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ORIGINAL ARTICLE

A Complete Embryonic Developmental Table of *Microhyla fissipes* (Amphibia, Anura, Microhylidae)

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Abstract Access to embryonic developmental stages is essential basic work for understanding how organisms develop. In this study, seven egg clutches (range 209–564 eggs) of ornamented pygmy frog *Microhyla fissipes* (Amphibia, Anura, Microhylidae) were obtained from seven breeding pairs in laboratory. One egg clutch of them was observed for the embryonic development, and the staging table of normal development was constructed based on morphological and physiological characteristics. Forty-five developmental stages were defined for *M. fissipes*, and two major developmental periods were designated: 1) early embryonic development period (stages 1–28), from fertilization to operculum completion stage, lasted for 82.6 hours at water temperature (WT) 23–25°C; 2) larval development period (stages 29–45), from operculum completion to tail complete absorption stage, took 38 days at WT 22–26.5°C, showing that the embryos of this species develop rapidly. In addition, the tadpoles were transparent, which is similar to those in field. These characteristics suggest that *M. fissipes* would be a good model to study developmental biology, adaptive mechanisms from aquatic to terrestrial phases, environmental toxicology, and human disease.

Keywords Microhylidae, embryonic stage, external characters, morphogenesis

1. Introduction

Among vertebrates, amphibians are known for their unique developmental cycle, especially metamorphosis, which has important implications in their life history (Werner, 1986). Accordingly, embryonic development (including early embryonic and larval development) is considered as a critical factor for understanding phylogeny, comparative embryology, and molecular mechanisms of development in amphibians (Hurney *et al*., 2015). In particular, since the larvae of anurans are morphologically distinct from the adults, it could be problematic in confirming the tadpoles through their adults. In addition, the morphological characteristics of tadpoles are unidentical at different developmental stages (Mohammad Ridzuan, 2013). Information, thus, on characteristics of tadpole development provides the basis for ensuring the same tadpole materials.

Most early staging tables were not comprehensive or undocumented the full embryonic period, as they only focused on either early embryonic or larval development separately (Pollister and Moore, 1937; Shumway, 1940; Taylor and Kollros, 1946). However, a more comprehensive staging table of embryonic development for anurans with notes on identification was first reported by Gosner (1960). Since then, more staging table studies of anuran embryonic development have been reported, which can provide comprehensive and systematic details for embryo developmental research of other species (Nieuwkoop and Faber, 1967; Iwasawa and Futagami, 1992; Shimizu and Ota, 2003; Xiong *et al*., 2010; Grenat *et al*., 2011; Mohammad Ridzuan, 2013; Narzary and Bordoloi, 2013; Aminutes *et al*., 2015). Nevertheless, such developmental descriptions are not yet available for all groups of anurans.

To date, there are at least 100 known developmental tables for anurans, but few of them are for microhylids (Rao, 1917; Mohanty-Hejmadi *et al*., 1980; Padhye

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and Ghate, 1989; Liu *et al*., 1995; Geng *et al*., 1995; Shimizu and Ota, 2003; Fabrezi *et al*., 2012; Narzary and Bordoloi, 2013). The absence of developmental data for microhylid frogs is one of the limitations in interpreting their evolutionary history (Fabrezi *et al*., 2012). *Microhyla fissipes* (Anura: Microhylidae) is a small-sized, widespread species which distributes in lowland scrub forest, grassland, agricultural land, pastureland and urban areas of Southeast Asia, Mainland and Taiwan of China (Matsui *et al*., 2005; Frost *et al*., 2017). Females spawn clutches of 243–453 eggs (diameter: 0.8–1.0 mm) and the embryos develop fast, with hatchlings taking 20-30 days to metamorphose (Fei *et al*., 2009). Furthermore, the tadpoles are transparent which provide facilities for observing the developmental physiological processes, such as heartbeat and gill-circulation (Fei *et al*., 2009; Liu *et al*., 2016). These characteristics suggest that *M. fissipes* could be an ideal model for researching the adaptive mechanism from aquatic to terrestrial phases, development biology, environmental toxicology, and human disease (Liu *et al.*, 2016). To compensate previous studies which only reported the early embryonic or postembryonic development of *M. fissipes* (Geng *et al*., 1995; Liu *et al*., 1995; Xu and Xu, 2012), a complete developmental staging table with live photographs, presenting all the morphological characters, was accomplished. This study is imperative for further researches of this species and provides important information to understand biodiversity of anuran development and the reciprocal influences between larval and adult body plans.

2. Materials and Methods

2.1 Animals Seven pairs of adult *M. fissipes*, collected from Shuangliu, Chengdu, China (30.5825° N, 103.8438° E) in June 2015, were used in this work. These pairs were labeled from I to VII, and housed in separate plastic containers (220 ×125 × 135 mm^3). These containers contained 20 mm deep de-chlorinated water and were decorated with small stones and mosses, making water-land area 3:2. Animal procedures were approved by the Animal Care and Use Committee of Chengdu Institute of Biology.

2.2 Induced breeding with LHRHa Embryos were produced by natural matings between hormonally-induced male and female frogs. Ovulation was induced by intraperitoneal injections of 0.1 ml Luteinizing Hormone Releasing Hormone analogue hormones (LHRHa) at 17:00 on 9 June, 2015, which was diluted to a concentration of 3μg/ml in sterile 0.65% NaCl. Each male and female was given a single dose LHRHa with 0.3 μg/g body weight (Kouba *et al*., 2009). After hormone injection, the brooders were released into their respective containers and raised at 28°C. The next day, 7 egg clutches were obtained and the number of eggs was counted.

2.3 Observation of embryonic development

2.3.1 Management of embryos and tadpoles Only one egg clutch was transferred to glass petri dishes (diameter: 160 mm) for observation of embryonic development. After hatching, every 60 tadpoles was transferred to a container (420 × 300 × 230 mm^3) with 150 mm depth de-chlorinated tap water and half of the water was replaced every two days. Tadpoles, when their yolks were entirely absorbed, were fed with the solution of boiled chicken egg yolk once a day. Water temperature was measured to the nearest 0.1°C with a thermometer around 4:30 pm every day.

2.3.2 Definition of embryonic development stages A stereo microscope (JSZ8T, Jiang Nan Yong Xin, Nanjing, China) with Mshot Image Analysis system (Mc50-N) was used to observe the embryonic developmental morphological characteristics, photograph embryos and tadpoles of each development stage, and measure their total length (TOL). Embryonic developmental stages were defined based on their morphological and physiological characteristics following published methods (Shumway, 1940; Gosner, 1960; Shimizu and Ota, 2003).

2.4 Data analysis Total number of laying eggs, fertilization ratio (total fertilized eggs / total released eggs), hatching ratio (total hatched eggs / total fertilized eggs), and abnormality ratio (total deformed embryos / total hatchlings) of each group were calculated. Polynomial regression was used to explore the relationship between TOL and embryonic development. Descriptive statistics was present with Mean ± SD.

3. Results

3.1 Development parameters Clutch sizes (n = 7) ranged from 209 to 564 eggs (337.86 ± 127.24). The mean fertilization ratio was 0.95 ± 0.02 (n = 6) and hatching ratio was 0.92 ± 0.05 (n = 4), while deformity ratio was 0.05 ± 0.01 (n = 4) (Table 1). Fertilized egg diameter was 0.976 ± 0.023 (n = 6, range 0.942–1.001 mm) and TOL varied considerable during the whole embryonic development (Table 2). Besides, a hump-shape relationship was presented between TOL and development, with reaching maximum at stage 40 (Figure

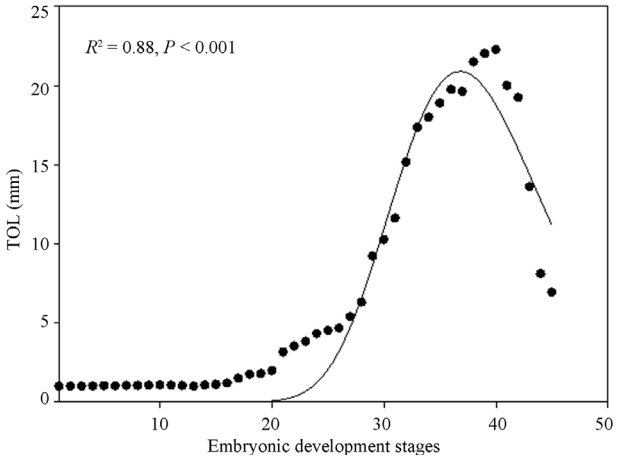

Figure 1 The trend of TOL in embryonic development. X-axle represents developmental stages while Y-axle denotes the total length (TOL) of embryos and larvals.

1). Embryos rotated clockwise or anticlockwise slowly within the vitelline envelope at stage 17 and the time of one full rotation ranged from 40–90 seconds (n = 6). Likewise, the observed *M. fissipes* embryos exhibited displaced radial, holoblastic cleavage pattern (n = 20).

3.2 Staging table of embryonic development A total of 45 stages were identified by examining the live embryos with stereo microscope (Table 2), and were sequentially described as follows. The three axes of a *M. fissipes* tadpole and 45 stages were given in Figure 2 and Figure 3, respectively.

Stage 1. Early cleavage (lasted 10 minutes): The eggs, floating on the water surface, with no fixed direction after spawning, and were covered with jelly coats. Animal hemisphere and vegetal hemisphere were brown and buff. All animal hemispheres rotated for the upper location after fertilization.

Stage 2. Late cleavage (lasted 22 minutes): A stage that was from fertilized egg to prior to the appearance of the first cleavage groove (Figure 3, 2a).

Stage 3. First cleavage (lasted 13 minutes): A stage characterized by appearance of the first cleavage (meridional), producing two cells of equal sizes, and cleavage groove was from animal hemisphere to vegetal hemisphere.

Stage 4. Second cleavage (lasted 15 minutes): Four equal sizes cells were formed with the second cleavage (meridional).

Stage 5. Third cleavage (lasted 15 minutes): This stage was a latitudinal cleavage (Figure 3, 5), producing four small dark brown cells above (animal hemisphere) and four large milky cells below (vegetal hemisphere).

Stage 6. Forth cleavage (lasted 15 minutes): This stage produced sixteen cells with different size (meridional).

Stage 7. Fifth cleavage (lasted 13 minutes): This stage produced thirty-two cells with different size and shape (latitudinal) and the animal hemisphere cells were smaller than vegetal hemisphere cells.

Stage 8. Morula (lasted 1 hour): This stage was the sixth cleavage, and all the cells size began to get smaller.

Stage 9. Early blastula (lasted 2 hours and 37 minutes): Cells size continued to reduce.

Stage 10. Late blastula (lasted 55 minutes): Further cleavages resulted in smaller cells and the boundaries between cells became blurry, creating a smooth surface.

Stage 11. Appearance of blastoporic lip: The vegetal hemisphere cells started to invaginate.

Stage 12. Early gastrula (lasted 1 hour and 30 minutes): The vegetal hemisphere cells continued to invaginate and

Table 1 Basic information of embryonic development of *Microhyla fissipes*, obtained at 23.5 ± 0.6 °C. The dashes (-) mean data missing. Individuals of group I and V were not used for analysis of hatching ratio and group II was not used for fertilization ratio as those samples were used to do histotomy and RNA extraction

Group	Egg clutch size	Fertilization ratio	Hatching ratio	Deformed ratio
I	443	95%	-	-
II	564	-	-	-
III	328	94%	96%	4%
IV	336	97%	96%	6%
V	237	95%	-	-
VI	209	93%	87%	5%
VII	248	97%	88%	3%
Mean ± SD	337.86 ± 127.24	95% ± 0.02	92% ± 0.05	5% ± 0.01

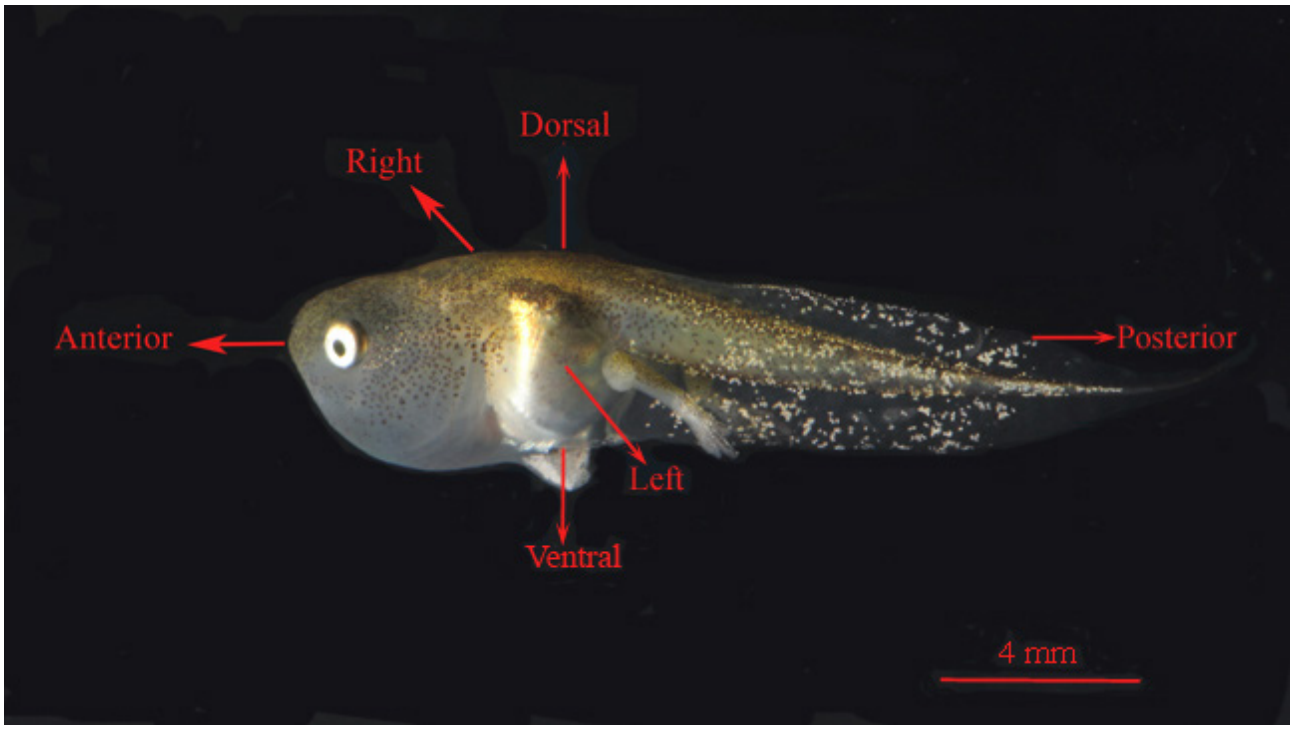

Figure 2 The anterior-posterior, dorsal-ventral and left-right axis of a fully developmental *Microhyla fissipes* tadpole.

blastoporic lip extended laterally, forming lateral lips.

Stage 13. Middle gastrula (lasted 1 hour and 20 minutes): Epiboly of the animal hemisphere progressively advanced and started flattening, creating a circular blastopore (Figure 3, 13a).

Stage 14. Late gastrula (lasted 1 hour and 48 minutes): Yolk plug became smaller (Figure 3, 14) and disappeared with the epiboly going on.

Stage 15. Neural plate (lasted 1 hour and 20 minutes): Embryo elongated slightly along longitudinal axis and the yolk plug was disappeared. The dorsal became flat and the color of embryo became light, forming a horseshoe-shaped embryo. Primary neural fold were distinct and secondary neural fold were slight.

Stage 16. Neural fold (lasted 1 hour and 41 minutes): The secondary neural fold and neural groove became distinct. Elongation proceeds in the embryo and dark pigmentation from chromophores became apparent on the dorsal side of the head.

Stage 17. Closure of neural fold (lasted 1 hour and 3 minutes): Neural fold merged in whole trunk.

Stage 18. Neural tube (lasted 1 hour and 45 minutes): Tail bud small but visible and slight uplifting at the position of future retina.

Stage 19. Tail bud I (lasted 1 hour and 48 minutes): Stomodeum and somatic structure were distinct. Tail bud elongated and dark pigmentation at the dorsal position.

Stage 20. Tail bud II (lasted 4 hours and 13 minutes): Both dorsal and ventral portions of tail fin were slightly developed (Figure 3, 20a, 20b).

Stage 21. Tail bud III (lasted 8 hours and 17 minutes): Tail fin and medial cloacal tail piece were very distinct.

Stage 22. Appearance of gill bud (lasted 10 hours and 47 minutes): Primary external gill slightly protruded (Figure 3, 22a) and the optic vesicles start to appear on the both side of head (Figure 3, 22b). Some swimming embryos can be detected.

Stage 23. Elongation of external gills (lasted 12 hours and 5 minutes): Primary external gill began to extend and ramify, and secondary external gill slightly protruded (Figure 3, 23a). The mouth opening was easily observable, and some embryos began to hatch at the later stage. In addition, the optic vesicles are distinct (Figure 3, 23b), and the individuals prefer to lie on the bottom.

Stage 24. Completion of external gills (lasted 5 hours and 56 minutes): Primary external gill reached maximum length and secondary external gill ramified. Optic vesicles now become black (Figure 3, 24b), and mouth movement was observed. Besides, tadpoles tended to flock together with head up. Black pointed stripes became obvious

Table 2 Embryonic developmental stages of *Microhyla fissipes* (stages 1–28 obtained at 23.5 ± 0.6 °C; stages 29–45 at 24.6 ± 0.8 °C).

Stage number	Stage	Time (h: m)	Total length (Mean ± SD) (n = 6, mm)	Stage number	Stage	Time (h: m)	Total length (Mean ± SD) (n = 6, mm)
1	Early cleavage	0:00	0.976 ± 0.023	24	Completion of external gills	54:31	4.299 ± 0.263
2	Late cleavage	0:10	0.978 ± 0.022	25	Opercular development I	60:27	4.490 ± 0.261
3	First cleavage (2 cells)	0:32	0.979 ± 0.043	26	Opercular development II	67:46	4.654 ± 0.325
4	Second cleavage (4 cells)	0:45	0.993 ± 0.005	27	Opercular development III	72:56	5.370 ± 0.392
5	Third cleavage (8 cells)	0:58	1.016 ± 0.004	28	spiracle completed	82:36	6.294 ± 0.709
6	Forth cleavage (16 cells)	1:13	1.005 ± 0.003	29	Limb I	8d	9.207 ± 0.245
7	Fifth cleavage (32 cells)	1:26	1.014 ± 0.005	30	Limb II	11d	10.251 ± 0.273
8	Morula	1:41	1.022 ± 0.006	31	Limb III	14d	11.603 ± 0.266
9	Early blastula	2:41	1.036 ± 0.011	32	Appearance of knee junction	17d	15.151 ± 0.061
10	Late blastula	5:18	1.053 ± 0.011	33	oar-shaped limb bud	19d	17.338 ± 0.141
11	Appearance of blastoporic lip	6:13	1.043 ± 0.068	34	Appearance of 4th and 5th toes	21d	17.973 ± 0.344
12	Early gastrula	6:54	1.016 ± 0.027	35	Appearance of 3rd toe	24d	18.877 ± 0.360
13	Mid-gastrula	8:24	0.968 ± 0.036	36	Appearance of 1st and 2nd toes	26d	19.735 ± 1.235
14	Late gastrula	9:44	1.050 ± 0.018	37	Growth of hind limb I	28d	19.604 ± 1.748
15	Neural plate	11:32	1.092 ± 0.057	38	Growth of hind limb II	31d	21.497 ± 0.668
16	Neural fold	12:52	1.172 ± 0.051	39	Appearance of metatarsal tubercle	33d	22.004 ± 0.471
17	Closure of neural fold	14:33	1.472 ± 0.098	40	Appearance of tubercles below digital	35d	22.267 ± 0.858
18	Neural tube	15:36	1.742 ± 0.087	41	Involution of cloacal tail piece	38d	20.460 ± 1.158
19	Tail bud I	17:21	1.767 ± 0.119	42	Emergence of forelimbs	40d	19.001 ± 1.769
20	Tail bud II	19:09	1.957 ± 0.074	43	Degeneration of tail I	41d	13.624 ± 1.883
21	Tail bud III	23:22	3.143 ± 0.409	44	Degeneration of tail II	42d	8.143 ± 0.269
22	Appearance of gill bud	31:39	3.502 ± 0.229	45	Completion of metamorphosis	43d	6.945 ± 0.259
23	Stretching of gills	42:26	3.797 ± 0.088				

dorsally from head to tail, and blood circulation in tails could be seen under a microscope.

Stage 25. Opercular development I (lasted 7 hours and 19 minutes): Opercular folds appeared and epiderm became to be transparent.

Stage 26. Opercular development II (lasted 5 hours and 10 minutes): Opercular folds continued to develop, started to cover gills. The stripe around the gills was increased.

Stage 27. Opercular development III (lasted 9 hours and 40 minutes): Mouth became larger and shifted to the anterior side of the head. The heart becomes visually observable from ventral side due to the transparent epidermis.

Stage 28. Completion of spiracle (lasted 3 days): Spiracle can be observed in the middle of posterior ventral position and embryos could keep balance in the water. Silver cells were obvious ventrally and the boundary between the back and the transparent parts were clear.

Stage 29. Limb I (lasted 3 days): Spur-like hind limb buds appeared and the golden spiral gut in the belly became obvious.

Stage 30. Limb II (lasted 3 days): The hind limb buds at this stage was characterized by the length was half of width (length of hind limb=1/2 width) (Figure 3, 30b).

Stage 31. Limb III (lasted 3 days): The hind limb buds length became approximately equal to the width (Figure 3, 31b).

Stage 32. Appearance of knee junction (lasted 2 days): Length of hind limb buds were 1.5 times of the width and knee junction was evident.


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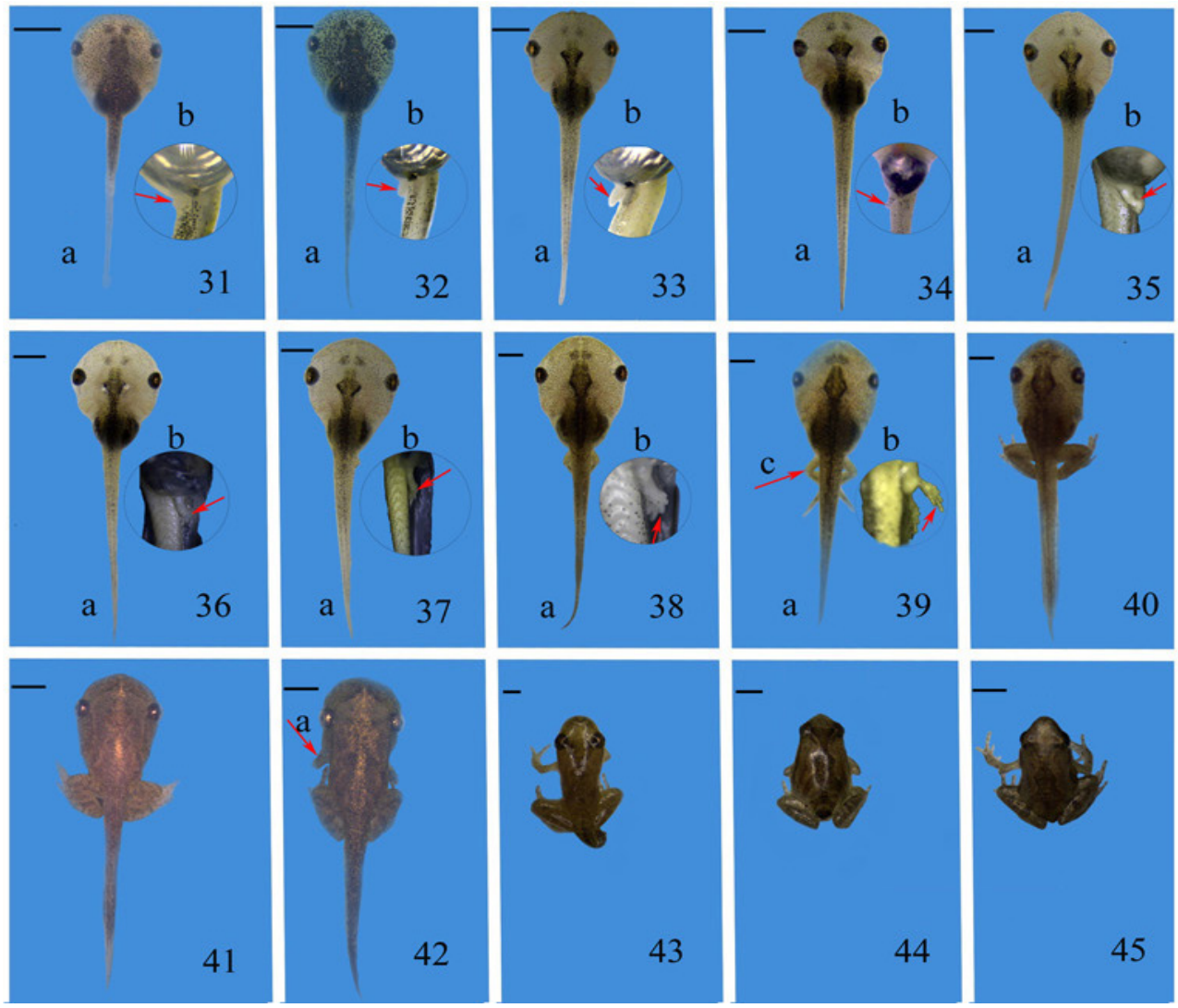

Figure 3 *Microhyla fissipes* embryonic developmental stages. Fertilization (1–2), 2-a show cleavage groove; segmentation (3–10); gastrulation (11–14), 13-a show blastopore, lateral view; neurulation (15–18), dorsal view; Tail development (19–21), 20-a, b show dorsal and ventral fin, 19-20 show dorsal view, 21 show lateral view; external gills development (22–24), lateral view, red arrows(b) show the development of optic vesicles, red arrows(a) show external gills development; Opercular development (25–27), dorsal view; completion of spiracle (28), dorsal view; limb bud (29–33)a, dorsal view, the insets, (30–33)b (red arrows) show limb bud, lateral view; toes development (34–38), a show dorsal view, b (red arrows) show toes differentiation, lateral view; appearance of metatarsal tubercle, 39b (red arrow) show metatarsal tubercle, lateral view, 39c(red arrow) show limb shape (like N), dorsal view; subarticular tubercles appearing (40), dorsal view; disappearance of vent tube (41), dorsal view; eruption forelimbs (42), a show forelimb, dorsal view; tail resorption (43–44), dorsal view; scale bars are 0.4 mm (1–28) and 2mm (29–45).

Stage 33. Oar-shaped limb bud (lasted 3 days): Length of hind limb buds doubles the width and the tip part of the hind limb was oar-shaped.

Stage 34. Appearance of 4th and 5th toes (lasted 3 days): The 4th and 5th toes appeared at the bud of the foot paddle.

Stage 35. Appearance of 3rd toe (lasted 2 days): The 3rd toe appeared, and the two thirds of the anterior tail fin developed while the last one third of the tail was finless.

Stage 36. Appearance of 1st and 2nd toes (lasted 2 days): 1st and 2nd toes appeared and foots were distinct. Toes differentiation was finished at this stage, which appeared 5 toes.

Stage 37. Growth of hind limb I (lasted 3 days): Except 1st and 2nd toes, others elongated and webbed. Two hind limbs could be seen from the dorsal part.

Stage 38. Growth of hind limb II (lasted 2 days): 1st and 2nd toes elongated, and the forelimbs reached at the outermost layer of the 8th spiral golden bowel covered by transparent pectoral skin ventrally.

Stage 39. Appearance of metatarsal tubercle (lasted 2 days): Metatarsal tubercle appeared and tail fin reached to the end of tail. The hind limbs were N-shaped dorsally (Figure 3, 39c), and it was easy to see the forelimbs covered by transparent pectoral skin. The 8th spiral golden bowel could be seen from the ventral and forelimbs reached to the 3rd circle which was far away from heart.

Stage 40. Appearance of tubercles below digital joints (lasted 3 days): Tubercles developed on toes just beneath joints and the shape of hind limbs was more like a froglet.

Stage 41. Involution of cloacal tail piece (lasted 2 days): Cloacal tail piece began to change at base and the tail fin which at end of tail started to degenerate. Forelimbs, covered by transparent pectoral skin, were distinct and reached above heart.

Stage 42. Emergence of forelimbs (lasted 1 day): Forelimbs erupted from the opercular fold (Figure 3, 42a), the shrinkage of head, the resorption of gills and the tail started to degenerate. Mouth became wider and the end of mouth was away from the anterior margin of eyes.

Stage 43. Degeneration of tail II (lasted 1 day): Tail was further diminishing and toe webs began to degenerate. Eyeballs started to protrude, and the lateral ends of mouth reached beneath anterior part of eyes and spiracle disappeared.

Stage 44. Degeneration of tail III (lasted 1 day): The tail continued to degenerate into a stub, and the lateral ends of mouth reached beneath posterior part of eyes.

Stage 45. Completion of metamorphosis: Tail degenerating completed and the tadpole became a froglet.

4. Discussion

This is the first comprehensive and complete embryonic development of *M. fissipes*. Here, 43 days (at WT 22.9–25.4°C) were needed from zygote to the end of metamorphosis (Figure 3, stages 1–45). With regard to early embryonic development (stages 1–28), our results mainly refer to the fertilization, cleavage, blastula, gastrulation, neurula, tail elongation, eye development, gill development and branching. Knowing the characteristics of this given terms is of importance for distinguishing each specific stage. For example, cleavage phase (stages 3–7) is mainly identified by cells number and stages 8–10 are differentiated by the size of cells. Besides, there are distinct features in some stages. For instance, latitudinal cleavage groove appears firstly at stage 5 and the zygote surface of stage 10 becomes smooth. The anterior-posterior and dorsal-ventral axis first become apparent in gastrulation phase (stages 11–14), while blastopore forms at stage 11 and small protruding plug of yolk cells gradually disappears at stage14. Accomplishing neural development at stages15–18, it is marketed by the elongation of the embryo, neural groove and neural tube formation. Eye development phase (stages 22–24) is identified by the tadpole optic vesicles' colour while the external gill development is finished in this phase. Then it is differentiated by the development of opercular and a mid-ventral spiracle which present at stage 28.

Limb formation and development, and metamorphosis are the two important processes of postembryonic development (stages 29–45). Normally, the hind limbs development and toes differentiation are utilized to identify stages 29–36 (Gosner, 1960; Shimizu and Ota, 2003), of which stages 29–32 are easily distinguished by the length/width ratio of the developing hind limb bud. Besides, the hind limb is oar-shaped at stage 33 (Figure 3, 33b), preparing toes differentiation. Hind limbs differentiation happens from stage 34 to 36, thus, toes number is utilized for confirming a stage at this phase and toes differentiation is finished at stage 36 (Figure 3, 36b). Stages 37–40 are identified through the appearance of metatarsal and subarticular tubercles, and the hind limbs dorsally forms N-shaped letter (Figure 3, 39c) at stage 39. Following stage 40, more drastic changes of metamorphosis begin. The disappearance of anal tube may appear at stage 41 or shortly thereafter. Distinctly tail absorption and metamorwphosis of head which indicate by mouth transformation are utilized to confirm the stages 42–45. Among these phases, stage 42 is special and important because the eruption of the forelimbs through the opercular wall (Zhao *et al*., 2016). At last, metamorphosis of this species is completed at stage 45.

Xenopus is an established and powerful model organism for the study of embryogenesis in vertebrates and their eggs and embryos are outstanding tools in basic biology and biomedical research (Liu *et al.*, 2016). The egg diameter of *M. fissipes* (0.9–1.0 mm) is between that of *Xenopus tropicalis* (0.7–0.8 mm) and *Xenopus laevis* (1–1.3 mm), which is large enough to readily permit microsurgical manipulation and injection (Hirsch *et al*., 2002). Regarding the sexual maturity time, *X. laevis* needs over a year to reach sexual maturity. Besides, it belongs to allotetraploid origin. Though the generation time of *X. tropicalis* is reported to be 3–4 months and it is belong to the diploid genome (Hirsch *et al*., 2002). *Xenopus*, however, lives in the water all its life, which lacks the information of the function, mechanism and evolution from aquatic to terrestrial phases (Liu *et al.*,

2016). Furthermore, *M. fissipes* has a much shorter generation time (about 9 months) and diploid genome (Fei *et al*., 2009), suggesting that, when comparing with *Xenopus*, *M. fissipes* could be an ideal model species to study adaptive mechanism from aquatic to terrestrial phases and embryo and organ development, especially lung and muscle growth (Liu *et al*., 2016).

Also, some other external morphology of *M. fissipes* tadpoles, like single mid ventral spiracle, dorsoterminal mouth, transparent body, quite rapid development and deep ventral tail fin (compared to the dorsal tail fin), are similar to other microhylid tadpoles (Padhye and Ghate, 1989; Bowatte and Meegaskumbura, 2011; Narzary and Bordoloi, 2013). These characteristics will provide a foundation for research into complex developmental phenomena, comparative embryology and phylogenetic analyses.

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