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Chronological Changes in the Somatic Development of *Hoplobatrachus rugulosus* (Wiegmann, 1834) (Anura: Dicroglossidae)

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ABSTRACT.– The rice field frog *Hoplobatrachus rugulosus* is widely distributed throughout the wetlands of Thailand. It is economically important and a potential experimental subject in many fields of research. To use its development as a biomarker, general information about normal morphological changes during metamorphosis is necessary. In this study, embryos and larvae of *H. rugulosus* were examined at every stage from fertilization until complete metamorphosis. Their developmental stages were identified using Gosner's (1960) staging system and the result showed that all morphological characteristics of *H. rugulosus* were corresponded to those in Gosner's system. This is the first study that report the period of embryonic and larval development at each stage of *H. rugulosus*. The embryonic phase occurred within 13-21 hours after fertilization, the hatchling phase continued within 36-44 hours after fertilization, the tadpole phase followed within 20-26 days post hatch, and the metamorph phase finished within 25-33 days post hatch. The rate of somatic development of *H. rugulosus* was relatively rapid comparing with those of other anurans. These results could be used as crucial developmental data for further research into this species.

KEY WORDS: developmental period, embryology, Gosner stage, *Hoplobatrachus rugulosus*, tadpole

INTRODUCTION

More than eighty years ago, staging tables of embryo and larva development in different species of anuran were published by several different researchers. In 1940, a study of the twenty-five prefeeding stages of *Lithobates pipiens,* as *Rana pipiens,* was published by Shumway, just after the publication of a study on temperature influenced egg development in *Lithobates sylvaticus,* as *Rana sylvatica* (Moore, 1939). Following these, a study of the twenty-five postfeeding stages of *Lithobates pipiens* was published by Taylor and Kollros (1946). However, the number of postfeeding stages was not the same as Shumway's table. Limbaugh and Volpe (1957) reported the complete staging table of *Incilius valliceps,* as *Bufo valliceps,* which was composed of 46 stages. In 1960, Gosner tried to simplify the staging table from the previous work of Shumway (1940), Taylor and Kollros (1946) and Limbaugh and Volpe (1957) and adopted this staging table to use for other species based on studies of larvae of toad species *Anaxyrus americanus,* as *Bufo terrestris americanus* and *Anaxyrus woodhousii,* as *Bufo woodhousei fowle* (Gosner and Black, 1958). Gosner's staging system was later adjusted and used as a supplement to other staging systems in identification of embryonic stages in a number of anuran species. Currently, Gosner's staging table is used as a standard system and is strongly recommended by several researchers (Altig and McDiarmid, 1999; Shimizu and Ota, 2003; Del Pino et al., 2004; Sayim and Kaya, 2008; Saha and Gupta, 2011; Pfalzgraff et al., 2015).

In Thailand, more than 175 species of amphibian fauna can be found (BEDO, 2016). There are several reports of their morphological and ecological data, but studies examining their life history and embryonic development are scarce. The rice field frog *Hoplobatrachus rugulosus* is an anuran amphibian in the family Dicroglossidae, the only member of the genus *Hoplobatrachus* found in Thailand (Chanard, 2003; Pansook et al., 2012). This native frog is widely distributed throughout the wet lands of Thailand and also in central, southern, and south-western China to Myanmar, Lao People's Democratic Republic, Viet Nam, Cambodia, and peninsular Malaysia (Diesmos et al., 2004; Frost, 2021). Recently, it was reported that the frog may be a cryptic species by molecular technique (Yu et al., 2015). The taxonomy as well as life history of this species still needs to be studied in different populations throughout their distribution range. *Hoplobatrachus rugulosus* has been used as an experimental animal for basic biological studies in Thailand, such as those looking into taxonomic diversity and geographic variation (Khonsue and Thirakhupt, 2001; Schmalz and Zug, 2002; Bain and Truong, 2004; Hasan et al., 2012), physiology (Ratanasaenga et al., 2008), behavior (Li et al., 2011) and pathology (Satetasit et al., 2009; Sailasuta et al., 2011). Furthermore, it also been used in studies of environmental endocrine disruption effects on metamorphosis and gonadal development (Ruamthum et al., 2011; Trachantong et al., 2013). *Hoplobatrachus rugulosus* tadpoles are suitable experimental subjects for many studies because they can be cultured and manipulated in the laboratory, they have a short metamorphosis period, and their body size and organs are large enough to observe morphological characteristics with the naked eye and under stereomicroscope. However, information about normal embryonic and larval development in this species has never been documented. Therefore, in this study morphological characteristics during embryonic and larval development were studied in *H. rugulosus* using photomicrography, and described according to Gosner's staging system (Gosner, 1960). The development of *H. rugulosus* is presented as a table of normal chronological development from fertilization to complete metamorphosis.

MATERIALS AND METHODS

Animal procurement was carried out at the Amphibian and Reptile Research Unit, Faculty of Science, Chulalongkorn University. In this study, *H. rugulosus* (bred type) were originally obtained from northern Thailand, which represented a single clade of *H. rugulosus* populations (Pansook et al. 2012). Six pairs of adult male and female *H. rugulosus* were used as breeders for stimulated fertilization. Somatic development during metamorphosis was studied during two breeding periods between April and August of 2015 and 2018. Fertilization was stimulated by injecting GnRH analogue (Suprefact, Frankfurt am Main, Germany) to induce spermination, ovulation and mating, as used in a previous protocol (Pariyanonth et al., 1985). A subcutaneous injection of 10 µg/1 kg body weight GnRH analogue was done in the abdominal area using a 27Gx1/2" needle. Each brood of tadpoles was raised in a 100L plastic container under natural light and temperature conditions (water temperature 27.5–28.1°C). A land area was provided when the tadpoles reached the hind limb development stages (stage 38-39) by setting the plastic container at an angle of 30 degrees to the ground. The water volume was 35 L for tadpoles and the water volume was reduced to 10 L for metamorphs. The water was changed every day to remove waste and maintain oxygen levels. Tadpoles and froglets were fed with commercial fish pellets once a day. Morphological changes from fertilization until complete metamorphosis were carefully observed under stereomicroscope, classified according to the characteristics of each stage of tadpole according to Gosner (1960), and photographed with an Olympus DP72 camera on an Olympus SZX16 stereomicroscope with Image Stacking Cell-D Auto-montage software. Tadpoles at Gosner stages 18–46 were euthanized by immersion in 0.25% ethyl 3-aminobenzoate methanesulfonate (MS-222) aqueous solution before observation. Eight to sixteen tadpoles were observed at each stage to collect the data of developing time. The average total length (TL) of embryos and tadpoles at stages 1–25 was measured using Image Stacking Cell-D Auto-montage software and the TL of tadpole and froglet at stages 26–46 was measured using a digital Vernier caliper. During the observation period, early-stage embryos (from fertilization to Gosner stage 20) were observed every 15 minutes. Following this, for Gosner stages 21–25 observation was done every hour and then for Gosner stages 26–46, every day. The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University (Protocol Review No. 1623002). All specimens were deposited at the Chulalongkorn University Museum of Natural History (voucher number CUMZ-A-7791 – CUMZ-A-8236).

RESULTS

Clutch sizes from the six pairs of breeders ranged from 600–1,800 eggs. The eggs were laid in water in large clumps. There were two layers of jelly envelope surrounding each egg. The average diameter of a spherical egg without envelope was 2.012-2.166 millimeters (n=8). The animal pole had a dark-brownish pigment, and the vegetal pole was white. According to the Gosner staging system, the developmental period of frogs is divided into 4 phases including embryo (stages 1–20), hatchling (stages 21–24), tadpole (stages 25–41), and metamorph (stages 42–46). The age ranges of each stage, as well as their morphological characteristics were determined as follows.

Embryo (stages 1–20, Table 1, Fig. 1**)**

The embryo phase began with fertilization (stage 1), then the fertilized egg entered mitotic cell division which marked the beginning of stage 2. The embryonic cells underwent multiple rounds of cleavage and became blastula without increasing their cell size (stages 3–9). Gastrulation occurred at stages 10–12. Neurulation was observed at stages 13–16. Tail bud development began at stage 17. Muscular response first occurred at stage 18. External gills began to develop at stage 19. Hatching began at stage 20.

Hatchling (stages 21–24, Table 2, Fig. 2**)**

In *H. rugulosus*, the hatchling phase began during stages 20–21. Then stages 22–24 comprised external gill development, pigmentation, and mouthpart development.

Tadpole (stages 25–41, Table 3, Figs. 2 and 3**)**

TABLE 1. Description of developmental stages 1–20 of *H. rugulosus* embryos according to morphological characteristics in Fig. 1. Stage = stage of embryonic development according to Gosner (1960); TL = total length of embryo; age = developmental period; haf = hours after fertilization.

Stage	Description	Range of TL (mm)	Range of age
1 (n=16)	***Fertilization stage***: The animal pole (pigmented area) was on top while the vegetal pole was in the lower part. This position allows the egg to go through the next stage of development.	2.012-2.166	0-10 mins after fertilization
2 (n=16)	***1-cell stage:*** A gray crescent was observed between the animal pole and vegetal pole of the fertilized egg, opposite the sperm penetration point. The upper part of the fertilized egg became flat.	2.044-2.088	
3 (n=16)	***2-cell stage:*** The embryo entered mitotic cell division which is the beginning of cleavage. The embryonic cell meridional divided into 2 equal blastomeres. The first cleavage furrow originated at the animal pole and proceeded to the vegetal pole.	2.013-2.118	
4 (n=16)	***4-cell stage*****:** The embryonic cells divided meridionally into 4 equal blastomeres. The second cleavage furrow was perpendicular to the first one, and passed from the animal pole to the vegetal pole.	2.073-2.102	10-25 mins after fertilization
5 (n=16)	***8-cell stage:*** The embryonic cells divided meridionally into 8 blastomeres. The third cleavage furrow was lateral to the second one. Cleavage at the animal pole was faster than that at the vegetal pole.	2.098-2.138	
6 (n=10)	***16-cell stage:*** At the animal pole, the embryonic cells divided meridionally into 16 blastomeres. At the vegetal pole, cleavage was delayed.	2.023-2.114	
7 (n=10)	***32-cell stage:*** The embryonic cells divided latitudinally into 32 blastomeres. Cleavage was still delayed at the vegetal pole.	2.097-2.160	0.5-1 haf
8 (n=10)	***Midcleavage/morula:*** The embryonic cells divided into more than 64 blastomeres. The blastomeres in the animal pole were smaller than those in the vegetal pole.	2.090-2.169	
9 (n=10)	***Late cleavage/blastula:*** Cell proliferation continued without any increase in the size of the blastula. Blastomeres divided into many smaller cells. The pigmented area at the animal pole extended over the vegetal pole; this process is called epiboly.	2.125-2.162	2-5 haf
10 (n=10)	***Dorsal lip:*** Involution of the cells from the animal pole toward the vegetal pole occurred. The dorsal lip appeared, indicating the beginning of gastrulation.	2.135-2.259	
11 (n=10)	***Yolk plug:*** The surface at the animal pole extended over the vegetal pole and the exposed area of the vegetal pole was reduced, yolk plug appeared. The embryo began to take an oval shape and its position began to change, creating anterior and posterior axes.	2.012-2.254	
12 (n=10)	***Late gastrula:*** A small protruding yolk plug appeared.	2.173-2.224	

TABLE 1. (Continue)

Stage	Description	Range of TL (mm)	Range of age
13 (n=10)	***Neural plate:*** The embryo became elongated. The dorsal area became flattening and formed a thick plate called a neural plate.	2.195-2.375	
14 (n=10)	***Neural folds:*** Neural folds were observed, created by the elevation of two ridges along the dorsal surface of the body; between these two ridges was the neural groove.	2.248-2.560	3-7 haf
15 (n=10)	***Elongation and rotation:*** The neural groove narrowed. The neural folds approached each other. The embryo elongated and rotated.	3.028-3.115	
16 (n=10)	***Neural tube:*** The neural tube formed by the closing of the neural fold. The embryo began to develop a head. Gill plates were observed.	3.741-4.164	5-9 haf
17 (n=10)	***Tail bud:*** The tail bud developed at the posterior end of the embryo. The adhesive gland (oral sucker) developed.	4.260-4.455	
18 (n=10)	***Muscular response, olfactory pits visible:*** Muscular response was noticeable. The embryo wriggled with spontaneous muscular movement. The vitelline membrane became thin. The olfactory pit developed at the ventral part of the head. The gill plates protruded.	4.302-4.707	11-13 haf
19 (n=10)	***Gill buds, heartbeat:*** External gill buds began to develop. The heartbeat was observed below the gills.	5.034-5.638	13-17 haf
20 (n=10)	***Gill circulation, tail elongation:*** The external gill plate divided into several ridges and increased in size to form external gill filaments. Gill circulation was also observed. The tail elongated. Hatching began. The violent wiggling of the embryo caused the surrounding gelatinous membrane to rupture, then the embryo spurted out from the membrane.	6.036-6.284	17-21 haf

The tadpole phase was the longest phase of development. The external gills disappeared at stage 25. Stages 26–30 consisted of hind limb bud development classified by the proportion of leg length to leg diameter. Stages 31–39 consisted of toe differentiation and development classified by indentations of toe formation and separation. Stage 40 was identified by the presence of foot tubercles and stage 41 was identified by forelimb formation.

Metamorph (stages 42–46, Table 4, Fig. 4**)**

The metamorph phase is when the tadpoles changed their physical appearance for terrestrial habitat. Metamorphs at stages 42–45 were identified by mouth development and those at stages 43–46 were identified by tail degeneration.

DISCUSSION

Anurans are a wildly distributed group of amphibians that can be found in several kinds of habitat. Because their embryonic development occurs in aquatic habitats, the adaptation of jelly coat formation is necessary for this group of amphibians. A report has shown interspecific variations of

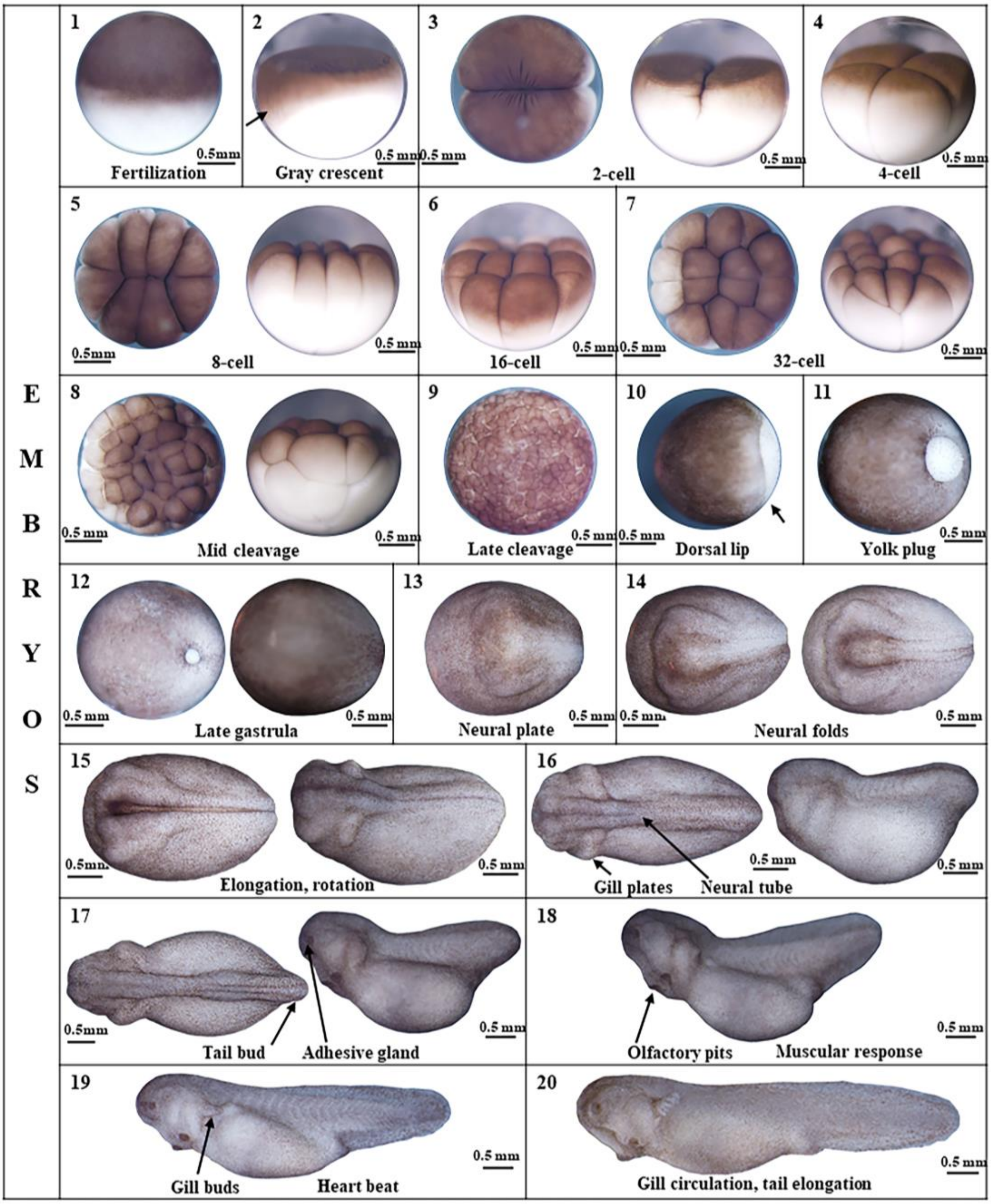

FIGURE 1. Micrographs of *H. rugulosus* at different embryonic phases (stages 1–20) with Gosner stage number (top left corner) and distinct key morphological characteristics according to Gosner (1960).

jelly coat patterns correlate with environmental conditions (Duellman and Trueb, 1994). In this study, the eggs of *H. rugulosus* were surrounded by two layers of jelly coat and laid in the form of large clumps in lentic habitats. This pattern is

TABLE 2. Description of developmental stages 21–24 of *H. rugulosus* according to the morphological characteristics in Fig. 2. Stage = stage of embryonic development according to Gosner (1960); TL = total length of embryo; age = developmental period; haf = hours after fertilization.

Stage	Description	Range of TL (mm)	Range of age
21 (n=10)	***Cornea transparent, mouth opening:*** The eyes of the tadpole developed. The cornea was transparent. The opening of the mouth part was observed. The tadpole was active and could swim spontaneously.	6.482-6.701	15-23 haf
22 (n=10)	***Tail fins transparent, fin circulation:*** Dorsal and ventral tail fins became transparent and capillary circulation was observed. Epidermis became transparent. The edge of the mouth became round. Pigmentation initially appeared in the dorsal part of the head and body.	6.705-7.113	21-26 haf
23 (n=10)	***Operculum covering gill bases, labia and teeth differentiation:*** Labia and teeth differentiated, and the adhesive gland degenerated. Operculum covered the external gill bases. The external gills were now fully developed. The gut also developed and the body trunk appeared asymmetrical when it was observed from the ventral aspect. Pigmentation patterns in the epidermis also appeared as yellowish-brown at the head, tail, and dorsal part of the trunk, mottled with black at the dorsal part of the tail and the body trunk. Iridescent speckles appeared on the dorsal area of the head and very distinctly on the skin area above the eyes.	7.177-8.323	31-35 haf
24 (n=10)	***External gill atrophy, operculum closure on right:*** The external gills contracted. Operculum folds covered the external gills and closed on the right side while the external gills on the left side remained uncovered. Iridescent pigment appeared faintly in the skin of the belly.	9.803-10.327	36-44 haf

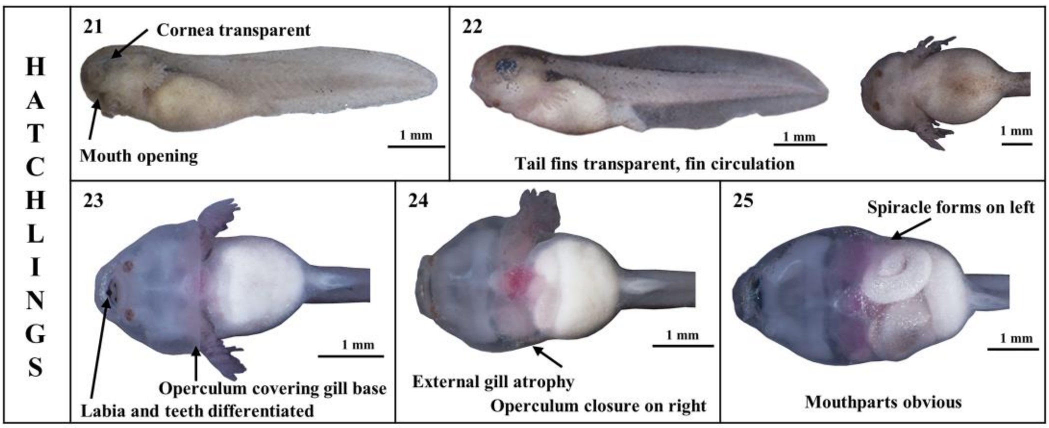

FIGURE 2. Micrographs of *H. rugulosus* at different hatchling phases (stages 21–24) and a tadpole phase (stage 25) showing Gosner stage number (top left corner) and distinct key morphological characteristics according to Gosner (1960).

normally found in *Rana* species (Duellman and Trueb, 1994). The clutch size of *H. rugulosus* was 600–1,800 eggs and egg diameter was approximately 2.012-2.166 mm. The differences in clutch size may be influenced by maternal effects; the size of the mother correlated with the number of eggs produced (Bernardo, 1996). *Hoplobatrachus rugulosus* is a large frog, the female weighing approximately 340–460 grams and able to produce large number of eggs per clutch. It is known that maternal characteristics can influence oviposition site, population size, clutch structure, egg site, energy content, and pigment in the ovum. Pattern of development, ecomorphology, and other factors can also influence the clutch size (Altig and McDiarmid, 2007).

In a key study by Gosner in 1960, *Incilius valliceps* and *Lithobates pipiens* were used as animal models to describe the stage characteristics of anuran development. In the current study, the morphological characteristics of *H. rugulosus* were used to identify stages corresponding to those in Gosner's system. The morphological characters of anuran tadpoles are various and different among species, corresponding to their ecomorphological diversity (Altig and McDiarmid, 1999). Some key characteristics are observable at different periods of the developmental timeline; some examples of these are shown in Table 5. *Hoplobatrachus rugulosus* tadpoles can be found in various kinds of aquatic habitat such as paddy fields, irrigation infrastructure, fishponds, floodplain wetlands, forest pools, pools, ponds, and other wet areas (Diesmos et al., 2004). The tadpoles have specific characteristics adapted for living in these habitats, including adhesive glands, spiracle opening position, eye position, and mouth part position. The adhesive glands develop before the hatchling stage (stage 17). The tadpole uses the glands to attach itself to the substrate after hatching from its jelly coat when it inhabits a fluvial environment. The position of the spiracle opening in stage 25 is a specific characteristic in each taxon of anuran. In *H. rugulosus*, the spiracle opening was found on the left side of the body. Spiracle characteristics depend on opercular fold fusion. In *H. rugulosus*, operculum fuses on the right side prior to the left side. Therefore, at stage 24 the tadpole only has external gills on the left side during the development of the lungs inside the body. When the lungs are developed, the left opercular fold fuses and forms a spiracle as well. Similarly, position of the eyes also correlates to the type of aquatic habitat. *Hoplobatrachus rugulosus* eyes are located at a dorsolateral position. This is suitable for living in both lentic and lotic systems. The mouth part of the anuran is one of the key characteristics used to identify its species during embryonic development. *Hoplobatrachus rugulosus* has a mouth part located at the ventral subterminal of the head portion. The characteristics of the oral apparatus of *H. rugulosus* correlates with its mode of feeding since this frog is a carnivore and has cannibalistic behavior (Altig and McDiarmid, 1999).

TABLE 3. Description of developmental stages 25–41 of *H. rugulosus* according to the morphological characteristics in Figs. 2 and 3. Stage = stage of tadpole development according to Gosner (1960); TL = total length of tadpole; age = developmental period; dph = days post hatch.

Stage	Description	Range of TL (mm)	Range of age
25 (n=8)	***Mouthpart obvious, spiracle on left:*** Mouthpart shifted to an upper position and was now located at the tip of the head. The operculum was completely formed. The left external gill atrophied and spiracle formation was observed.	10.734-12.639	1-2 dph
26 (n=8)	***Hind limb bud development I:*** Developing hind limb buds became visible for the first time. The length (L) of limb buds was shorter than half of their diameter (D) ($L < ½D$). Iridescent pigmentation was observed on the tail.	11.900-13.557	2-3 dph
27 (n=8)	***Hind limb bud development II:*** The hind limb buds increased in size. The length of the limb buds was equal or longer than half of their diameter ($L \geq ½D$).	12.984-15.592	3-5 dph
28 (n=8)	***Hind limb bud development III:*** The hind limb buds elongated. The length of the limb buds was equal or longer than their diameter ($L \geq D$).	13.060-14.845	4-6 dph
29 (n=8)	***Hind limb bud development IV:*** The hind limb buds elongated. The length of the limb buds was equal or longer than one and a half times their diameter ($L \geq 1½D$).	18.773-21.943	6-8 dph
30 (n=8)	***Hind limb bud development V:*** The hind limb buds elongated. The length of the limb buds reached twice their diameter ($L=2D$).	22.121-25.157	7-9 dph
31 (n=8)	***Toe differentiation and development—foot paddles:*** Foot paddles developed with slight indentations at the paddle margin.	24.510-27.470	8-10 dph
32 (n=8)	***Toe differentiation and development—first indentations:*** The foot paddles showed indentations of the 4th and 5th toes.	22.091-25.160	8-11 dph
33 (n=8)	***Toe differentiation and development —second indentations:*** The foot paddles showed indentations between the 5th and 4^{th,} and the 4th and 3rd toes.	23.570-28.530	9-12 dph
34 (n=8)	***Toe differentiation and development—third indentations:*** The foot paddle margins showed indentations between the 5th and 4th, 4th and 3rd, and the 3rd and 2nd toes.	26.190-29.883	10-13 dph
35 (n=8)	***Toe differentiation and development—fourth indentations:*** The foot paddle margins showed indentations between all five toes. Knee joints began to develop.	29.267-33.227	11-15 dph
36 (n=8)	***Toe differentiation and development—the 3rd – 5th toes separated:*** The 3rd – 5th toes separated. The margin of the 5th toe web was directed toward the tip of the 2nd toe.	32.650-36.930	13-17 dph

TABLE 3. (Continue)

Stage	Description	Range of TL (mm)	Range of age
37 (n=8)	***Toe differentiation and development —all toes separated:*** The toes elongated and all toes separated. The margin of the 5th toe web was directed toward the tip of the 1st toe.	36.033-38.740	14-18 dph
38 (n=8)	***Toe differentiation and development—metatarsal tubercles:*** The hind limbs increased in size. Inner metatarsal tubercles appeared at the lateral side of the metatarsal area above the 1st toe. The oral structure was clearly developed and could be used as an important characteristic to identify the tadpole's species.	36.873-40.721	17-21 dph
39 (n=8)	***Toe differentiation and development—subarticular patches:*** Subarticular patches appeared as light patches on the inner surfaces of the toes.	37.840-41.597	19-22 dph
40 (n=8)	***Foot tubercles, vent tubes presented:*** The hind limbs were now well developed. Foot tubercles were observed beneath the joints on the toes. Vent tubes were present and not yet reduced.	39.100-44.853	19-23 dph
41 (n=8)	***Forelimbs visible, mouthpart atrophied, vent tube lost:*** Forelimb development was observed under the cover of the operculum. The mouth part atrophied. The keratinized jaw sheaths started shedding. Cloacal tail piece shrank and vent tubes completely disappeared.	46.137-51.547	20-26 dph

Several studies reported that the developmental periods were different between species of anuran. The metamorphosis of *Hydrophylax leptoglossa,* as *Rana leptoglossa,* was completed within 68–72 days (Saha and Gupta, 2011) while that of *Polypedates teraiensis* was 58 days (Chakravarty et al., 2011). In Madagascar, metamorphosis of *Mantidactylus betsileanus* was completed within 89 days (Scheld et al., 2013). There are reports of different developmental periods of the same species that live in different areas. The metamorphosis of *Microhyla ornata* in Japan was completed within 40 days (Shimizu and Ota, 2003) while that of *M. ornata* in India was 49 days (Narzary and Bordoloi, 2013). In this study, *H. rugulosus* tadpoles took 58–88 hrs (1–2 dph) to develop to stage 25, which was quite fast compared to other species in the anuran order. Grosjean et al. (2004) suggested that the adaptations of *Hoplobatrachus* could explain population succession in this genus. Adult frogs could breed in temporarily or permanently flooded areas, and tadpoles could develop quickly. In this study, metamorphosis of *H. rugulosus* was completed within 26-34 days which is before the gonads were sexually differentiated (Traijitt et al., 2020). The rapid rate of development in this species may provide benefits for escaping from stressful conditions such as predators (Calsbeek and Kuchta, 2011), dryness (Gomez-Mestre et al., 2013), and diseases (Warne et al., 2011) which have also been reported in other frog species. The length of the developmental period depends on species intrinsic factors and environmental conditions (Duellman and Trueb, 1994). The environmental factors influencing *H. rugulosus* tadpole development include

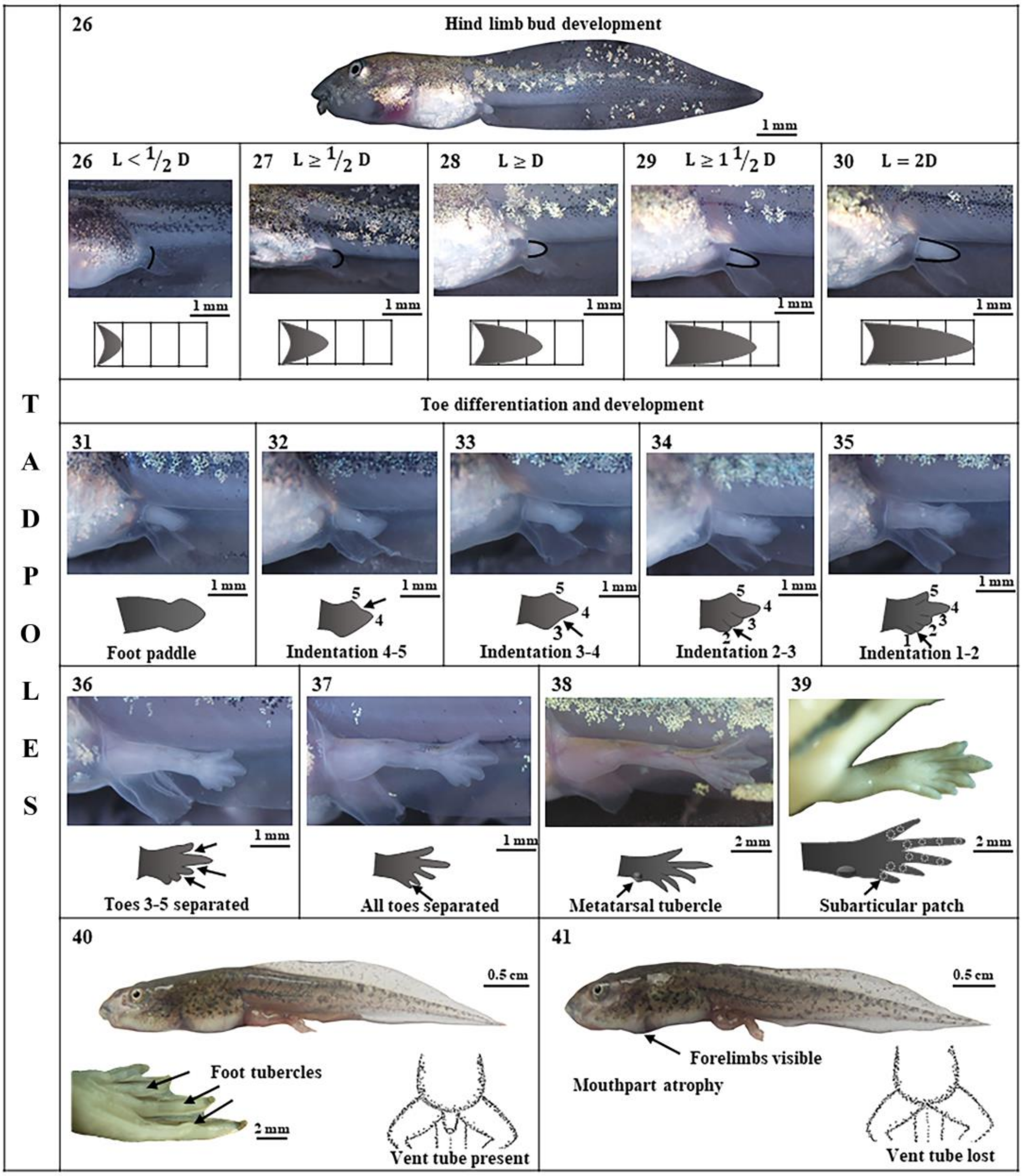

FIGURE 3. Micrographs of *H. rugulosus* at different tadpole phases (stages 26–41) showing Gosner stage number (top left corner) and distinct key morphological characteristics according to Gosner (1960).

temperature (Liamtong et al., 2019, Tang et al., 2020), diet (Somsueb and Boonyarat-palin, 2002, Ding et al., 2015), salinity of water (Nakkrasae et al., 2016), and population density (Wei et al., 2014).

TABLE 4. Description of developmental stages 42–46 of *H. rugulosus* according to the morphological characteristics in Fig. 4. Stage = stage of tadpole development according to Gosner (1960); TL = total length of tadpole; age = developmental period; dph = days post hatch.

Stage	Description	Range of TL (mm)	Range of age
42 (n=8)	***Forelimbs emerge, mouth angle anterior to nostrils:*** The forelimbs emerged. The shape of the mouth changed. From the lateral view, the end of the mouth angle was located anterior to the nostril. The keratinized jaw sheaths spurted out from the mouth part.	44.196-50.373	20-26 dph
43 (n=8)	***Mouth angle located between nostril and eye:*** From the lateral view, the end of the mouth angle was located between the nostril and the anterior end of the eye. The eyeballs began to protrude. The tail atrophied.	40.867-43.627	22-26 dph
44 (n=8)	***Mouth angle located beneath eye, tail greatly reduced:*** From the lateral view, the end of the mouth angle reached the middle point of the eye. The tail began to shrink. Dorsal and ventral fins gradually disappeared.	31.020-34.692	23-27 dph
45 (n=8)	***Mouth angle located posterior to eye, tail stub:*** From the lateral view, the end of the mouth angle was posterior to the eye. The tail shrank greatly, but remained as a small tail stub.	24.462-26.887	24-28 dph
46 (n=8)	***Tail resorbed / complete metamorphosis:*** The tadpole became a froglet. The tail completely disappeared.	19.188-20.847	25-33 dph

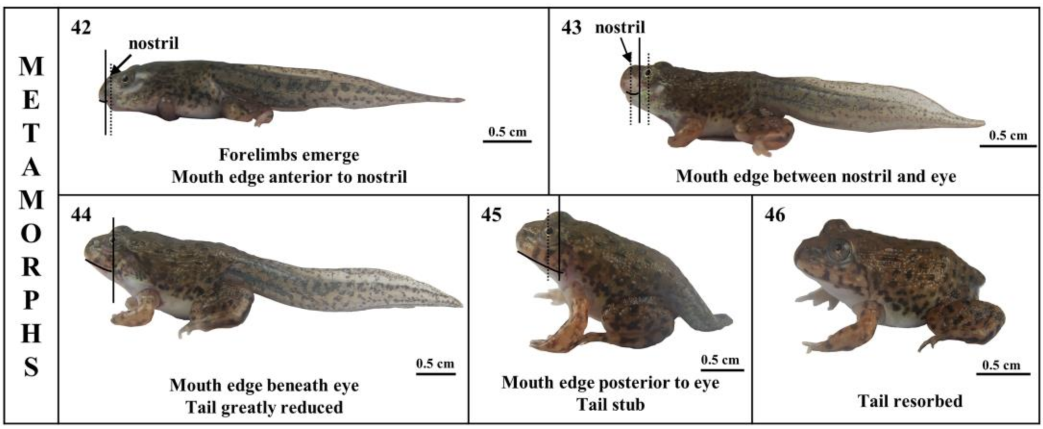

FIGURE 4. Micrographs of *H. rugulosus* at different metamorph phases (stages 42–46) showing Gosner stage number (top left corner) and distinct key morphological characteristics according to Gosner (1960).

In Thailand, more than 175 species of amphibians are listed (BEDO, 2016) but the study of their developmental stages and metamorphosis was limited. This is the first

TABLE 5. Comparison of staging characteristics of tadpoles in anuran species

Characters \ Species	***Lithobates pipiens*** (Shumway, 1940)	***Hyla arborea*** (Sayim and Kaya, 2008)	***Mantidactylus betsileanus*** (Scheild et al., 2013)	***Microhyla ornata*** (Shimizu and Ota, 2003)	***Microhyla ornata*** **(**Narzary and Bordoloi, 2013**)**	***Polypedates teraiensis*** (Tamuly and Dey, 2014)	***Hydrophylax leptoglossa*** (Saha and Gupta, 2011)	***Hoplobatrachus rugulosus*** (This study)
Fifth cleavage (Stage 7)	Irregular	Regular	N/A	Irregular	Irregular	Irregular	Regular	Irregular
Rotation (Stage 15)	Ciliary rotation	No ciliary rotation	N/A	N/A	N/A	Ciliary rotation	Ciliary rotation	Ciliary rotation
Tail bud (Stage 17)	No arched form	Arched form	No arched form	Arched form	Arched form	No arched form	No arched form	No arched form
External gill	Present	Present	Absent	Present	Present	Present	Present	Present
Hatchling	Stage 20	Stages 20–21	Stages 23–25	Stage 21	Stage 20	Stage 20	Stages 20–21	Stages 20–21
Days of development	N/A	N/A	89 days	40 days	49 days	58 days	68-72 days	26-34 days

report which provides a staging table with time data for the normal development of *H. rugulosus* under natural light and temperature in the laboratory during breeding season (April to August). *Hoplobatrachus rugulosus* developed from the first cell division until complete metamorphosis within 26–34 days, which is a rapid rate comparing with those of other anurans. The information of the developmental rate and somatic stages of *H. rugulosus* in this study is an important life history characteristic that will be crucial for developmental research of this species in the future. The results may also be useful for applying to other taxa that have similar ecomorphological patterns, but interspecific differences should be considered. It is also useful in terms *H. rugulosus* being experimental subject for environmental toxicology research such as teratogenicity tests. Furthermore, these findings can be applied to frog farming, for example to find suitable conditions that can increase commercial benefits.

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