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RESEARCH ARTICLE

Developmental Aspects of the Direct-Developing Frog *Adelophryne maranguapensis*

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Summary: Direct development in amphibians is characterized by the loss of aquatic breeding. The anuran *Adelophryne maranguapensis* is one example of a species with direct development, and it is endemic to the state of Ceará, Brazil. Detailed morphological features of *A. maranguapensis* embryos and the stages of sequential development have not been described before. Here, we analyzed all available genetic sequence tags in *A. maranguapensis* (*tyr* exon 1, *pomc* and *rag1*) and compared them with sequences from other species of *Adelophryne* frogs. We describe the *A. maranguapensis* reproductive tract and embryonic body development, with a focus on the limbs, tail, ciliated cells of the skin, and the egg tooth, which were analyzed using scanning electron microscopy. Histological analyses revealed ovaries containing oocytes surrounded by follicular cells, displaying large nuclei with nucleoli inside. Early in development, the body is unpigmented, and the neural tube forms dorsally to the yolk vesicle, typical of a direct-developing frog embryo. The hindlimbs develop earlier than the forelimbs. Ciliated cells are abundant during the early stages of skin development and are less common during later stages. The egg tooth appears in the later stages and develops as a keratinized microridge structure. The developmental profile of *A. maranguapensis* presented here will contribute to our understanding of the direct-development model and may help preserve this endangered native Brazilian frog. genesis 54:257–271, 2016. © 2016 Wiley Periodicals, Inc.

Key words: *Adelophryne maranguapensis*; embryo; reproductive tract

INTRODUCTION

The ancestral life history of most amphibians is biphasic, meaning that embryos develop into a feeding larva that swims in an aquatic habitat and undergoes metamorphosis to reach the adult form (Callery, 2006; Duellman and Trueb, 1994). However, amphibians have evolved a wide variety of reproductive strategies in response to different habitats and living conditions, which is particularly true for species in tropical rainforests (Desnitskii, 2004). Ontogenic strategies can differ widely from the biphasic ancestral model, as represented by species such as *Xenopus laevis* (Chipman, 2002; Elinson and del Pino, 2012). The most divergent ontogeny is direct development, in which amphibians omit the free-living larval stage, laying their large yolk-enriched eggs on damp ground or on plant leaves. The embryo develops in moist environments, is

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characterized by the presence of a large yolk reserve, and hatches from the egg as a miniature adult. Members of all three orders of Amphibia (Caudata, Anura, and Gymnophiona) display direct development. However, their modes of embryonic development and the morphological properties that occur during these developmental stages are poorly understood. Such information could provide important clues regarding adaptive changes made to deal with specific environments and help explain why this reproductive model was successfully retained among anurans. Direct development could represent the diversification of a biological process used to cope with certain environmental challenges, with developing embryos exhibiting specific morphological features to assure the evolutionary success of their lineages (Callery, 2006).

One example of a species with direct development is the anuran *Adelophryne maranguapensis*, a little-known species endemic to a small remnant of the Atlantic Forest on Maranguape Mountain in the state of Ceará, northeastern Brazil. *Adelophryne maranguapensis* was first described by Hoogmoed *et al*. in an exploratory study (1994). Currently this species is included in the endangered category on the global Red List due to its presence in a region subject to human impact and its small range of less than 5,000 km^2 (www.iucnredlist.org).

The genus *Adelophryne* belongs to the family Eleutherodactylidae and the subfamily Phyzelapryninae, together with the genus *Phyzelaphryne*. It is thought that the genus *Adelophryne* arose in the northeastern Amazon basin and dispersed into the Atlantic Forest around 23-16 MYA, originating three geographically circumscribed clades: Northeast Amazon Clade (NAMC), Northeast Atlantic Forest Clade (NAFC), and Southeast Atlantic Forest Clade (SAFC). Therefore, the genus *Adelophryne* has an allopatric distribution reaching from the northern Amazon to relict areas of the Atlantic Forest. *Adelophryne maranguapensis* belongs to NAFC clade, and it also constitutes a monophyletic group, distinguished from other Terrarana species by the morphology of its terminal digits (Fouquet *et al*., 2012).

As *A. maranguapensis* has never been successfully mated or spawned in the laboratory, its sequential early and late embryonic development is almost entirely unknown. In addition, it is not clear whether its developing structures are comparable with other direct-developing frogs, such as *Eleutherodactylus coqui* (Thomas, 1966), a species with well-documented morphogenesis and gene expression (Gross *et al*., 2011; Townsend and Stewart, 1985; Townsend *et al*., 1981), or with the indirect-developing *X. laevis*. Therefore, we compared the sequence tags from the *tyr* (exon 1), *pomc* and *rag* genes with those of other known frog species. We describe the embryonic development of this species in sequential developmental stages, and we also describe in detail the development of the limbs, tail, skin, ciliated cells, and egg tooth, which were observed by scanning electron microscopy. Taken together, our findings detail crucial novel developmental aspects of *Adelophryne maranguapensis* and reveal numerous key early and late morphological features of this endangered species.

The developmental and evolutionary profiles of *A. maranguapensis* presented here contribute to our understanding of the direct development model and may help preserve this endangered native Brazilian frog.

RESULTS

Comparative Dendrogram (*tyr* exon 1, *pomc*, and *rag* Sequence Tags)

The tropical forests of South America are thought to host more species of amphibians than anywhere else in the world (Fouquet *et al*., 2012; Myers *et al*., 2000). The genus *Adelophryne* comprises eight species (*A. adiastola, A. baturitensis, A. gutturosa, A. maranguapensis, A. meridionalis, A. mucronatus, A. patamona* and *A. pachydactyla*), three of which are found in scattered locations in northern Amazonia (*A. adiastola, A. gutturosa* and *A. patamona*) and three in relict patches of the Brazilian Atlantic Forest (*A. baturitensis, A. maranguapensis* and *A. pachydactyla*) (Fouquet *et al*., 2012). The amphibian species from the Atlantic forest are characterized by old divergences and highly conserved morphological properties, which makes them difficult to study based only on their morphology. Moreover, the species from clades that share morphological properties have restricted distributions in isolated locations (Fouquet *et al*., 2012). To compare the gene sequences of *A. maranguapensis* with other frog species possessing direct and indirect development, we analyzed expression sequence tags (ESTs) of the *tyr* (exon 1), *pomc*, and *rag* genes from different *Adelophryne* species using the Neighbor Joining method (Saitou and Nei, 1987). Molecular data concerning the *Adelophryne* genus is poor, and indeed, there is only sequence information for the three genes described in this study. Therefore, we constructed a dendrogram for the three genes available for *A. maranguapensis* (*tyr*, *pomc*, and *rag1*). We observed that *tyr, pomc* and *rag1* are conserved among the five *Adelophryne* species analyzed, and as expected, they show divergence from the indirect-developing *Xenopus laevis* (Fig. 1A). In particular, the *tyr* gene displays at greater than 70% sequence identity with its orthologs (Fig. S1, Supporting Information). Comparative analyses were also performed for the *pomc* and *rag1* genes, and they showed similar patterns of conservation (Fig. 1A). Species and their regional origins are shown in Figure 1B. Interestingly,

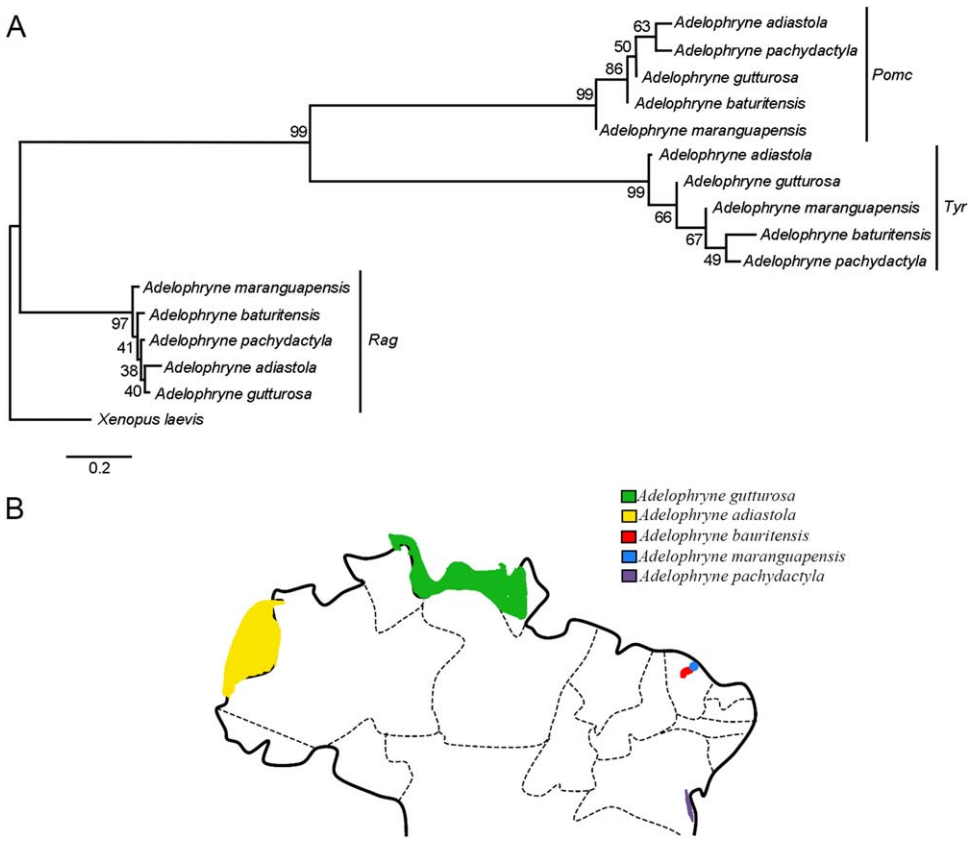

FIG. 1. Phylogenetic and geographical comparisons between *A. maranguapensis* and other South American species. (**A**) Dendrogram comparing the *tyr, pomc*, and *rag1* genes among *Adelophryne* species. (**B**) Biogeographical distribution of select South American frogs; the colors in the map refer to the species in the dendrogram.

the short evolutionary distance between *A. maranguapensis* and *A. baturitensis* correlates with their geographical proximity.

Clutches and the Female Reproductive Tract

A. maranguapensis adults are rarely found, which explains the genus name *Adelophryne*, meaning "invisible frog" (Hedges *et al*., 2008). Macroscopic analysis of the reproductive tract reveals a pair of ovaries and oviducts that occupy most of the abdominal cavity (Fig. 2A). Histological sections showed that the ovary is covered by a simple squamous epithelium and is filled with connective tissue and several follicles at different stages of maturation; the oviduct is lined with a luminal epithelium with ciliated cells and a wall of glandular cells (Fig. 2B). The oocytes show a large nucleus containing multiple large nucleoli near the nuclear membrane and are surrounded by follicular cells (Fig. 2C,D). The morphological features of the specimens accord with the original description, and the reproductive tract is similar to those of other anurans (Dumont, 1972; del Pino, 1989). Clutches of 6–8 eggs are deposited near the water and accumulate in the axils of bromeliad leaves, and in general, adult frogs are not found with the embryos. However, in two clutches, we found a female with early-stage embryos, suggesting some degree of parental care (Fig. 3A). We confirmed the presence of *A. maranguapensis* females on these clutches using previously described morphological characteristics (Hoogmoed *et al*., 1994). The snout-vent length of the *A. maranguapensis* female was found to range between 17 and 22 mm.

Embryonic Developmental Stages

To characterize the embryonic development of *A. maranguapensis*, eggs in early stages of development were collected on Maranguape Mountain during the rainy season. The embryos were maintained in the laboratory under controlled temperature (23°C) and humidity conditions to mimic their natural habitat. Of the described species on Maranguape Mountain, the only anuran that is a bromeligen species is *A. maranguapensis*. Furthermore, eggs at different stages of development and from different clutches were maintained in the laboratory, and ultimately reached the later stages of development and hatched as *A. maranguapensis* froglets, with comparable morphology to the adults. On at least two occasions we observed a female depositing

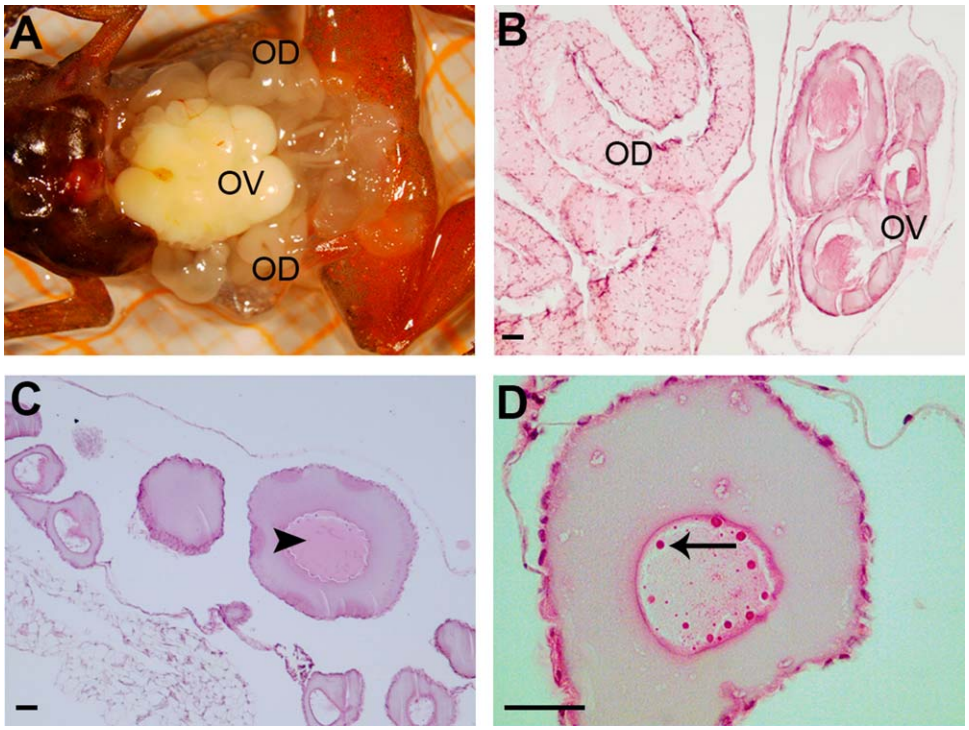

FIG. 2. *Adelophryne maranguapensis* reproductive tract. (**A**) Dissected female, showing the ovary and oviduct. (**B**) Histological section showing the oviduct and the ovary with oocytes. (**C**) Ovarian follicles showing the nucleus (black arrowhead) of the oocyte. (**D**) The nucleoli of the oocyte are shown (black arrow). Ovary (OV); Oviduct (OD). Scale bars: B–D: 50 μm.

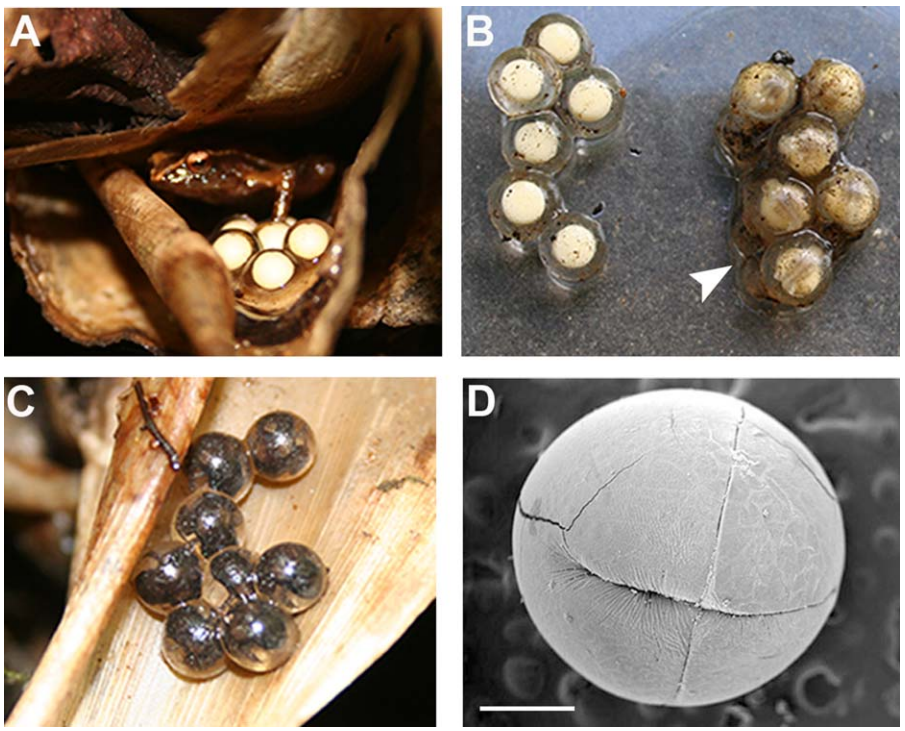

FIG. 3. *Adelophryne maranguapensis* eggs. (**A**) Female on a clutch in early stages of development. (**B**) Early and intermediate (white arrowhead) embryonic development. (**C**) Eggs in a late stage of development on bromeliad leaves. (**D**) Scanning electron micrograph of embryo at the second cleavage stage of development. White arrows indicate the cleavage furrows. Scale bar: H: 500 μm.

eggs on bromeliad leaves, ensuring that the embryos were from this species.

We described the embryonic stages of *A. maranguapensis* based on sequential external morphological changes. Because of the limited number of embryos collected and the different clutches, the timing of the stages was not described. We have classified the stages according to the table of stages described for *Eleutherodactylus coqui* (Townsend and Stewart, 1985).

Early embryos showed a uniform appearance, were unpigmented (white color) and were enveloped externally by a thin vitelline membrane and a thick layer of transparent jelly with a sticky consistency that covered the vitelline membrane; the membrane and jelly covered the eggs from one spawning only (Fig. 3A,B). As the body of *A. maranguapensis* embryos develops on the top of the yolk, the embryo body is visible through the transparent jelly layer, and the eggs show a darker pigmentation, which marks the middle and final phases of development (Fig. 3B,C).

Stage 2. During the earliest stage of embryonic development, the second cleavage groove is apparent (Fig. 3D and Fig. S2, Supporting Information). Scanning electron microscopy of these embryos allowed for observation of the cleavage planes. The first cleavage occurs vertically along the animal-vegetal axis of the egg. The cleavage furrow extends to the midpoint of the egg, forming two large blastomeres on the animal pole. The second cleavage plane can be seen before the first furrow reaches the vegetal pole of the egg, and it is also vertical, albeit perpendicular to the first cleavage plane. After the second division, four large blastomeres are formed (Fig. 3D and Fig. S2, Supporting Information). The furrows are present only on the animal pole of the embryo, following the holoblastic unequal cleavage model, a common feature of all amphibians. We considered zygote formation to be the first stage of *A. maranguapensis* embryonic development. The cleavage stage shown in Figures 3D and S2, Supporting Information was classified as Stage 2, which last until the gastrula stage. Note that this is different from the initial stages of *E. coqui* development, in which first stage is classified as lasting from zygote until blastopore formation and the second stage from blastopore until the first appearance of the neural fold. We classified these stages differently because it was not possible to visualize the initial steps of neural tube formation, such as the appearance of a neural plate or neural fold, and the neural tube is already closed when it is first apparent. Measurements performed on cleavage stage embryos using scanning electron microscopy (SEM) images allowed us to estimate the embryos size to be ~3.0 mm without jelly and the vitelline membrane. Other early embryonic phases, such as the blastula, gastrula, neural plate, and neural fold, were not observed in the collected embryos.

Stages 3 and 4. Later embryos showed a closed neural tube dorsally to the large yolk mass; the anterior region is enlarged compared with the posterior end, and the limb buds emerge as small protuberances laterally to the neural tube (Fig. 4A). We considered this to be Stage 4, as we assumed earlier gastrula and neural tube stages must have occurred prior to this, which we considered as Stage 3, although we did not observe these steps in the embryos we analyzed. Different from the developmental mode proposed for *E. coqui*, at the Stage 3 the neural tube is already closed, and at Stage 4 the first evidence of limb buds appears.

Stage 5. The proposed Stage 5 of *A. maranguapensis* development is characterized by the appearance of the head and tail, the limb buds emerging as differentiated structures, and the onset of the embryo pigmentation, with a few melanophores present on the surface of the embryo body (Fig. 4B). The emergence of early limb buds in *A. maranguapensis* embryos occurs earlier than in *E. coqui*, in which the limb buds appear "detached" from the primary axis of the body (Richardson *et al*., 1998; Hanken *et al*., 2001). The presence of melanophores was not cited at this stage for *E. coqui*, and the hindlimb buds start to elongate in *E. coqui*.

Stage 6. At Stage 6 the anterior limb buds and the posterior limb buds continue growing on the lateral sides of the body. The primary axis of the embryo body is visible on the dorsal midline (Fig. 4C). Anteriorly, the cranial neuropore (CN) is still apparent (Fig. 4C′). Increased pigmentation can be observed along the dorsal midline. The optic vesicles are also apparent at this stage, and the first indication of the presence of endolymphatic calcium deposits is observed (ECD) (Fig. 4C′–C″). Similarly, the ECD is first visible in *E. coqui* at Stage 6.

Stage 7. Stage 7 is marked by the spreading of pigmentation throughout the entire embryo body, with the exception of the yolk mass and the tips of the limb buds and tail. The pigmentation delimits the edge of the body wall, or secondary cover of the embryo. The limb buds start to elongate, and the hindlimbs are more flattened dorsoventrally. The tail shows a central axis and small fins, and it makes up approximately one-third the length of the embryo (Fig. 4D). At Stage 7 of *E. coqui* development, the hind limb buds show foot paddles, and the tail is more developed and with well vascularized fins.

Stage 8. At Stage 8, the hindlimbs are longer than the forelimbs, the tail is ~1/3 of its final length, the pigmented secondary cover is expanding, and there are many ciliated cells; the gills are absent (Fig. 4E). In *E. coqui*, Stage 8 is characterized by a clear pupil and a gray iris in the eye, digit rudiments are first noticeable on the feet, and the tail is 1/2 of its final length.

Stage 9. During Stage 9, the hindlimbs undergo a medial constriction along the proximodistal axis,

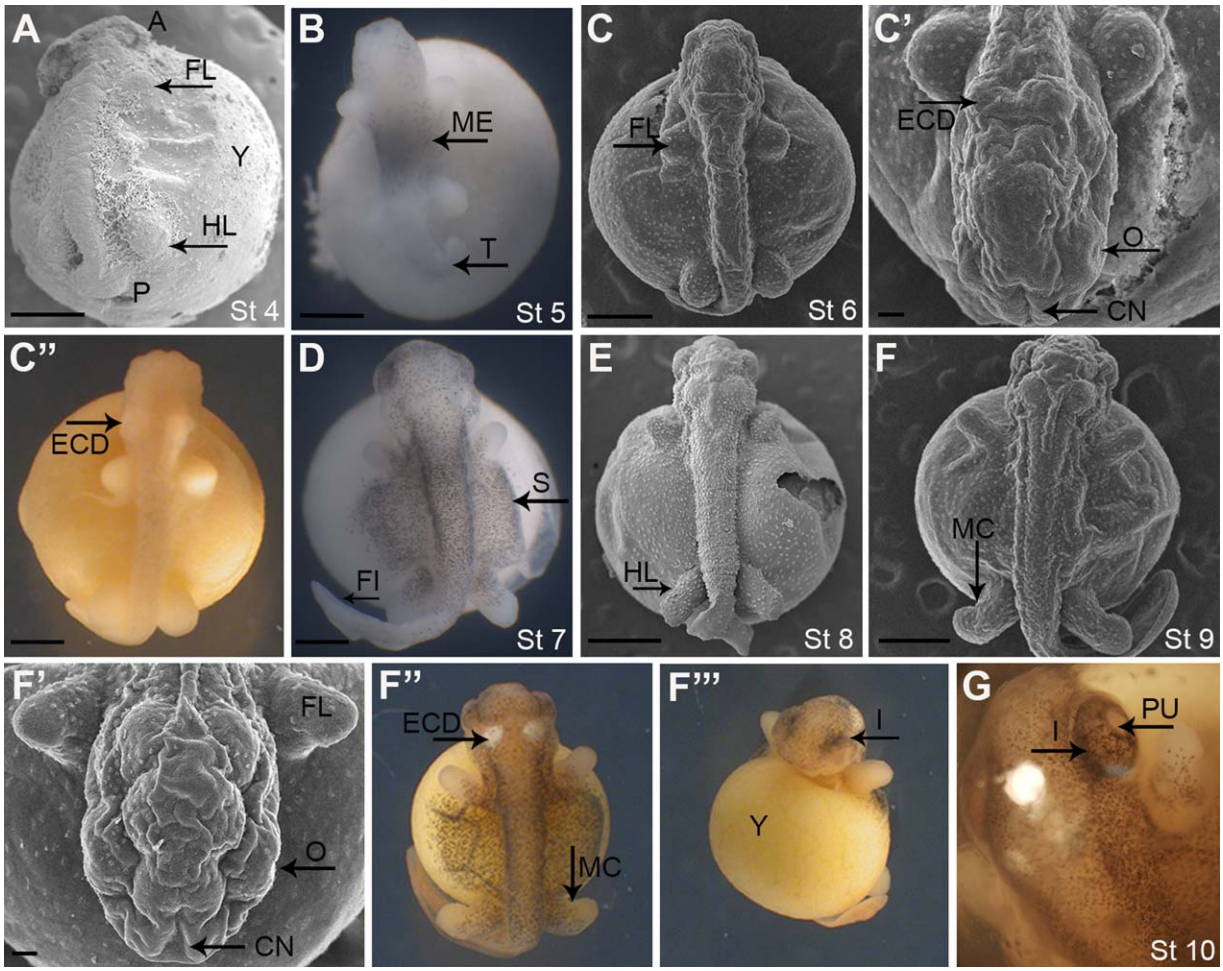

FIG. 4. *Adelophryne maranguapensis* early development. (**A**) Scanning electron micrograph at Stage 4 showing the neural tube forming dorsally on the yolk. (**B**) Stage 5, showing early pigmentation. (**C**) Stage 6, with the limb buds growing close to the neural tube. (**C′**) Head morphology of a Stage 6 embryo showing the open cranial neuropore. (**C″**) Stereomicroscopy image showing the embryo at Stage 6 with the beginning of endolymphatic calcium deposit formation. (**D**) Stage 7 embryo showing spreading pigmentation. (**E**) Stage 8 embryo with elongating hindlimbs. (**F**) An embryo at Stage 9 showing hindlimbs with medial constriction. (**F′**) Head morphology of a Stage 9 embryo presenting well defined optic vesicles. (**F″**) Stereomicroscopic image of a Stage 9 embryo showing endolymphatic calcium deposits as round white dots. (F‴) The yolk remains unpigmented at Stage 9. (**G**) At Stage 10, the eyes are pigmented. Anterior region (A), medial constriction (MC), endolymphatic calcium deposits (ECD), forelimb buds (FL), fin on tail bud (FI), hindlimb buds (HL), iris (I), melanophore (ME), cranial neuropore (CN), optic region (O), posterior region (P), pupil (PU), edge of secondary cover (S), tail bud (T), yolk mass (Y). Scale bars: A, C, E, F: 500 μm; B, D: 1 mm; F′: 100 μm.

demarcating the distal autopodium (foot paddle); the forelimbs are longer but remain smaller than the hindlimbs (Fig. 4F). The cranial neuropore is more ventral, and the optic vesicles are more evident (Fig. 4F′). In this stage, the entire dorsal surface of the embryo is pigmented, although the limb tips and the ventral egg surface [i.e., the yolk (Y)] remain unpigmented. The forelimb buds are in the process of elongating. Endolymphatic calcium deposits (ECD) are seen as rounded bodies posterior to the eyes on the dorsal surface of the embryo (Fig. 4F″–F‴). The tail is 1/3 of its final length. In *E. coqui* development, during Stage 9, the tail is ~2/3 of its final length, and digits are discernible on the feet.

Stage 10. At Stage 10, the eyes are more laterally positioned, with pigmented irises and unpigmented pupils. The tail is 1/3 of its final length, and the digits have begun to differentiate (Fig. 4G). At Stage 10 in *E. coqui*, the pupil is dark, and the iris is dark gray to black; the toes are 1/3 of their final length, and the tail reaches full length.

Stage 11. At Stage 11, the embryo is strongly pigmented, except for the ventral surface of the yolk mass, and the borders of the secondary cover are visible. The egg tooth is apparent on the upper lip; the tail is composed of two thin layers of highly vascularized tissue. The hindlimb buds show the five indentations of the autopodium (I, II, III, IV, V), corresponding to digits I,

FIG. 5. *Adelophryne maranguapensis* late development. (**A**) Ventral view of Stage 11 and the beginning of digit development (insert). (**A′**) Dorsal view of stage 11. (**B**) Ventral view of Stage 12 showing that the tail has completely developed. (**B′**) Dorsal view of stage 12. (**C**) Ventral view of Stage 13 with the yolk being internalized. (**C′**) Dorsal view of Stage 13. (**D**) Ventral view of Stage 14 showing the digits separated and the yolk completely internalized. (**D′**) Dorsal view of stage 14. (**E**) Ventral view of Stage 15 showing the tail regressing. (**F**) Dorsal view of Stage 15 with adult external morphology fully formed. Scale bars: A, A′, B, B′, C, C', D, D′, E: 1 mm.

II, III, IV, V (Fig. 5A–A′, insert). The embryos of *E. coqui* at Stage 11 show an expanded secondary cover, the tail is fully developed, and no egg tooth can be seen.

Stage 12. At Stage 12 of *A. maranguapensis* development, the pigmented area, which marks the presence of the body wall or secondary cover, almost completely covers the ventral surface of the embryo. The forelimb digits are formed but the interdigital membranes are still evident on the fore and hindlimbs. The digits of the hindlimbs, particularly digit IV, are elongated. At this stage, the tail is fully developed (Fig. 5B–B′). Stage 12 of *E. coqui* development is marked by a secondary cover that is fully expanded, and the egg tooth can be seen.

Stage 13. During Stage 13, the yolk mass is almost completely internalized, and the border of the secondary cover is entirely fused along the midline. The eyes are prominent and appear similar to those in the adult animal. The tip of the egg tooth shows keratinization. The endolymphatic calcium deposits are no longer visible on the embryo's head (Fig. 5C–C′). At stage 13, *E. coqui* embryos, the yolk mass is totally incorporated within the embryo, the toepads are present, the tail has regressed, and the ECD is less evident.

Stage 14. At Stage 14, the yolk is completely surrounded by the body wall, which encloses the ventral surface of the embryo (Fig. 5D–D′). The body pigmentation pattern is the same as in the adult, the short jaw becomes longer and pigmented, and the nasolacrimal cleft is closed. The interdigital membranes are disappearing, and four digits are visible on the forelimb and five digits on the hindlimb. The stylopodium, zeugopodium, and autopodium are completely distinct along the limbs. The third digit is the longest on the hands, and the fourth digit is the longest on the feet (Fig. 5D–D′). The drop shape of the digit tips is evident, which is a characteristic of the *Adelophryne* genus. *E. coqui* embryos at Stage 14 of development show eyes with adult coloration, toes that are fully developed, tails that are fast regression, ECDs that are masked by pigmentation and a visible egg tooth.

Stage 15. At Stage 15, the embryos show elongated limbs and a shortened tail, the interdigital membranes are no longer apparent, and the egg tooth is completely developed (Fig. 5E). At the end of stage 15, a froglet is formed, with features similar to the adult animal, such as the absence of a tail. These fully formed embryos hatched in the laboratory and were able to crawl on soaked cotton. The crawling behavior may have been caused by premature hatching, as froglets that hatch in their natural environment immediately start to jump (Fig. 5F). At Stage 15, both *A. maranguapensis* and *E. coqui* embryos show most adult external and internal structures, and hatching occurs at any time during Stage 15.

Limb Development

The limb development in *A. maranguapensis* begins in Stage 4 with the formation of small buds on the lateral sides of the body. The forelimbs emerge anteriorly, near the head, and begin to elongate around Stage 5 (Fig. 6A). The limb buds elongate, and at Stage 9, a few ciliated cells can be observed at the tip of the forelimb (Fig. 6B). As the forelimbs elongate, the ciliated cells come to cover the entire limb surface, although they

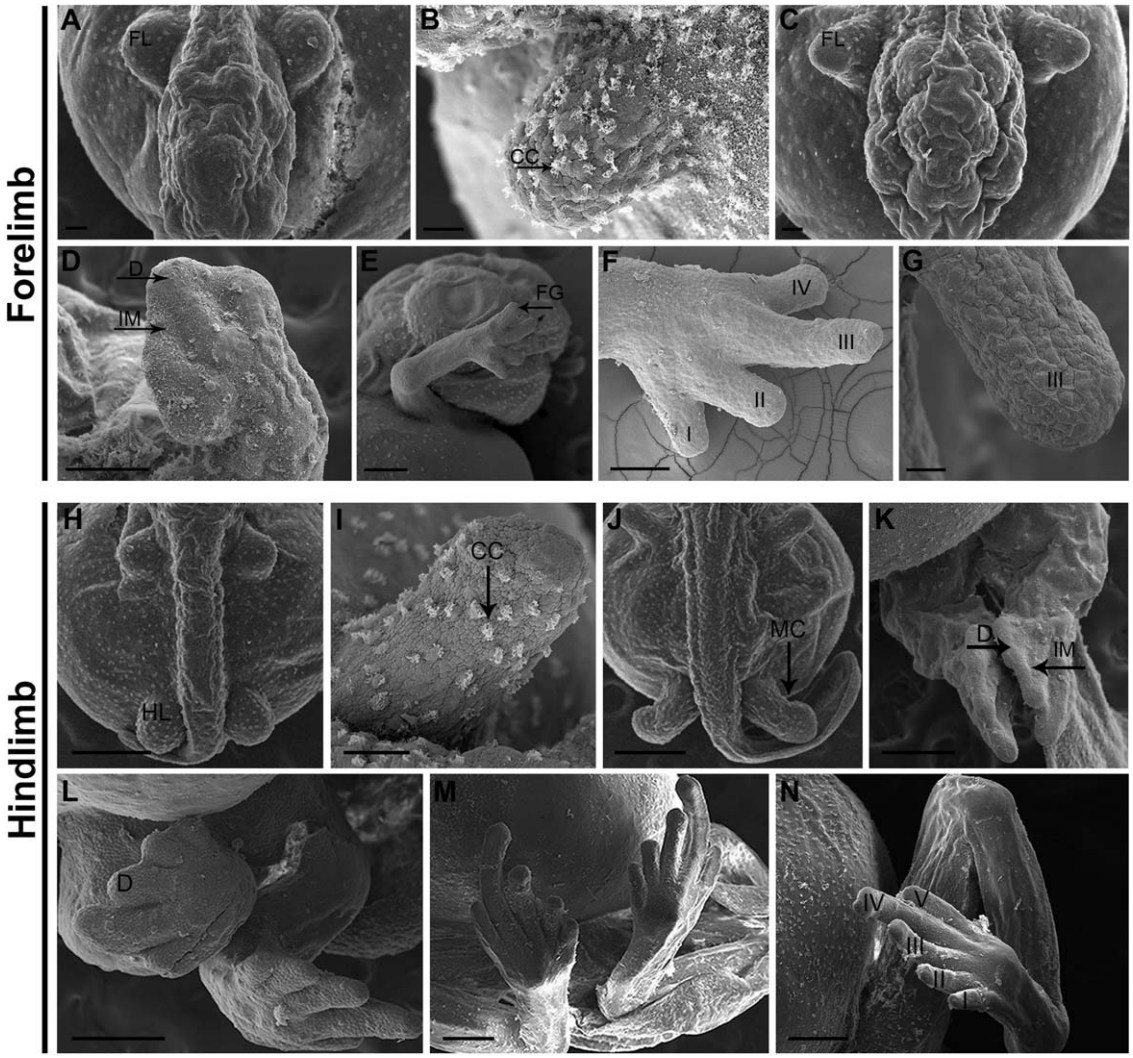

FIG. 6. *Adelophryne maranguapensis* forelimb and hindlimb development. (**A**) Early forelimb bud. (**B**) Forelimb bud with ciliated cells. (**C**) Elongation of limb bud at Stage 9. (**D**) The autopodium with digits and interdigital membrane. (**E**) Separated digits at Stage 15. (**F**) Digits I, II, III, and IV at Stage 15. (**G**) Digit III shows few ciliated cells. (**H**) Early hindlimb bud. (**I**) Hindlimb bud with ciliated cells. (**J**) Hindlimb with medial constriction at Stage 9. (**K**, **L**) Digits of hindlimb with interdigital membrane at Stage 13. (**M**) Separated digits at Stage 14. (**N**) Digits I, II, III, IV, and V at Stage 15. Ciliated cells (CC), digit (D), forelimb bud (FL), finger (FG), finger I (I), finger II (II), finger III (III), finger IV (IV), finger V (V), interdigital membrane (IM), hindlimb (HL), medial constriction (MC). Scale bars: A, C, F, I: 100 μm; B: 50 μm; D: 150 μm; E: 200 μm; G: 20 μm, H, J: 500 μm; K, L, M, N: 250 μm.

are less apparent on the tip (Fig. 6C). Around Stage 13, the interdigital membranes are conspicuous, and the digits are apparent (Fig. 6D). At Stage 15, the digits are fully discernible and the interdigital membranes have disappeared (Fig. 6E). The forelimbs are composed of four digits, of which the third digit is the longest (Fig. 6F). During the final steps of forelimb formation at Stage 15, the surface is no longer covered by ciliated cells (Fig. 6G).

The hindlimbs emerge posteriorly around Stage 4 and are formed earlier than the forelimbs throughout embryonic development. The hindlimbs emerge as small protuberances and then elongate (Fig. 6H). At Stage 7, the hindlimbs are flattened and longer than the forelimbs (Fig. 6I), and at Stage 9, a medial constriction is evident, indicating the distal autopodium (Fig. 6J). At Stage 13, the interdigital membranes are present attached to the prominent digits (Fig. 6K). At Stage 13, the hindlimbs continue to elongate and the interdigital membranes disappear (Fig. 6L). At Stage 14, the digits become separated due to regression of the interdigital membrane, and they become fully elongated. The hindlimbs are composed of five digits, of which the fourth digit is the longest. Ciliated cells are no longer evident during this and succeeding stages (Fig. 6M, N).

Ciliated Cells on the Body Surface

From Stage 4 onward the embryo body becomes covered by ciliated cells, forming clusters of cilia (Fig. 7A).

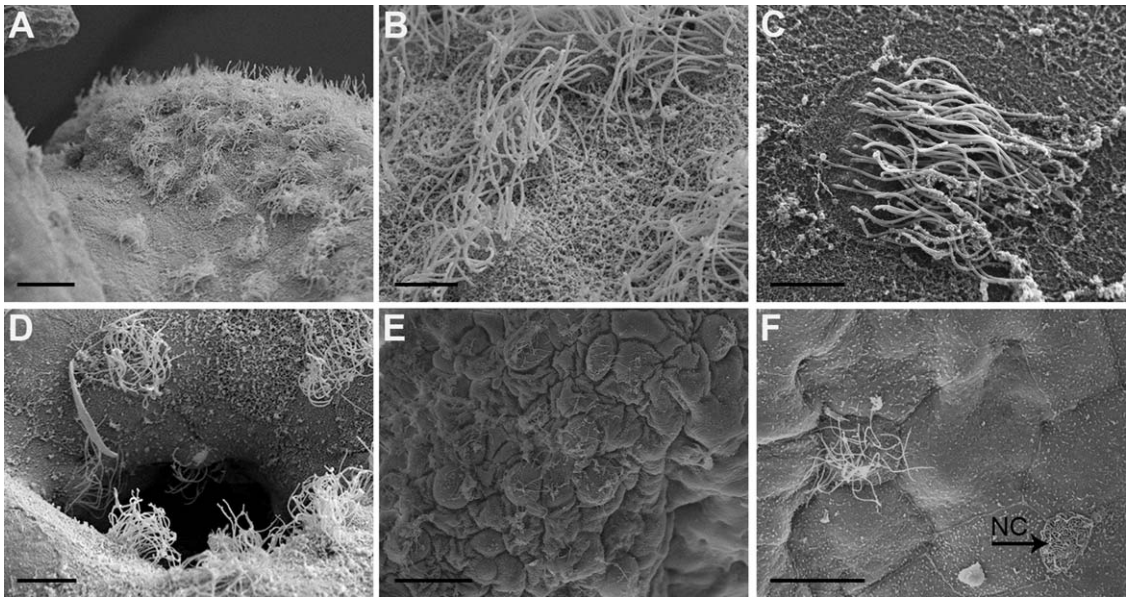

FIG. 7. Ciliated cells on body surface of *Adelophryne maranguapensis*. (A) Clusters of ciliated cells at stage 14. (**B**) Elongated cilia from ciliated cells. (**C**) Rounded morphology of the ciliated cell. (**D**) Ciliated cells in the nasal cavity. (**E**) Loss of cilia was observed at Stage 15. (**F**) Ciliated cell without cilia (NC). Scale bars: A: 20 μm; B, C: 5 μm; D: 10 μm; E: 25 μm; F: 30 μm.

Scanning electron microscopy revealed that some cells have multiple cilia and are scattered among nonciliated cells; as described elsewhere, the epidermis of *X. laevis* likewise has many ciliated and nonciliated cells (Hayes *et al*., 2007). The skin surface of *A. maranguapensis* has multiple cilia (Fig. 7B, C), and as the embryo develops this layer of ciliated cells covers the entire embryo surface, including the nasal cavity (Fig. 7D). After limb formation is complete, some ciliated cells regress, and during the final steps of embryonic development only a few ciliated cells could be seen (Fig. 7E, F). The ciliated cells were numerous around the nostrils, mouth, eyes, chin, and tail, but they were less common on the limbs and yolk.

Tail Morphology

The tail develops early in *A. maranguapensis* embryos; as the neural tube forms dorsally on the yolk mass, the tail is formed and elongates quickly. The tail is a heavily vascularized structure composed of two thin tissue layers, the fins (Fig. 8A), in which an intricate network of blood vessels can be observed (Fig. 8B). The external surface of the tail is covered by ciliated cells interspersed with nonciliated cells (Fig. 8C). The ciliated cells of the tail skin had a morphology distinct from that on the body surface, showing an elongated structure (Fig. 8D, compare Fig. 7C).

Egg Tooth Formation

The egg tooth is visible from Stage 11 onward as a small, single-cuspidate tooth on the upper lip of the embryo (Fig. 9A). The cells on the edge of the protuberance are modified, likely by keratinization (Fig. 9B–D). At Stage 13, the development is complete (Fig. 9D–E). Morphological details of the egg tooth revealed by scanning electron microscopy revealed a keratinized tip with compact cells on the proximal side (Fig. 9F–H). The keratinized cells are morphologically similar to the microridges (Fig. 9H).

Table 1 lists morphological features according to stages of development in *E. coqui* and *A. maranguapensis* as described in this study and in a study by Townsend and Stewart in 1985. The table shows the similarities and differences between the two species with respect to the formation of embryonic structures, which could represent heterochrony among species. To emphasize the sequential morphological features, we plot the main characteristics of *E. coqui* and *A. maranguapensis* embryonic development (Fig. S3, Supporting Information).

DISCUSSION

The external morphological changes observed by scanning electron microscopy during the embryonic development of *A. maranguapensis* can be compared to the developmental staging table of *Eleutherodactylus coqui* (Townsend and Stewart, 1985). The external surface features of the *A. maranguapensis* embryo we examined included the ciliated cells of the skin, the limbs, the tail and the formation of the egg tooth.

The terrestrial eggs of *A. maranguapensis* are ~5 mm wide, as measured in the field with the jelly coat and the vitelline membrane intact (Cassiano-Lima

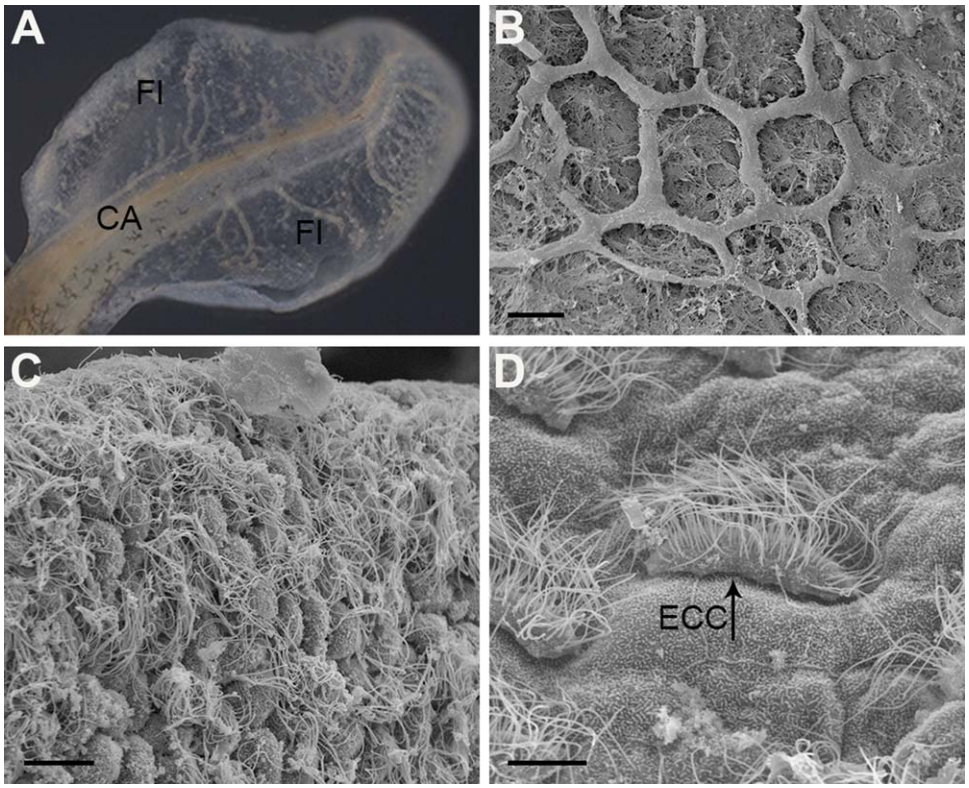

FIG. 8. Tail development of *Adelophryne maranguapensis*. (**A**) Tail with central axis (AC) and fins (Fl). (**B**) Branching vascularization of the tail fins. (**C**) Ciliated cells on tail surface. (**D**) Elongated morphology of ciliated cells found on the tail (ECC). Scale bars: B: 20 μm; C: 10 μm; D: 7.5 μm.

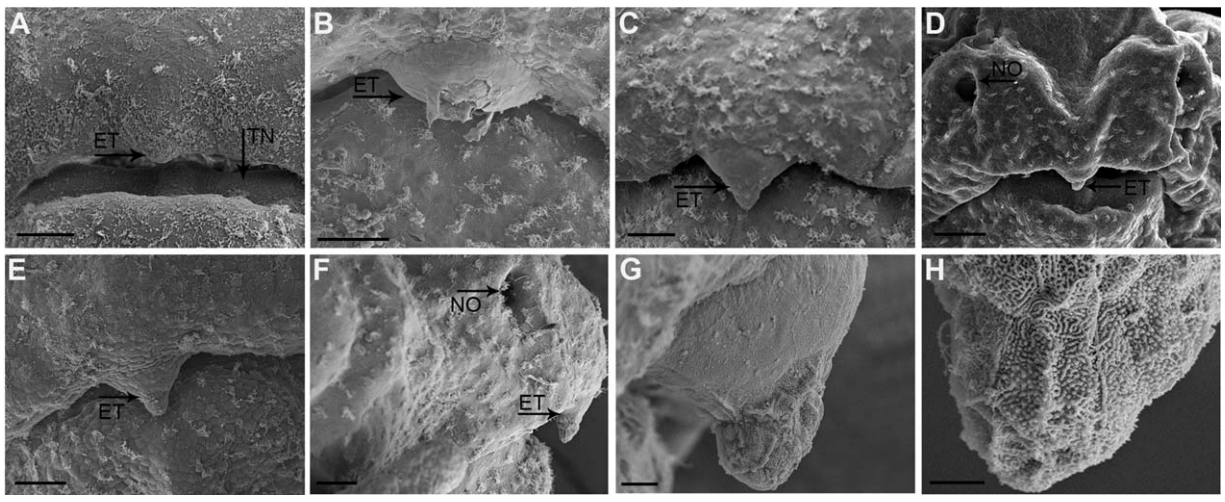

FIG. 9. *Adelophryne maranguapensis* egg tooth development. (**A**) Early development of the egg tooth at Stage 11. (**B**) Early tooth keratinization. (**C**–**E**) Ventral view of the egg tooth from different embryos. (**F**) Lateral view of the egg tooth showing the distal and proximal surfaces. (**G**) Lateral view of the egg tooth in the final stage of development. (**H**) Tip of the egg tooth, showing cells with microridges. Egg tooth (ET), nostril (NO), tongue (TN). Scale bars: A, D, E, G: 50 μm; B, C: 100 μm; F: 25 μm; H: 5 μm.

et al., 2011), and they are unpigmented until Stage 4 (Figs. 2 and 3). *Adelophryne maranguapensis* spawns in bromeliads in the treetops, which protects embryos from potentially damaging sunlight. According to Elinson and del Pino (2012), the aquatic eggs contain small pigment granules due to yolk accumulation; these granules may restricted to a position around the nucleus of dividing blastomeres. The yolk-enriched egg

Table 1
Adelophryne maranguapensis *and* Eleutherodactylus coqui *(Townsend and Stewart, 1985) Morphological Features During Embryonic Development*

Stage	*Adelophryne maranguapensis*	*Eleutherodactylus coqui*
01	Zygote	Zygote until blastopore formation
02	Cleavages	From blastopore until first evidence of neural fold
03	Gastrula and Early neurula stage	Neural groove closes to form the neural tube; limb buds absente
04	Neural groove closed; Eye bulges unpigmented; Gill arches no visible; Limb buds rounded; Tail bud presente; Ciliated cells present	Neural groove closed; Eye bulges unpigmented; Gill arches present (no gills); Limb buds rounded; Tail bud present; Ciliated cells present
05	Eye bulges unpigmented; Gills absent; Limb buds rounded; Tail bud elongate; Melanophores present	Eyes prominent and unpigmented; Gills present; Hind limb buds elongate; Tail bud elongate; Melanophores are not mencioned
06	Eye bulges unpigmented; Gills absent; Limb buds rounded; Small tail bud curved; Endolymphatic calcium deposits (ECD);	Pupil clear and iris gray; Gills with blood circulating; Hind limb buds with slight constrictions; Tail with central axis and small fins; Endolymphatic calcium deposits (ECD);
07	Pigmentation of the eye starting; Hind limb buds dorso-ventraly flattened; Tail with central axis and small fins; Pigmented secondary cover present	Pupil clear and iris gray; Hind limb buds with foot paddle; Tail developed and with well vascularized fins; Pigmented secondary cover present
08	Dark iris's upper half; Hind limb bud flattened; Tail 1/3 of its final length; Pigmented secondary cover expanding; Many ciliated cells; Gills absent	Pupil clear and iris gray; Nubs of digits first evident on feet; Tail 1/2 of its final length; Pigmented secondary cover expanding; Ciliated cells are not mentioned; Gills regression in size
09	Dark iris's upper half; Medial constriction evident; Tail 1/3 of its final length; ECD present; Secondary cover expanding; Melanophores scattered throughout surface's embryo; Ciliated cells present	Pupil clear and iris dark gray; Digits discernible on feet; Tail 2/3 of its final length; ECD present; Secondary cover expanding; Melanophores scattered throughout surface's embryo; Ciliated cells are not mentioned
10	Pupil clear and iris dark; Nubs of digits on the feet; Tail 1/3 of its final length; ECDs are not expanding; Secondary cover expanding	Pupil darkness and iris dark gray to black; Toes 1/3 of its final length; Tail full length; ECD's expansion; Secondary cover expanding
11	Pupil gray and iris darkening; Digits on feet and hands; Tail full length; Secondary cover expanding; Egg tooth appears	Pupil darkening and iris black; Toes 1/2 of its final length; Tail full length; Secondary cover expanding; No egg tooth
12	Pupil gray and iris dark; Digits's elongation; Tail full length; Secondary cover expanding; Egg tooth present	Pupil dark and iris lightens; Toes 2/3 of its final length; Tail full length; Secondary cover fully expanded; Egg tooth appears
13	Pupil dark; Digits's elongation; Tail full length; ECD not visible; Yolk almost totally incorporated within the embryo	Iris lighter; Toepads present; Tail's regression; ECD less evident; Yolk totally incorporated within the embryo
14	Pupil shining; Digits's elongation; Tail full length; ECD not visible; Egg tooth present; Ciliated cells regressing; Limbs ossification;	Eyes with adult coloration; Toes full length; Tail's regression; ECD masked by pigmentation; Egg tooth present
15	Adult external and internal morphology formed	Adult external and internal morphology formed

supplies the embryos and is an essential feature of direct development (Ninomiya *et al*., 2001), unlike in aquatic-feeding larvae (Elinson and Beckham, 2002).

A. Maranguapensis Cleavage

The cleavage furrows in the *A. maranguapensis* egg culminate in the formation of four blastomeres, which are visible only at the animal pole of the embryo. Cleavage in *A. maranguapensis* follows the unequal holoblastic pattern observed in all amphibians (Elinson and del Pino, 2012). The cleavage furrows extend slowly to the vegetal pole, because of the resistance caused by the large amount of yolk. Townsend and Stewart (1985) reported that in *E. coqui* the initial cleavages follow a reticulated pattern. It was not possible to observe continuing divisions in the *A. maranguapensis* eggs, although we noticed divided cells in the vegetal pole of later stages of development (not shown). In *E. coqui*, the first horizontal cleavage arises at the 16-cell stage, generating eight small cells in the animal pole and eight large cells in the vegetal pole, identifying the borderline between the animal and vegetal poles (Ninomiya *et al*., 2001). The large size of the early blastomeres in relation to the total size of the egg suggests the possibility of differences in the size of the blastomeres, the position of the cleavage furrow (horizontal or equatorial), and its subsequent development in *A. maranguapensis* as compared with *E. coqui*. According to Elinson and del Pino (2012), further analyses in non-model species would be useful for determining egg size and cleavage patterns, as well as the timing of each cleavage. The molecular properties of embryogenesis in yolk-enriched eggs, such as in *E. coqui*, are shifted toward the animal pole relative to metamorphosing frogs, such as *X. laevis* (Beckham *et al*., 2003). Other early development events such as blastocoel formation, gastrulation movements, yolk-plug formation and neurula remain to be

investigated and should be identified to confirm that the initial development of *A. maranguapensis* follows the pattern described for *E. coqui*.

Limb Bud Development

The sequential events involved in limb formation in *A. maranguapensis* and *E. coqui* are likely similar. In *E. coqui*, these include the following: (1) early neural tube closure without limbs (Townsend and Stewart, 1985); (2) continued closure of the neural tube during the appearance of hindlimb buds (Richardson *et al*., 1998; Hanken *et al*., 2001); and (3) a closed neural tube with the presence of hind and forelimb buds (Townsend and Stewart, 1985). We were unable to observe whether the neural tube closes without the limb buds present. However, the hindlimbs formed more rapidly than the forelimbs throughout embryonic development. Comparing *A. maranguapensis* limb, eye and tail formation with *E. coqui* revealed potential heterochrony. These structures might start development at the same stages, but they proceed more rapidly in *E. coqui*; for example, tail formation probably begins earlier in *A. maranguapensis*, although it develops more rapidly in *E. coqui*.

In *X. laevis* the limb apical ectoderm has been described histologically as three layers of ectodermal cells organized in a ridge-like structure (Tarin and Sturdee, 1971). Richardson *et al*. (1998) did not find a similar structure in *E. coqui*. Even without the formation of a ridge, the apical ectodermal cells appear to affect the morphogenesis of the bud because they are able to express the gene *distal-less*, and loss of this gene in late stages causes metatarsal and tarsal phalangeal defects in the hindlimb buds (Hanken *et al*., 2001). The scanning electron microscopy analyses of *A. maranguapensis* limb buds did not show the apical ectodermal ridge, as described for *X. laevis*, and the limb buds appeared similar to those of *E. coqui*. According to Hanken *et al*. (2001), changes in the mechanisms of limb formation between direct development and biphasic developmental modes represent a phylogenetic component that can be shared by all members of a lineage as a result of their common ancestry. The absence of an apical ectodermal ridge in *E. coqui* and *A. maranguapensis* may represent a shared feature among direct-developing frogs, although further studies involving molecular data and more species should be conducted to clarify whether this aspect is related to the evolutionary success of this reproductive model in frogs.

Egg Tooth Formation

The egg tooth was first observed at Stage 11 in *A. maranguapensis* as a small tooth with a single cusp, formed on the upper lip of the embryo (Fig. 8). First, the bulge of the egg tooth arises, and then the cells located on the tip of the protuberance become modified, mostly likely by keratinization. Townsend and Stewart (1985) noted the presence of the egg tooth at Stage 14 in *E. coqui*, although they did not define whether this is when it is first apparent. The egg tooth is a marker of direct-developing frogs that compose the taxon Terrarana (Hedges *et al*., 2008). Through head movements during hatching, the tooth cuts the egg capsule to allow the froglet to emerge (Duellman and Trueb, 1994).

Endolymphatic Calcium Deposits and Ciliated Cells in *A. Maranguapensis* Development

Endolymphatic calcium deposits (ECDs) are structures associated with auditory organs and the deposition of calcium carbonate for skeletal calcification appears at Stage 6 in *A. maranguapensis*. The ECDs correspond to a portion of the endolymphatic duct, which is extremely enlarged in anurans. This pair of symmetric dorsal points are present in the aquatic anuran larvae before metamorphosis (Duellman and Trueb, 1994). Endolymphatic sacs were also observed in *E. coqui* embryos from Stage 6 onward, and they appear to be homologous to those present in aquatic anuran larvae (Townsend and Stewart, 1985).

Ciliated cells were found in *A. maranguapensis* from Stage 4 onward. The ciliated cells gradually begin to disappear around Stage 14. These cells persist in the face and tail, but are smaller in number, until the last stage observed by electron microscopy (Stage 15) (not shown). Richardson *et al*. (1998) reported the presence of ciliated cells in *E. coqui* from Stage 4 onward, but provided no details. Ciliated cells are present in both biphasic and direct-developing frog embryos. Hayes *et al*. (2007) identified ciliated cells, goblet cells that produce mucus, and small secretory cells of unknown function in the epidermis of *X. laevis*. Nokhbatolfoghahai *et al*. (2006) investigated 20 species of anurans, including a direct-developing frog *Pristimantis urichi*, and concluded that the cilia are an ancestral larval feature that persists in direct-developing frogs. These authors suggested that ciliated cells might function to prevent the retention of microorganisms and cell debris on the epidermis of the embryo; for *P. urichi*, they described the presence of large numbers of ciliated cells in the pharyngeal region and on the tail fins, which are structures associated with respiration, as is the tail of *A. maranguapensis*. This feature, which is shared by direct- and indirect-developing species, suggests that it is a conserved and essential characteristic for the survival of anuran embryos.

Environmental Influences on the Embryo Morphology

The direct-developing embryo has a large yolk mass and exhibits similar external morphology, derived from

convergent evolution, in different direct-developing lineages, and it is thought to be a reproductive mode derived from the ancestral biphasic reproduction (Elinson and del Pino, 2012). The terrestrial environments where direct-developing frogs reproduce are humid, although peculiarities of the spawning microenvironment may result in unique selective pressures in different species, reflecting features of their ancestors and derivative forms. An important difference between *A. maranguapensis* and *E. coqui* is the absence of external gills (considered an apomorphy) in the former and the presence of external gills (considered a plesiomorphy) in the latter. Therefore, the presence or absence of external gills reflects the microenvironmental differences in the amount of external oxygen available to diffuse through the jelly layer.

The heterochronies found when comparing embryonic development in *A. maranguapensis* and *E. coqui*, such as tail, limb, and eye formation, do not affect the general plan of the adult body (Table 1). The explanation for this phenomenon could be the presence of a modular embryonic development program, in which individual modules can unfold without greatly affecting other modules. A group of developmental events (e.g., formation of eyes, ears, limbs, etc.) could occur at different times during embryogenesis in different species, with the final general adult body plan being largely similar (Chipman, 2002). Indeed, embryonic development in anurans shows great evolutionary plasticity, and changes in ontogenetic processes are common, although they do not significantly affecting the adult form. By contrast, the modular nature of anuran embryonic development may have allowed for these and similar changes, leading to the successful development of embryos in widely differing spawning locations (Chipman, 2002; Chipman *et al*., 2000).

There are many similarities and embryonic heterochronies in the family Eleutherodactylidae, which do not affect the overall body plan of the adult organism. The presence of external gills, considered an ancestral trait in *E. coqui* embryos, indicates that this genus may have arisen before the genus *Adelophryne* in the evolutionary history of the family Eleutherodactylidae. According to Elinson and del Pino (2012), the genus *Eleutherodactylus* shares a common ancestor with other genera of the monophyletic clade Neobatrachia at ∼55 MYA, and this clade in turn shares a common ancestor with the biphasic *Xenopus* clade at ∼230 MYA. According to Fouquet *et al*. (2012), the species of *Adelophryne* that populated the Atlantic Forest of northeastern Brazil diversified from other *Adelophryne* species at ∼10 MYA.

Adelophryne maranguapensis is the only member of the genus for which several clutches have been collected, enabling detailed morphological studies of its embryonic development. As a model for further studies of direct-developing frogs of the Atlantic Forest, this study will aid in the protection and conservation of these endangered species.

MATERIALS AND METHODS

Embryos

Embryonic specimens of the direct-developing frog *Adelophryne maranguapensis* were collected in the Serra de Maranguape, a mountainous area approximately 920 m above sea level, located in the state of Ceará, northeastern Brazil, under permits issued by IBAMA (permit number: 22909-1). The clutches were removed from the natural habitat during the rainy season (January to May) from 2010 to 2012, totaling 24 clutches and 121 embryos. The clutches were found near the Hummingbird river stream (03°53'44.3"S; 38°43'18.8"W, 890 m a.s.l.). They were located only on the upper surfaces of leaves of the bromeliad *Guzmania lingulata*, at a mean height of 1.76 m above the ground. The eggs were always found near the axils of the leaves, where they were directly wetted by rainwater; the eggs were never found in the bromeliad tank water itself. During two collections, we directly observed the female laying eggs on the leaves of bromeliads, unequivocally showing that these embryos were from the attributed species. In addition, previous herpetological studies have shown that *A. maranguapensis* is the only species that lays eggs in bromeliad leaves in the Maranguape mountain (Cassiano-Lima *et al*., 2011; Haddad and Prado, 2005; Toledo *et al*., 2012; Vieira *et al*., 2009;). The embryos were collected from early to the final stages of development. We attempted to grow the eggs clutches under laboratory conditions. Egg clutches collected at different stages of development, and they were kept in a humid chamber on filter paper moistened with 0.1× Steinberg's solution at room temperature (∼23°C). During two of these attempts, the eggs reached later stages of development and hatched as *A. maranguapensis* froglets. However, none of these survived more than few hours after hatching. The froglets grown in the laboratory showed comparable morphology to *A. maranguapensis* adults. Live or fixed specimens were photographed using a Leica S8APO stereomicroscope.

Histology

For histology, females collected at the same sites as the embryos were killed with 2% lidocaine and dissected to expose the reproductive tract using a Nikon SMZ1500 stereomicroscope. Bouin's fixative was used for light microscopy studies. After fixation, the ovary and oviduct were dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut in 6-μm-thick slices, most of which were

stained with hematoxylin-eosin using standard procedures. The slides were analyzed using a Zeiss Primo Star Trinocular microscope, and images of the selected sections were collected using the AxioVision software program.

Scanning Electron Microscopy (SEM)

Embryos were de-jellied either chemically (3% cysteine, pH 7.8, for 2 min) or manually using watchmaker's forceps. The majority of embryos were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1*M* sodium phosphate buffer for 2 h at room temperature. The embryos were dehydrated using a graded ethanol series ethanol, critical-point dried (Bal-Tec, CPD 030), and mounted on aluminum stubs. The embryos were sputter-coated with gold (Leica EM SCD050) and then examined using a Quanta 250 electron microscope.

Phylogenetic Analysis

The neighbor-joining method was used to reconstruct the phylogenetic relationships of *A. maranguapensis*. All nodes were supported significantly (>95%) in bootstrap analyses using neighbor-joining and maximum likelihood. Neighbor-joining phylogenetic trees with 1000 bootstrap iterations were then generated with the Mega 5.0 program. Sequences used in this analysis of *TYR* were obtained from GenBank: *Adelophryne gutturosa*, EU186772.1; *Adelophryne pachydactyla*, JX298216.1; *Adelophryne maranguapensis*, JX298207.1; *Adelophryne bauritensis*, JX267679.1; *Adelophryne adiastola*, JX298221.1; *Brachycephalus ephippium*, JX267681.1; *Eleutherodactylus planirostris*, JX298238.1; *Ceuthomantis smaragdinus*, EU186771.1; and *Oreobates barituensis*, JF809892.1.

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