to five hours later. The fertilized eggs obtained by this procedure were reared at 21 ± 1°C, with natural ambient lighting, in de-chlorinated and gently aerated tap water. In order to obtain a sequence of developmental stages, groups of 10 embryos were removed at any one

time, and the rest were left in the clutch for further development. During the development of the embryos, a thermoregulation device was used to stabilize the ambient temperature. The experiment was set in 3 groups based on the observation of 30 embryos from two mating pairs in different sets of spawning eggs. Live embryos were reared in petri dishes in their jelly coatings were observed and photographed using a stereomicroscope (Olympus SZ61) attached to a digital camera (Camedia C-5060) every 10 minutes to describe the stages of species' embryonic development according to Gosner (1960). Sampled pairs were released back to nature.

3. RESULTS

The embryonic development monitoring study started on 10 February 2015 and lasted about 18 days. In 30 zygotes embryonic stages were studied and the mean time (age in an hour) for each stage was obtained. Embryos hatched at stages 20 - 21 about 3rd – 4th days after fertilization. The embryonic development process of *H. savignyi* from fertilization to the 25th stage lasted about 157 hours (7 days) at 21±1 °C (Table 1).

This study presents temporal data and the morphologic characteristics of embryonic development prefeeding stages (from fertilization to 25th) of *H. savignyi*. Like other frogs their egg is telolecithal with a large amount of yolk. Fertilization is monospermic and they undergo unequal holoblastic cleavage. Embryonic developmental stages have been divided into four major categories: (1) Fertilization, two stages (2) Cleavage, seven stages (3) Gastrula, three stages (4) Tadpole, seven stages. Developmental features include the tail bud, indicated initially by a strong upward arching of the back. Embryo hatching occurs at stage 19, when the cornea is just beginning to become transparent. However, it is not until the end of stage 20 and the beginning of stage 21 that the cornea becomes fully clear. At stage 20, circulation begins in the caudal fin, but the caudal fin is not transparent as in the Gosner stages and remains dark.

Embryonic Developmental Stages Categories

Fertilization stages

Stage 1. The spherical egg has differentiation including an animal and a vegetal pole (Figure 1A).

Stage 2. The gray crescent, seen as a pigmented area, is visible between the animal and vegetal poles (Figure 1B).

Cleavage stages

Stage 3. Meridional furrow, advancing from animal pole to vegetal pole, divides the egg in equal halves (Figure 1C).

Stage 4. The second meridian furrow divides the egg into four cells from the animal pole to the vegetal pole at a right angle to the first (Figure 1D).

Stage 5. The third division, consisting of eight unequal cells, is at latitude slightly above the equator (Figure 1E).

Stage 6. Eight cells divide vertically to form sixteen cells (Figure 1F).

Stage 7. Sixteen cells divide latitudinally to form thirty-two cells (Figure 1G).

Stage 8. The number of cells exceeded 64 (Figure 1H).

Stage 9. The animal polar surface has a granular appearance (Figure 2A).

Gastrulation stages

Stage 10. The dorsal lip of the crescent-shaped blastopore is formed on the animal polar surface (Figure 2B).

Stage 11. It reduces the exposed area of non-pigmented macromeres surrounded by the lateral lips of the circular blastopore with epibolism of micromeres at the vegetal pole (Figure 2C and D).

Stage 12. The embryo is slightly elongated and the dorsal surface of the embryo flattens to form the *neural plate* (Figure 2E).

Stage 13. The posterior part of the embryo enlarges. The neural plate forms a wider neural groove in the cerebral region (Figure 2F).

Rotation. Sperm entry affects the rotation of the cell's cortex relative to the inner cell mass. This rotation moves the determinants on the vegetal side of the egg towards the animal-vegetal boundary and the gray crescent appears (Figure 2G).

Stage 14. The neural folds are completely fused to form the neural tube (Figure 2H).

Stage 15. The tail is wider than the length of the bud and extends dorsoposteriorly (Figure 3A).

Stage 16. The muscular response is produced by unilateral flexion of the head, which is well defined by optic bulges and prominent protrusions of

the gill plates. As the embryo elongates, the tail begins to curl to the right or left (Figure 3B).

Stage 17. The heartbeat is seen below and behind the gill bud. A pair of external gill buds emerged from each gill plate. Dorsal and ventral fins are translucent. The stomodeal pit is triangular in shape (Figure 3C).

Stage 18. In branched gills, circulation is seen as the movement of bodies through the external gill filaments (Figure 3D).

Tadpole stages

Stage 19. The olfactory pit becomes prominent. The cornea of the optic lobes begins to become transparent. The stomodeum, which has no feeding activity yet, becomes triangular to form a simple mouth. The anterior end of the head bulges the vitelline membrane. At this point the membrane ruptures, the larva comes out and settles at the bottom (Figure 3E).

Stage 20. The caudal fin circulation begins at the base of the anterior part of the fin and moves slowly through the vessel (Figure 4A).

Stage 21. The operculum has a small fold of skin and covers the base of the external gills (Figure 4B).

Stage 22. The opercular fold covering the external gills on the right is fused with the skin of the abdomen on the right. The upper and lower lips around the mouth become prominent and keratinized (Figure 4C).

Stage 23. The operculum closes and the gills disappear. Spiracle occurs. The feeding of the tadpole begins (Figure 4D).

Stage 24. Hind limbs formed and straightened mediolaterally to form a foot paddle.

Stage 25. Formation of all toes in the posterior parts.

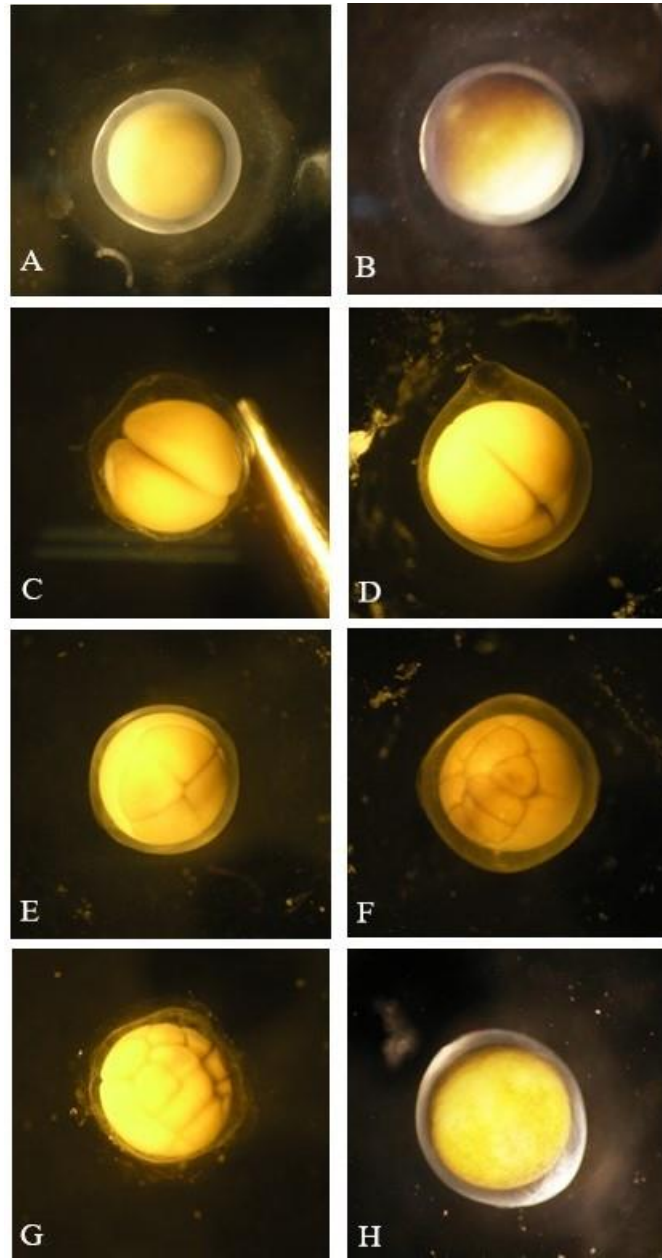


FIGURE 1. **A.** Fertilization, **B.** Gray crescent, **C.** Two cell stage, **D.** Four cell stage, **E.** Eight cell stage, **F.** Sixteen cell stage, **G.** Thirty-two cell stage, **H.** Mid-cleavage.

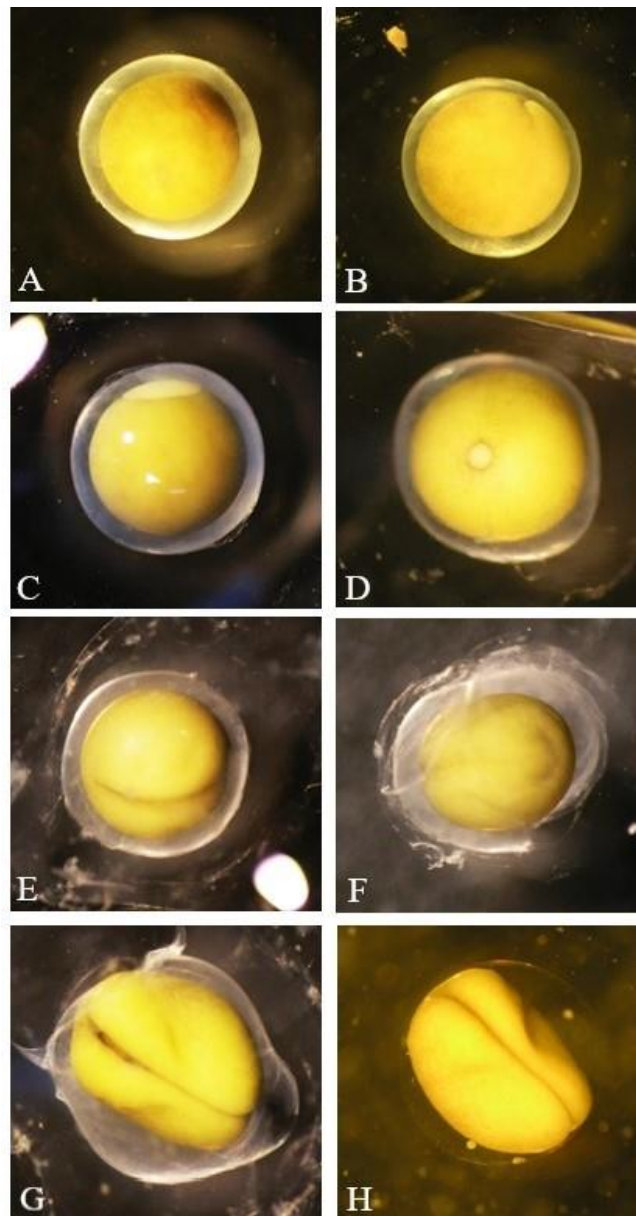


FIGURE 2. **A.** Late cleavage, **B.** Dorsal lip, **C.** Mid-gastrula, **D.** Late gastrula, **E.** Neural plate, **F.** Neural fold, **G.** Rotation, **H.** Neural tube.

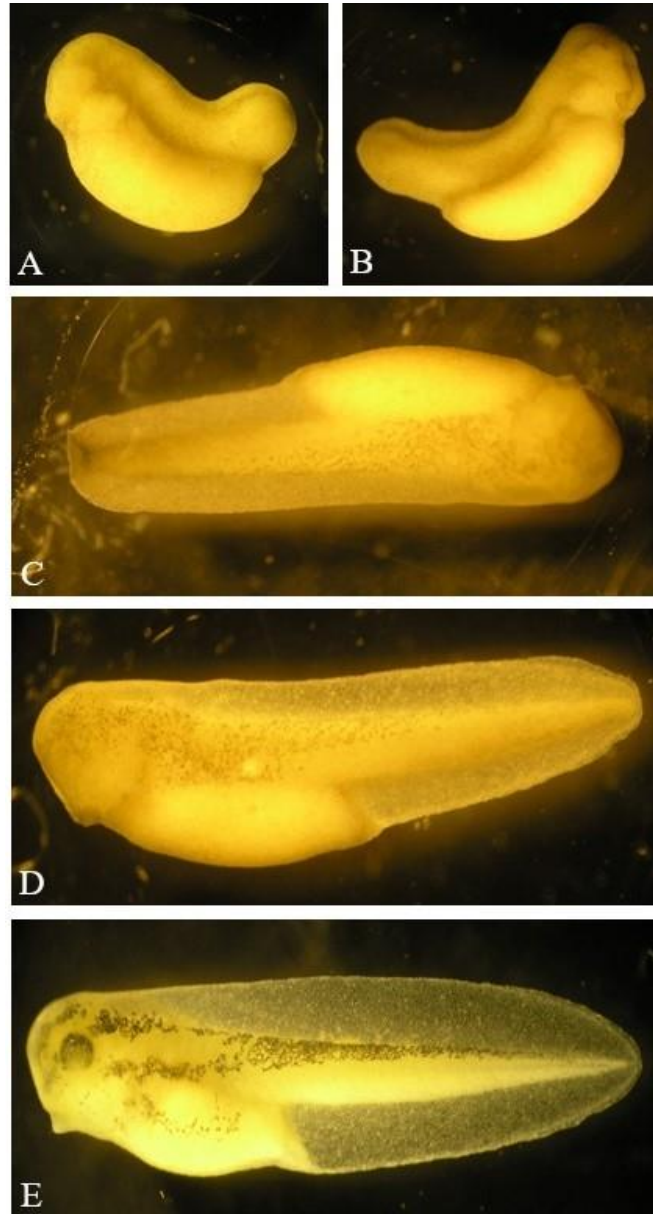


FIGURE 3. **A.** Tail bud, **B.** Muscular response, **C.** Heartbeat, **D.** Gill circulation, **E.** Cornea transparent

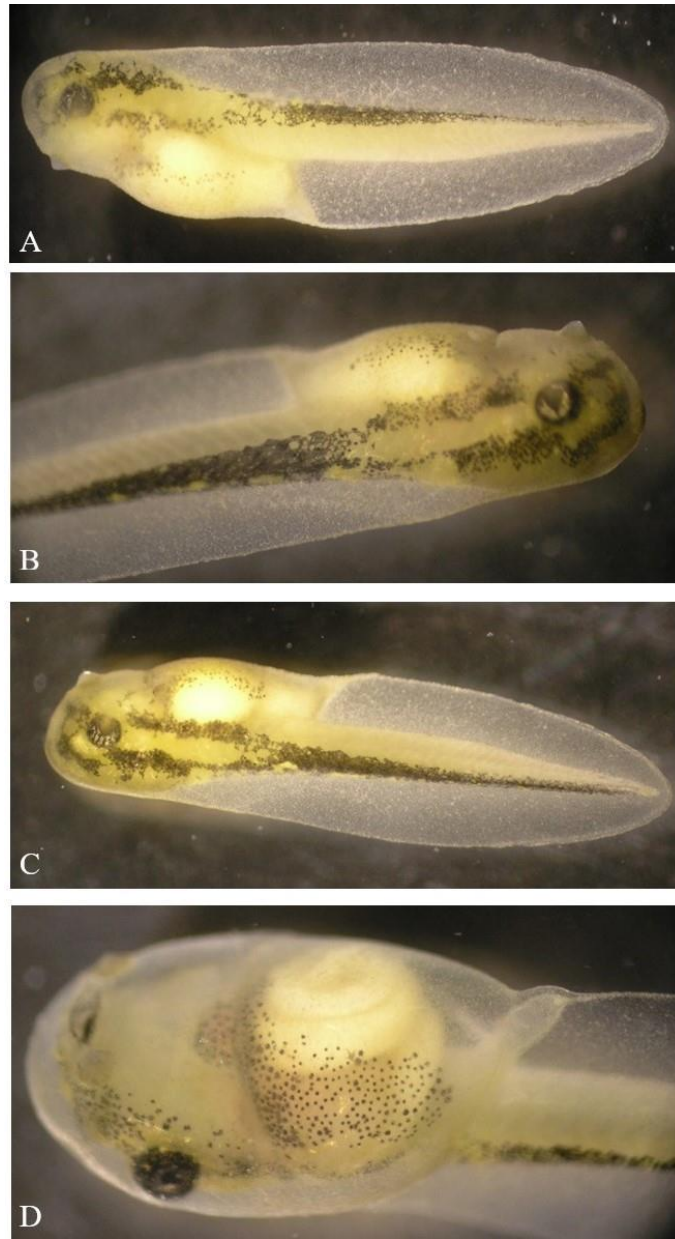


FIGURE 4. **A.** Tail fin circulation, **B.** Operculum fold, **C.** Operculum closed on right, **D.** Operculum closed on the left and a spiracle formed.

TABLE 1. The embryonic stages of *H. orientalis*, *H. annectans* and *H. savignyi* follow Gosner stages (1960).

Stage	Characteristics of embryos Gosner stages (1960)		Age in hours		
			<i>H. orientalis</i>	<i>H. annectans</i>	<i>H. savignyi</i>
1	Fertilization		0	0	0
2	Second polar body released		0.30	0.55	0.45
3	First cleavage (meridional); 2 blastomeres	Cleavage	2.00	1.50	3.00
4	Second cleavage (meridional); 4 blastomeres		2.20	2.20	4.00
5	Third cleavage (latitudinal); 8 blastomeres		3.00	2.45	4.30
6	Fourth cleavage (meridional); 16 blastomeres		3.30	3.20	5.30
7	Fifth cleavage (latitudinal); 32 blastomeres		4.10	3.52	7.00
8	Mid-cleavage; early blastula		7.30	7.54	7.30
9	Late cleavage; late blastula		9.30	12.10	11.30
10	Involution at dorsal lip of blastopore; beginning of gastrulation	Gastrula	16.00	15.05	20.30
11	Mid-gastrula; large yolk plug		21.00	16.50	23.00
12	Late gastrula; small yolk plug		25.30	34.40	26.00
13	Dorsal flattening; formation of neural plate	Neurula	28.30	38.20	33.30
14	Early neurula stage; neural folds approach each other		33.30	40.50	35.30
15	Mid neurula stage; neural folds coalesce; body begins to elongate		34.30	42.50	37.00
16	Formation of the neural tube; body elongated		35.30	60	39.30
17	Tail bud; adhesive organs begin to develop		38.30	70.07	41.00
18	Muscular response; differentiation of gill arches; olfactory pits form		52.30	72.29	54.00
19	Heart beat		73.30	102.29	65.30
20	Gill circulation begins		88.00	117.29	76.30
21	Cornea transparent		112.00	143.59	92.33
22	Tail fins become transparent; circulation begins in fins		117.00	172.29	101.00
23	Opercular fold covers base of gills		139.30	185.29	119.00
24	Opercular fold closes on the right side		163.30	748.00	132.30
25	Opercular fold closes on the left side; spiracle forms		211.30	1136.00	157.00
Duration of the embryonic development from fertilization to 25th stage			8.8 days	94.6 days	7 days
Temperature			20 ± 1°C	16 – 22 °C	21±1 °C
Ovum size (mm)			1.4	1.52	1.4

4. CONCLUSIONS

The pattern of development of hylid species is generally the same as Ranidae. Although comparing our results by embryonic development of *H. orientalis* and *H. annectans* show morphologic similarity, the duration of each stage and inter stages time is different in the three species.

The embryonic development process of *H. savignyi* from fertilization to the 25th stage lasted about 157 hours (7 days) at 21 ± 1 °C but this period for *H. orientalis* was about 211.30 hours (9 days) at 20 ± 1 °C. This rate in *H. annectans* is slower and the process lasts 1136 hours (94.6 days) at 16 – 22 °C (Table 1).

The rate of development to different stages may depends primarily on temperature and secondly on ovum size [30]. While these three species are from the same genus, the ovum size is the same in *H. orientalis* and *H. savignyi* (1.4 mm) and a bit larger in *H. annectans* (1.52 mm). The rate of embryonic development in *H. savignyi* is slower than *H. orientalis* up to 18th stage but from 19th stage the rate increases in *H. savignyi* and completes the process earlier than *H. orientalis*.

It is known that the development rate in anurans with larger eggs is slower than smaller ones [31, 32, 33, 34], which may occur due to oxygen limitations in the larger eggs which slows the rate of embryonic tissue synthesis [35]. Generally, there is a correlation in animals [36], smaller eggs may result in faster hatching, and it's also possible for smaller offspring to be produced after hatching [32, 37]. However, more research studies are needed on the relationship between egg size and post-hatch development between species. After hatching, the offspring can use environmental resources, which affects the rate of development [38]. The comparison of temporal data on embryonic development of *H. savignyi*, *H. orientalis* and *H. annectans* shows a slower embryonic development rate in *H. annectans* (1136 hours = 94.6 days) that could be related to its larger ovum size in this species.

Of course, in addition to the size of the eggs, other external factors such as the environmental temperature also play a crucial role in the rate of development during the incubation [30, 39, 40] that must be taken into account. Even after hatching, the egg size can still have a direct impact on the development time of the amphibian offspring.

Considering that the world is experiencing unprecedented anthropogenic changes [41], including global climate change [42], and since amphibians are sensitive to changes in their environment [43], studying the impact of

these temperatures' changes on the development and growth of amphibians in and throughout their early life history is critical to conservation activities [44].

Most laboratory studies that investigate the effects of the environment on development have taken place under stable environmental conditions. Naturally occurring thermal environments can vary widely, so it is not clear that working models in stable environments provide an adequate representation for the variations. Temperature is the most important factor affecting embryonic development [45, 30]. For this reason, there have been studies on the embryogenesis of many amphibian species since the 1900s [46, 47, 48, 49, 50, 28]. Several studies in the literature have investigated the effects of diel temperature fluctuation on amphibian development, which particularly is sensitive to variable environmental conditions [51, 52]. Extensive studies about the effects of temperature fluctuations on the developmental rate of ectotherms show that these variations may increase, decrease, or have no effect on development rates [52, 53, 54]. The result of research on models and experiential data reveals that temperature fluctuations are ecologically significant when individuals are temporarily exposed to temperatures within their physiologically relevant temperature range [55, 56, 58, 59] and that fluctuations between cooler and warmer temperatures can have different or opposing impacts on developmental traits [53, 56].

Low temperatures can slow metabolic rate and development, and reduce growth, swimming and feeding activity [59]. The temperature effect may be the reason why the development period of *H. annectans*, whose embryonic development was examined in the laboratory between 16 – 22 °C, was completed in a longer time. The completion times of cleavage and gastrula stages seem to be almost parallel to each other in all three hylid species (Table 1). The difference is towards the end of the neurulation. After the neural tube is formed, the rate of embryonic development slows down in the *H. annectans* species (Table 1). In *H. savignyi*, embryonic development progresses more slowly to muscle response than *H. orientalis* whereas it accelerates after the 18th stage (Table 1). As a result, the *H. savignyi* species completed its embryonic development faster than the other two hylid species (Table 1). Completing the embryonic development process earlier may be advantageous for adapting *H. savignyi* to warmer zones where it lives and increasing the survival chance of the species.

Author Contribution Statements ŞK- conceptualization, analysis, writing, EN- validation, editing, review, writing, EY- validation, editing, review, writing, UK- review.

Declaration of Competing Interests The authors declare no conflict of interest.

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