Uttar Pradesh Journal of Zoology

Volume 44, Issue 5, Page 15-24, 2023; Article no.UPJOZ.2478
ISSN: 0256-971X (P)

Stages of Development and Metamorphosis of Tree Frog Rhacophorus Bipunctatus (Amphibia, Anura, Rhacophoridae)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.56557/UPJOZ/2023/v44i53438

Editor(s):
(1) Dr. Belisario Dominguez-Mancera, University Veracruz, Mexico.
Reviewers:
(1) Suresh Chandra Joshi, Kumaun University, India.
(2) Jacob Mueti Ngwava, National Museums of Kenya, Kenya.

Original Research Article

Received: 22/01/2023
Accepted: 25/03/2023
Published: 03/04/2023

ABSTRACT

Amphibians are the highest vertebrate group to retain an essentially naked egg and the only vertebrate group which has an aquatic larval stage and a terrestrial adult phase. One of the prominent life history characteristics common to most living amphibians is the presence of an aquatic larval period, which immediately follows the initial embryonic development after fertilization and ends with the completion of metamorphosis. Metamorphosis in anurans involves resorption of the tail, development of the front and hind limbs and large changes in most organ system. Proper staging of the larval period is therefore necessary for accurate comparison of developmental stages in different organisms. Hence, several workers [1-3] have reported on tables of normal stages of development for a number of species. Keeping in view the paucity of information on the development and metamorphosis of tree frogs from Meghalaya, it was decided to study the

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development and metamorphosis of the tree frog *Rhacophorus bipunctatus* at Cherrepunjee, Meghalaya. The present study was conducted during the breeding season (April to August). In the present study it was found that, the development and metamorphosis of *Rhacophorus bipunctatus* was completed in about 59 – 60 days under natural environment as well as in the laboratory condition. This tree frog was observed to be dependent on the rain fed pools at the breeding sites to complete their life cycle.

Keywords: Rhacophorus bipunctatus; development; cleavage; metamorphosis.

1. INTRODUCTION

"Among vertebrates, amphibians have been found to be convenient material for analysis of development. Amphibians are the highest vertebrate group to retain an essentially naked egg and the only vertebrate group which has an aquatic larval stage and a terrestrial adult phase. One of the prominent life history characteristics common to most living amphibians is the presence of an aquatic larval period, which immediately follows the initial embryonic development after fertilization and ends with the completion of metamorphosis" [4,5]. "Metamorphosis in anurans involves resorption of the tail, development of the front and hind limbs and large changes in most organ system" [1]. Anuran development has been investigated by several researchers and normal table of development are available for a few number of amphibians. Twenty five developmental stages of a microhylid, *Ramanella variegate* were described [2]. [6] described the direct development in the rhacophorid frog *Philautus variabilis* [3]. Studied the normal development of *Microhyla ornata* [7]. Reported that it took 42 – 57 days for *Leptodactylus fallax* to complete its metamorphosis [8.] Studied the normal table of embryonic development in the four toed salamamder, *Hemidactylium scutatum*. A complete embryonic developmental table of *Microhyla fissipes* have been reported [9] and [10] studied on the development of amphibians. Similarly, [11] described the stages of development and metamorphosis of tree frog *Polypedates leucomystax*. "Studies on the successive ontogenic stages to record the normal developmental table are important in understanding the ecology of a species and for planning conservation measures" [12].

2. MATERIALS AND METHODS

To study the development and metamorphosis of *Rhacophorus bipunctatus*, the investigation was conducted at study sites in the natural environment located at Cherrapunjee, Meghalaya, North East India. These study sites were temporary rain fed pools, which were found to be excellent breeding grounds and served as good habitat for the development and metamorphosis of this tree frog. Observations on the development and metamorphosis of *Rhacophorus bipunctatus* was conducted at two sites (Cherrapunjee) and also under laboratory. The time of spawning and fertilization was taken as 0 hours. Various stages of the development and metamorphosis were recorded and staging was done according to [13]. Foam nests were collected during the breeding phase (April – August) just after they were constructed at the study sites and brought to the laboratory for further studies. The time of egg laying was taken as the time of fertilization. Development of the eggs was observed with the help of a stereoscopic dissecting binocular microscope during field observations. The study sites were visited at regular intervals to observe and record the development and metamorphosis of *Rhacophorus bipunctatus* in the natural environment.

Some of the foam nests collected from the study sites were maintained in a plastic tray containing pond water to allow further development and metamorphosis, which were observed under a stereoscopic dissecting binocular microscope (Meopta DM 2366223). The time of onset of each new stage was noted and some developmental stages and tadpoles were anesthetized and then were fixed in 4% formaldehyde. "Staging of the new anuran embryos and larvae of *Rhacophorus bipunctatus* was carried out on the basis of a new external morphological change as per the criteria described by" [13]. Photographs of the developmental stages were taken with the help of a binocular (Magnus MLX) with photographic attachments. Photographs of the different developmental and metamorphic stages were taken at different magnification. The hatched tadpoles were reared in a plastic tray containing pond water and fed daily with boiled cabbage and algae under laboratory conditions (temperature 20^0C – 22.5^0C).

3. RESULTS

*Rhacophorus bipunctatus*was found to construct its foam nest attached to vegetation always above the water surface or the leaves of a plant hanging over a water body. A brief account of the stages of development and metamorphosis are given below -

- **Stage 1 – Fertilized egg:** The fertilized egg was spherical in shape and light yellow in colour and measured 3.0 (Fig. 1).
- **Stage 2 – Stage 6:** It was observed that when the eggs were removed from the foam nest after 1 hour of spawningand fertilization, early embryonic stages of development (stage 2 – stage 6) could not be observed due to the presence of large amount of yolk. In addition, removal of the eggs from the foam nests at stage 1 before 5 hours (stage 2 – stage 6) was found to obstruct further development. Therefore, the eggs were removed from the foam nest only after 5 hours of spawning and fertilization (after stage 6) to observe further development.
- **Stage 7 – Thirty two cell stage:** Five hours after fertilization, the eggs were found to reach the cleavage stage 7. The egg was round in shape measuring 3.20 mm and composed of a number of blastomeres (Fig. 2).
- **Stage 8 – Mid cleavage:**Seven hours after fertilization, the embryo developed further reaching mid cleavage stage 8 and appeared as a compact ball of cells. The number of blastomeres could not be clearly identified due to the presence of large amount of yolk (Fig. 3).
- **Stage 9 – Late cleavage:**The late cleavage was recorded after 14 hours 5 minutes. At this stage, the blastomeres were further divided and appeared smaller in size and the egg was considered as the blastula stage (Fig. 4).
- **Stage 10 – Dorsal lip:** During this stage, the embryo changed its shape and became slightly oval. Involution of cells at the dorsal lip of blastopore indicated the beginning of gastrulation, which was observed after 25hours 15 minutes (Fig. 5).
- **Stage 11 – Mid gastrula:** The blastopore appeared semi-circular and protruded constisting protruded consisting of the yolk plug the yolk plug after 30 hours 30 minutes.
- **Stage 12 – Late gastrula:** The size of the blastoporal lip was reduced, and the yolk plug was observed to be smaller in size and diminished after 35 hours and 30 minutes
- **Stage 13 – Neural plate:** The embryo became slightly elongated and the neural plate developed as a tubular area on the dorsal surface after 37 hours 5 minutes (Fig. 6).
- **Stage 14 – Neural fold:** This stage was marked by further elongation of the embryo and measured 4.30 mm. Distinct elevation of the two lateral ridges separated by neural groove was observed. Neural folds became distinct after 40 hours and 25 minutes (Fig. 7).
- **Stage 15 – Rotation:** The embryo was oval in shape and measured 4.45 mm. Neural folds were distinct and approached and met each other, and the neural groove formed after 43 hours 45 minutes (Fig. 8).
- **Stage 16 – Neural tube:** The embryo was further elongated and the neural folds were fused completely to form the neural tube after 45 hours 40 minutes. The embryo measured 4.56 mm (Fig. 9).
- **Stage 17 – Tail bud:** This stage was marked by the formation of the tail bud at the posterior end. The tail was observed to curve to the left side after 53 hours and 15 minutes. The embryo measured 4.75 mm and the yolk was still present (Fig. 10).
- **Stage 18 – Muscular response:** Anterior region was well defined with prominent optic bulges after 60 hours 50 minutes. Embryo showed muscular response and measured 4.78 mm (Fig. 11)
- **Stage 19 – Heart beat:** Pulsation of the heart was seen below and behind gill bud, and yolk was still present.Rudimentary gill buds became prominent and the tail was greatly elongated after 71 hours and 20 minutes. The embryo measured 5.51 mm (Fig. 12).
- **Stage 20 – Gill circulation:** After 82 hours45 minutes gills were developed and branched into gill filaments. Opercula fold covered the base of the gills, and oral sucker became well developed. Melanophores appeared on the dorsal surface, and the embryo measured 6.64 mm (Fig. 13).

- **Stage 21 – Cornea transparent:**The larva hatched out of the foam nest after 4 days and tail became straight and elongated. Gills had developed. Cornea was transparent. Oral suckers and nasal pits became prominent and the larva measured 8.25 mm (Fig. 14).
- **Stage 22 – Tail fin circulation:** Tail fins become transparent. Melanophores present on the dorsal surface dispersed towards the ventral surface and tail region. Mouth became distinct and the tadpole measured 9.34 mm after 6 days (Fig. 15).
- **Stage 23 – Opercular fold formation (Opercular and pigmentation):** The operculum developed after 8 days. Gills length shortened. Small and large melanophores developed on the body surface. The tadpole measured 10.55 mm (Fig. 16).
- **Stage 24 – Opercular fold closed on right side:**After 12 days, right operculum fold closed and left external gill shortened, and the larva measured 12.25 mm (Fig. 17).
- **Stage 25 – Opercular fold closed on left side:**The left operculum fold was observed to close after 19 days. Operculum formation was completed, and spiracle was formed on the left side. Gills disappeared, and the tadpole measured 13.45 mm. The tadpole started feeding during this stage, and only a few rows of teeth were present (Fig. 18).
- **Stage 26 – Hind limb bud development:** Limb buds appeared at the junction of the trunk and tail on either side of the cloacal tail after 22 days. Coiled intestine became visible. The length of the limb bud was smaller than its diameter and the tadpole head length was 10 mm and the tail length was 8 mm.Melanophores were observed to be present on the tail region. The oral structure was further developed than stage 25 with a number of teeth rows on the upper and lower labium which were not so well distinct (Fig. 19).
- **Stage 27 – Length of limb bud equaled half its diameter:** After 25 days the length of the limb bud increased in size and its length equaled to half of its diameter. The tadpole head length was 10mm and the tail length was 18 mm. Melanophores appeared to be more denser than in the early stages, and the oral structure was similar to that of stage 26 (Fig. 20).
- **Stage 28 – Length of limb bud equaled to its diameter:** Length of limb bud equaled its diameter after 27 days. The tadpole head length was 10 mm and the tail length was 19 mm. Melanophores were also found to be present at the base region of the limb bud. In the tail region, melanophores were found to be concentrated in small patches. Oral structure was found to be the same as observed in the stages 26 and 27 (Fig. 21).
- **Stage 29 – Length of limb bud equaled to one and half time its diameter:** After 30 days, the length of limb bud increased to one and half time its diameter. The tadpole head length was 10 mm and the tail length was 21 mm. Melanophores was observed to be dispersed in the limb bud region, and no differences were observed in the oral structure (Fig. 22).
- **Stage 30 – Length of limb bud was twice its diameter:** The length of limb bud further increased and was slightly curved after 33 days. The tadpole head length was 11 mm and the tail length was 21 mm. Dispersion of melanophores was found to be denser at the tail region and had dispersed towards the tip of the limb bud. There were no changes in the oral structure (Fig. 23).
- **Stage 31 – Foot paddle (Toe differentiation and development):** The distal end of the limb bud curved slightly and was spatula-shaped forming the foot paddle after 35 days. The tadpole head length was 11 mm and the tail length was 22 mm. At this stage, melanophores were also found to be present at the tip of the limb bud. There were some changes in the oral structure and the teeth rows were also found to be well developed. From this stage onwards till stage 39 maximum number of teeth rows was observed on the upper and lower labium with complete dental formula (Fig. 24).
- **Stage 32 – First indentation:**The foot paddle became slightly indented on the dorsal side, which marked the margin of the fourth and fifth toe after 37 days. The tadpole head length was 12 mm and the tail length was 22 mm. At this stage melanophores started to disperse toward the tip of the limb bud. Oral structure was the same as that in the earlier stage (Fig. 25).

- **Stage 33 – Second indentation:** After 38 days, the margin of the foot paddle became indented on the ventral side behind the prominence of the fourth toe, which marked the third, fourth and fifth toes. The tadpole head length was 12 mm and the tail length 23 mm. Melanophores were now observed on the regions, which marked toe formation. Oral structure still remained the same as that in the earlier stage (Fig. 26).
- **Stage 34 – Third indentation:** The margin of foot paddle became indented on the ventral side behind the third toe, which marked the prominence of second, third, fourth and fifth toes after 39 days. The tadpole head length was 12 mm and the tail length was 24 mm. Patches of melanophores were observed at the tail region, and melanophores were found to be denser at the base of the limb bud region. There were no changes in the oral structure (Fig. 27).
- **Stage 35 – Fourth indentation:** The margin of foot paddle became indented behind the second toe demarcating the prominence of first toe and all five toes were demarcated from each other after 40 days. The tadpole head length was 13 mm and the tail length was 24 mm. At the base of the limb bud, melanophores had decreased and moved towards the region of toe formation. Oral structure remained the same (Fig. 28).
- **Stage 36 – First and second toes joined:** After 41 days, first and second toes were still joined, while the third, fourth and fifth toes were separated. The tadpole head length was 13 mm and the tail length was 26 mm. Only few melanophores were found scattered at the base of the limb bud concentrated in the tip region. Oral structure still remained the same (Fig. 29).
- **Stage 37 – Toes separated:** All five toes were completely separated and webbed after 42 days. The tadpole head length was 14 mm and the tail length was 27 mm (Fig. 30).
- **Stage 38 – Metatarsal tubercles appeared:** There was appearance of metatarsal tubercles after 44 days, and the tadpole head length was 14 mm and tail length was 27 mm (Fig. 31).
- **Stage 39 – Sub articular tubercles appeared:** Sub articular tubercles appeared after 46 days, and the tadpole head length was 14 mm and the tail length was 29 mm (Fig. 32).
- **Stage 40 – Toes pad complete:**Toes were fully webbed and distal ends with developing disc developed after 49 days. The tadpole head length was 15 mm and the tail length was 29 mm. Teeth started to shed from this stage onwards (Fig 33).
- **Stage 41 – Cloacal tail piece reduced:** After 51 days, cloacal tail piece of the tadpole started to reduce in size. The tadpole head length was 15 mm and the tail length was 27 mm. Head of tadpole is more or less circular. Forelimbs visible and few rows of teeth were observed at the upper and lower labium (Fig. 34).
- **Stage 42 – Both limbs developed:** Tadpoles with well developed both fore limbs and hind limbs were observed after 52 days. The head of the tadpole changed in shape and was broader at the anterior end, measured 16 mm, tapered towards the tail region and the tail length measured 27 mm. Melanophores were clearly seen in patches at the tail region and even in the fore and hind limbs. Further shedding of teeth was observed (Fig. 35).
- **Stage 43 – Resorption of tail begins:** The tail of the tadpole tail started to regress after 54 days. The tadpole head length was 16 mm and the tail length was 25 mm. Melanophores had decreased in the tail region. Complete shedding of the teeth was found (Fig. 36).
- **Stage 44 – Tail shortened:**There was gradual loss of the tadpole tail after 57 days and was now shorter. The tadpole head length was 17 mm and the tail length was 16 mm. Formation of tongue was observed (Fig. 37).
- **Stage 45 – Tail stub:** After 59 days, tail was completely lost and only a stub remained which measured 2 mm, and the tadpole measured 22 mm. The formation of tongue was completed (Fig. 38).
- **Stage 46 – Metamorphosis completed:** Tail was completely absorbed and adult characteristics appeared after 59 – 60 days (example, dorsal surface leafy green and ventral is yellowish, and webs were red in colour). Metamorphosis was completed, and the froglet measured 21 (Fig. 39).


Fig 1: Fertilized egg


Fig 2: Thirty two cell


Fig 3: Mid cleavage

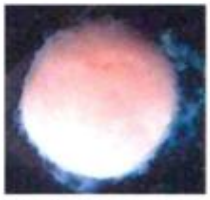
Fig 4: Late cleavage


Fig 5: Dorsal lip


Fig 6: Neural plate


Fig 7: Neural fold


Fig 8: Rotation


Fig 9: Neural tube

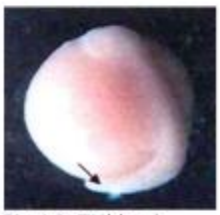
Fig 10: Tail bud


Fig 11: Muscular response


Fig 12: Heart beat

Fig 13: Gill circulation

Fig 14: Cornea transparent

Fig 15: Tail fin circulation

Fig 16: Opercular fold formation

Fig 17: Opercular fold close on the right

Fig 18: Opercular fold close on the left

Fig 19: Hind limb bud

Fig 20: Length of limb bud equaled half its diameter

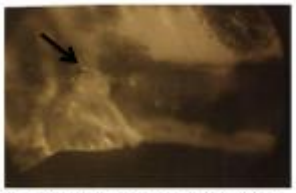

Fig 21: Length of limb bud equaled to its diameter

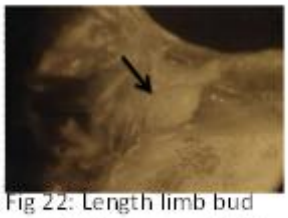

Fig 22: Length limb bud equaled to one and half its diameter

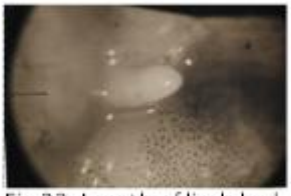

Fig 23: Length of limb bud equal to twice its diameter

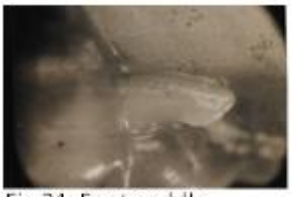

Fig 24: Foot paddle

Fig 25: First indentation

Fig 26: Second indentation

Fig 27: Third indentation

Fig 28: Fourth indentation

Fig 29: 1st and 2nd toes joined

Fig 30: Toes separated

Fig 31: Metatarsal tubercle

Fig 32: Subarticular tubercle

Fig 33: Toes pad complete

Fig 34: Cloacal tail piece reduce

Fig 35: Both limbs develop

Fig 36: Resorption of tail begins

Fig 37: Tail shortened

Fig 38: Tail stub

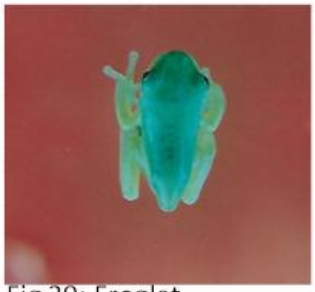

Fig 39: Froglet

4. DISCUSSION

On the basis of the present findings, the development and metamorphosis of *Rhacophorus bipinctatus* was completed in about 59 – 60 days under the natural environment aswell as laboratory conditions. Hatching was observed after 4 days of fertilization, which indicates a rapid developmental rate. The eggs of *Rhacophorus bipinctatus* were found to be soft and delicate, and yellow in colour, and separation of the eggs were difficult due to the sticky nature of the foam nests. The eggs of *Rhacophorus bipinctatus* contained a large amount of yolk. As a result, few early embryonic stages (stage 2 – stage 6) were difficult to observe and identify due to the soft and sticky nature of the foam nests. When the eggs were removed from the foam nests before hatching, the embryos did not develop further. Deuti [14] also reported that in *Chirixalis simus*, the fertilized egg develop normally only in the foam nests. Earlier workers [15] have also reported that foam nests protect the eggs and embryos from predators and desiccation.

During development and metamorphosis, comprehensive and dramatic changes took place where the aquatic tadpole was transformed into a terrestrial adult. Some of these changes were development of the hind limb and fore limbs, which were observed after 52 days. The oral structure of the tadpole was observed to develop further and complete teeth rows were observed during this phase. Teeth rows began to shed from stage 40 onwards, which marked the onset of metamorphosis, and there was resorption of the tail. Complete absorption of the tail took place after 59 – 60 days which marked the end of metamorphosis. Just and Kraus-Just [16] have also reported that during the larval period amphibians, anurans in particular, exhibit a series of dramatic morphological changes like tail formation, perforation and closure of the spiracle, limb formation, tail reduction, etc. The changing appearance of embryos, especially during organogenesis necessitates a method of quantifying the progress of development.

5. CONCLUSION

Hatching was observed after four days of fertilization indicating rapid developmental rate, which might be advantageous for this tree frog in ephemeral habitats which allow larvae to metamorphose quickly and escape desiccation. The foam nests seem to provide optimal conditions for the early development and ensure successful normal hatching of the larva. The small clutch size, large size of the egg and presence of large amount of yolk in *Rhacophorus bipunctatus* might not only be be an adaptation to its arboreal mode of life and higher altitude habitat but also an adaptationto ensure high chances of survival of the embryo away from water bodies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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