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Direct-developing frogs: ontogeny of *Oreobates barituensis* (Anura: Terrarana) and the development of a novel trait

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Abstract. Within Anura, direct development involves ontogenetic changes of the biphasic ancestral pattern. The recent partitioning of the genus *Eleutherodactylus*, along with the proposition of the unranked taxon Terrarana, has renewed an interest to the morphological and ecological diversity among direct-developing frogs. The morphological changes during embryonic development of *Oreobates barituensis* is similar to those of other Neotropical direct-developing species, including the reduction or absence of several larval and embryonic characters (e.g., external gills and adhesive glands), heterochronic changes (e.g., early developing limbs and late persistence of ciliated epidermal cells), and the appearance of new structures (e.g., egg tooth). The tail achieves an extraordinary peramorphic development (encloses the entire embryo), and the location of its expanded part is interpreted as a heterotopic change resulting in a novel trait. An enveloping tail with apparently non-heterotopic fins, combined with the absence of gills, has been only reported for a species of the related genus *Craugastor*, and these morphologies suggest an informative perspective for the study of evolution of direct development in terrararanans.

Keywords: embryo, heterochrony, heterotopy, peramorphic tail.

Introduction

In amphibians with direct development the embryonic period comprises of features that typically occur during the larval period and metamorphosis in amphibians with ancestral, complex life histories (Wake, 1989). In anurans, direct development has evolved independently at least ten times, including more than once in some lineages (Hanken, 1999; Heinicke et al., 2009). Development has been considerably altered compared to the biphasic ancestral state and involves three major aspects: 1) some typical larval characteristics never develop, such as adhesive and hatching glands, lateral line organs, and a long coiled intestine; 2) other structures are heterochronically shifted such as limbs or pharyngeal arches development; and 3) new structures can arise such as the egg tooth (e.g., Hanken et al., 1997a, b; Callery et al., 2001; Elinson, 2001).

Among anurans, the genus *Eleutherodactylus*, and particulary *E. coqui*, is regarded as the classical example of direct development and it is stated to exhibit the most pronounced ontogenetic changes relative to the ancestral, metamorphic ontogeny (Townsend and Stewart, 1985; Elinson, 1990; Hanken et al., 1997a, b; Hanken, 1999; Callery et al., 2001; Elinson, 2001; Kerney et al., 2010; Singamsetty and Elinson, 2010). Recent studies have partitioned this genus and proposed the unranked taxon Terrarana with four or five families and 940 species that probably share direct development (Hedges et al., 2008; Heinicke et al., 2009; Frost, 2011; Pyron and Wiens, 2011). In spite of this enormous diversity, development in this group has been thoroughly described only in about 20 species (e.g., Sampson, 1904; Noble, 1925; Lynn, 1942; Gitlin, 1944; Lynn and Lutz, 1946; Jameson, 1950; Adamson et al., 1960; Valett and Jameson, 1961; Townsend and Stewart, 1985; Elinson et al., 1990; Pombal

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Jr., 1999; Nokhbatolfoghahai et al., 2005, 2006, 2010; Nokhbatolfoghahai, 2006).

The 16 species in the genus *Oreobates* are distributed primarily in the Andes of northwestern South America, and two cryptic taxa, *Oreobates discoidalis* and *O. barituensis*, that inhabit the subtropical humid montane forest in northwestern Argentina represent one of the southernmost ranges of terrarananans (Akmentins and Vaira, 2009). The biology of *Oreobates* species, particularly reproductive biology and development, is poorly known because of the low population densities and erratic calling behaviour (Padial et al., 2008; Akmentins, 2011).

We describe the oviposition sites and embryonic ontogeny of *Oreobates barituensis*. Based on the wide diversity in sizes, adult morphologies, and habitats within Terrarana, we anticipate that the comparison with the developmental patterns of *Eleutherodactylus coqui* and other direct-developing frogs will continue revealing spatial and temporal variations, including the evolution of novel traits.

Materials and methods

We collected three terrestrial clutches of *Oreobates barituensis* in Arazay (Salta, Argentina – 22°19′3.54″S, 64°43′3.25″W; 1675 m.a.s.l. – December 2009). Eggs collected in the field were incubated at ambient temperature (20-25°C) in a plastic container with disaggregated, clay rock that was lightly moistened with a water spray once a week. Eggs were examined daily and 4-5 individuals were fixed in 10% formalin every 5 days. Voucher material is deposited in the Herpetological Collection of the Museo de Ciencias Naturales (MCN 1363; Universidad Nacional de Salta, Argentina). Embryos were staged by the table of Townsend and Stewart's (1985) (TS from here) based on *Eleutherodactylus coqui* and differences were taken into account. Morphological features were observed with a stereomicroscope, and pictures were obtained with a digital camera. Measurements were taken with dial callipers (0.02 mm). Descriptions of surface ciliation and scanning photomicrographs were made for specimens at TS6 and 12 at LASEM (Laboratorio de Microscopía Electrónica de Barrido, ANPCyT/UNSa/CONICET; Salta, Argentina; Bozzola and Russell, 1999). For histological cross-sections of the tail, two embryos of *O. barituensis* at TS12 and a dissected tail of a tadpole of *Scinax acuminatus* at Gosner (1960) Stage 37 (Hylidae, MCN 987) used for comparative purposes, were dehydrated, embedded in paraffin, and sectioned at 7 μm. Sections were stained with haematoxylin and eosin following the protocol by Martoja and Martoja-Pierson (1970).

In order to compare the number of eggs in the clutches with the number of post-vitellogenic oocytes in gravid females' oviducts, we dissected three adults [one female (MCN 1364) collected in the same locality of Arazay and two females (MCN 1365) collected in Las Condoreras locality, Jujuy Province, Argentina].

Results

The clutches of *Oreobates barituensis* were located within a narrow crevice in an almost vertical wall of clay rock with scarce herbaceous vegetation cover, a high percentage of bare soil, and little arboreal cover. The highly hygroscopic clay rock provided an obscure, moist microhabitat for development of embryos. A calling male was found in the entrance of the crevice and three non-vocalizing males were found about 30 cm from the first one. In two of the clutches embryos were at TS4, so we estimate clutches were at maximum five days old (table 1). The numbers of eggs in the clutches were similar to the numbers of post-vitellogenic

Table 1. Oviposition site and clutch features of *Oreobates barituensis* from the Arazay locality. Vertical measurement relates to the distance between the clutch and the entrance of the crevice, and horizontal distance is taken from the edge of the vertical wall. Mean egg diameter includes the jelly layers.

	Clutch 1	Clutch 2	Clutch 3
Vertical distance	30 cm	25 cm	20 cm
Horizontal distance	15 cm	15 cm	15 cm
Distance between clutches	–	40 cm to clutch 1	50 cm to clutch 2
Clutch size	20	22	28
Mean egg diameter (range)	6.4 mm (6-7.2)	6.8 mm (6-7.4)	6.4 mm (6.1-6.8)
Embryo stage	TS11	TS4	TS4

Table 2. Number of post-vitellogenic oocytes in females of *Oreobates barituensis*. Measurements in parentheses are mean size of oocytes.

Locality	Snout-vent length	Oocytes in left ovary	Oocytes in right ovary
El Arazay	3.8 cm	9 (3.73 mm)	8 (3.98 mm)
Las Condoreras	3.6 cm	11 (3.65 mm)	4 (2.45 mm)
Las Condoreras	4 cm	19 (3.53 mm)	8 (3.71 mm)

oocytes present in the oviducts of dissected females (table 2).

The nonpigmented eggs of *Oreobates barituensis* had a translucent egg capsule and were weakly attached to each other at their contact points, each ovum had up to six, clear jelly layers and the vitelline membrane; the external layer was very thin, dry and covered with clay particles, and it could be easily removed. In several specimens, scattered yolk granules appeared inside the inner membrane. We estimate developmental duration (from fertilization to hatching) to last between 35 and 40 days (fig. 5A). Embryo size increased about 2 times (TS4 average diameter = 2.88 mm; TS14 = 5.94 mm). Hatchlings had an average SVL of 6.51 mm and posthatching growth involves an increase of 5.8 times the hatchling size.

Cephalic region

By TS4 (fig. 1A), the head is differentiated from the rest of the body and only the mouth cavity can be recognized as a shallow stomodeal depression with a slight ridge below. Embryos lack gill slits and possess three pairs of gill arch bulges which are not visible in later stages. No external gills develop. By the next stages, the mouth changes from a subterminal to a terminal position. In the posterior region of the head the otic capsules differentiate. At TS5 the eyes are already prominent and have a clear pupil and a grey iris which gets darker in the next stages (fig. 1B, C). Adhesive glands are absent. By TS9-10, the snout is rounded. The distal end of the lower jaw is recessed behind the upper jaw which possesses a white, bifid egg tooth (fig. 1F). Non-protruding nostrils are small and oval, and a lacrimal groove extends from each nostril to the eyes. The angle of the mouth changes from the anterior margin of the eye to its final location posterior to the eye at TS14. As development progresses, the egg tooth becomes larger and darkly keratinized (figs 1G, 4B). The tympanic membrane forms, and by TS13 the lower eyelid is distinct and the pigmented upper eyelid differentiates. At hatching, the snout is pointed (fig. 2), the distal end of the upper and the lower jaws are at the same level, the distance between both eyes reduces, and the lacrimal groove is no longer visible. The keratinized egg tooth is still present in some hatchlings. Endolymphatic calcium deposits were not detected in any of the specimens examined, but this could be a fixation artifact as suggested by Townsend and Stewart (1985).

Limbs

Both fore- and hind limbs appear at TS4 as rounded buds distinctive from the trunk (fig. 1A). Limbs project perpendicular to the trunk as they grow, and by TS5 a dermal fold continuous with the epidermis of the body covers the base of each forelimb (fig. 1B). In the next stage, limb bud elongation is evident. Hind limbs are slightly advanced in development regarding forelimbs with a new proximal segment defined by the appearance of both anterior and posterior constrictions that delimit the autopodium (fig. 1C). Stylopodia, zeugopodia and autopodia are delimited by TS9-10 (fig. 1F). All toes and fingers have differentiated, with a progressive appearance of interdigital indentations. The elongation of digit IV (primary axis) is noticeable (fig. 1F, G). Dermal folds acquire their maximal coverage and enclose half of the forelimbs (fig. 1F). After all fingers and toes are formed and have lengthened, inner metatarsal (TS11) and metacarpal (TS12) tuber-

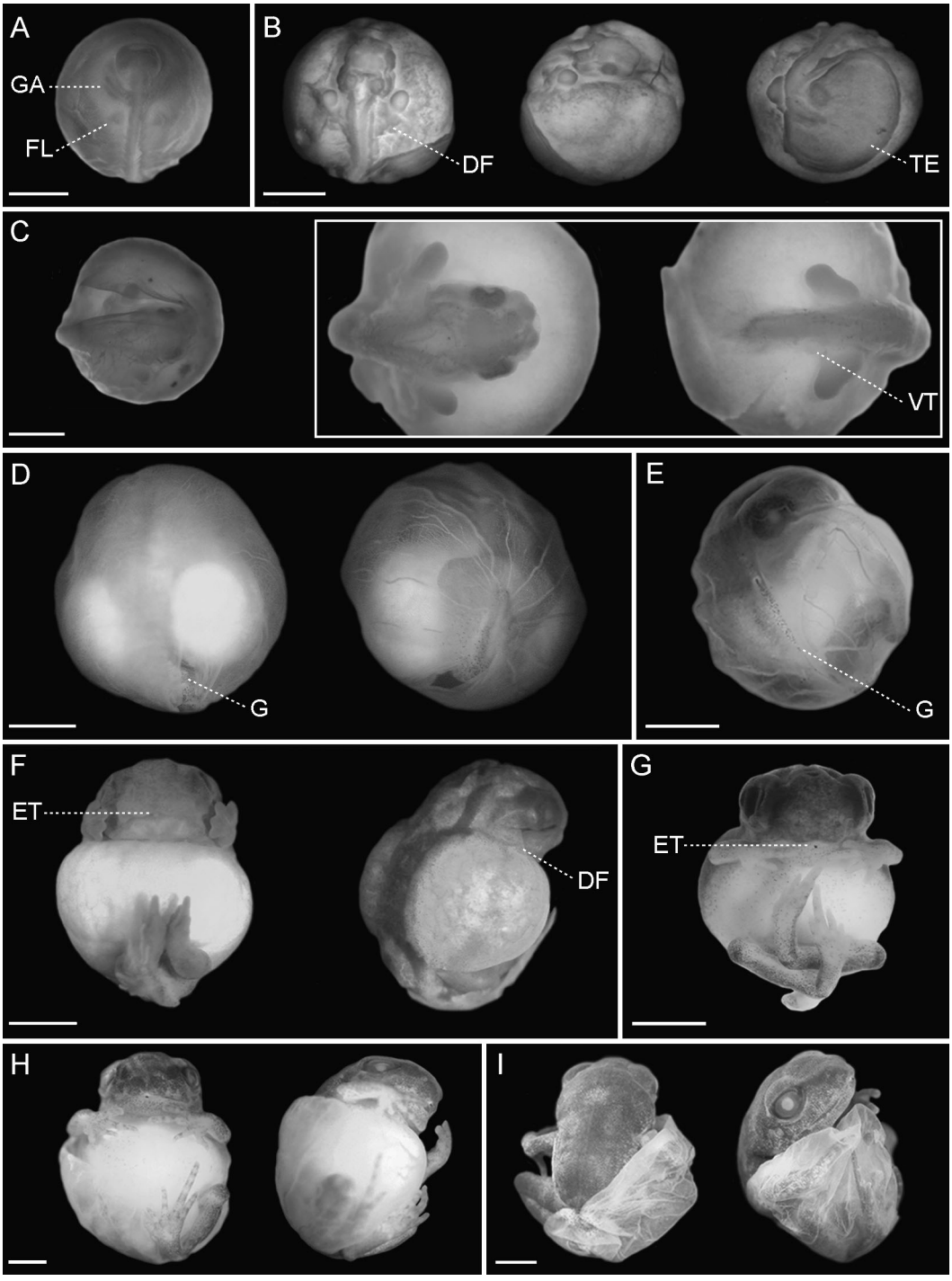

Figure 1. External development in pre-hatching stages of *Oreobates barituensis.* Jelly layers have been removed and contrast was increased with methylene blue. Stages of Townsend and Stewart (1985; TS) are indicated. (A) TS4, dorsal view. Note bulges of the gill arches and forelimbs. (B) TS5, dorsal, anterolateral, and rear views. Note the dermal fold at the base of the forelimb, and the lateral extensions of the tail. (C) TS6, dorsal view. The lateral extensions of the tail surround

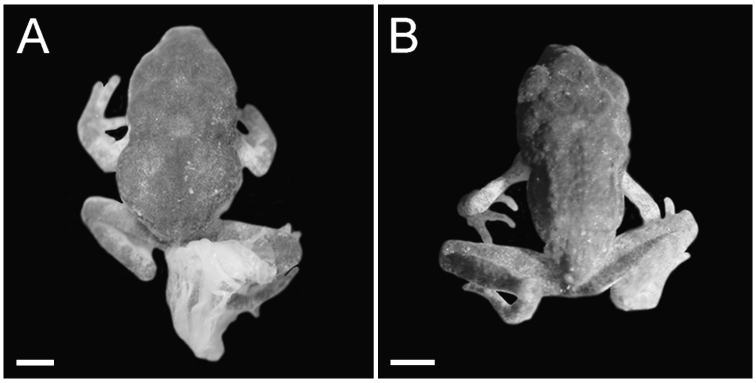

Figure 2. Post-hatching specimens of *Oreobates barituensis.* (A) Specimen with a vestige of the tail. (B) Specimen with the tail completely resorbed. Scale bars = 1 mm.

cles are present. Before hatching, forelimb skin and body integument meet. Digits enlarge and subarticular tubercles develop.

Tail

The tail bud appears at TS4 as a rounded, compact bump rising from the rest of the body and the yolk mass. It can be curved indistinctly to either the right or the left side of the embryo and no fins are discernible. By TS5, the tail has two well-differentiated parts: a stout and curved axial core (sensu Nokhbatolfoghahai et al., 2010) resting on the yolk mass, and a flat and circular expansion that extends laterally (mainly to the right side) and caudally around the surface of the yolk mass to cover hindlimb buds (fig. 1B). A small dextral vent tube appears under the tail and in close contact with the hindlimb bud. The axial core lengthens and straightens at TS6 and well-vascularized lateral extensions grow ventrally and pass dorsally over the head to almost cover the entire embryo (fig. 1C); their margins meet on one side of the embryo to leave a small gap. By TS10, the margins of the lateral extensions fuse and a small opening can be seen in the dorsocaudal or lateral region (fig. 1D). Histological sections through the tail at TS12 show the axial core (including the notochord, the dorsal spinal cord, and blocks of muscles) and tail extensions (fig. 3). The extensions are laterally disposed relative to the axial core, dorsal to the horizontal axis of the notochord. They are composed of a thin epidermis and connective tissue; small blood vessels appear underneath the epidermis and large vessels are immersed within the connective tissue. Muscle blocks are reduced in *O. barituensis* compared to the pond tadpole *Scinax acuminatus* (fig. 3). Before hatching, the tail regresses in size (fig. 1H, I). The lateral extensions first appear translucent and show little or no vascularization but still surround the embryo completely; later, the core shortens and the extensions regress by folding to the inside in a caudal direction. Six of the 11 hatchlings we examined still exhibited vestiges of the tail extensions (fig. 2A).

Body pigmentation and ciliated cells

At TS4, the embryo has no pigmentation and is slightly clearer than the yolk mass. In the

the embryo completely. Pictures within the square are details of the anterior and posterior parts once the tail extensions have been removed. (D) TS10-11, dorsal and posteroventral views. Note the highly vascularized tail extension with a small gap. (E) TS12-13, lateral view. Note the small gap in the tail extensions. (F) Same specimens as in D, with the tail extensions removed. Note the egg tooth and the dermal fold. (G) Same specimen as in E, with the tail extensions removed. Note the keratinized egg tooth. (H) TS13, ventral and lateral views, showing the tail regressing. (I) TS14-15, dorsal and lateral views, showing more advanced tail regression. DF: dermal fold; ET: egg tooth; FL: forelimb; G: gap in the enveloping tail; GA: gill arches; TE: tail extension; VT: vent tube. Scale bars = 1 mm.

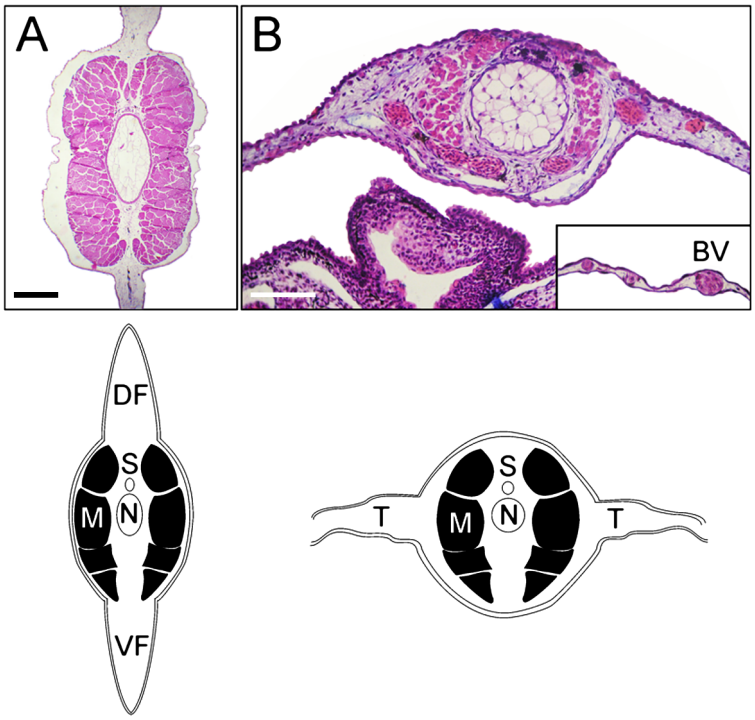

Figure 3. Tail cross sections. (A) *Scinax acuminatus* pond tadpole. (B) *Oreobates barituensis*, tail distal portion with a detail of large blood vessels in the lateral extensions. The spinal cord/notochord axis is in line with the fins in (A) but perpendicular to the lateral extensions in (B); also note the large blood vessels in contact with the epidermis in *Oreobates*. BV: blood vessel; DF: dorsal fin; M: muscles; N: notochord; S: spinal cord; T: tail extension; VF: ventral fin. Scale bars = 1 mm (A) and 0.1 mm (B). This figure is published in colour in the online version.

next stage, head, trunk, and tail are slightly pigmented while limb buds lack melanophores. Circular or oval ciliated cells are regularly distributed on the lateral region of the head and trunk, dermal fold, limb buds, and yolk mass (fig. 4A). Body pigmentation that increases as development progresses results in a dispersed pattern of scattered melanophores on the head and the trunk. Hind limbs are lightly pigmented dorsally. By TS11, pigmentation has only progressed over the ventral side of the fore- and hind limbs and on the lower jaw. At TS12 pigmentation produces an almost uniform dark pattern, and ciliated cells can still be seen near the nostrils (fig. 4B), upper and lower jaws and mandibular joint, and the medial region of yolk mass. Before hatching, the entire embryo exhibits a dark dorsal pigmentation pattern with a thin white sagittal line extending from the middle of the eyes to the origin of the tail.

Discussion

Our data are the first record of oviposition site and early development of *Oreobates barituensis*. This species lays eggs at the same sites where the territorial males vocalize, and males defend vocalization sites from conspecific males by aggressive vocalizations and physical aggression (Akmentins, 2011). The small amount of information on the reproductive biology of this and related species makes it difficult to draw any general conclusion from the findings of multiple clutches in the proximity of a vocalizing male, but parental care (in several variants such as egg and tadpole attendance, clutch brooding, and froglet trans-

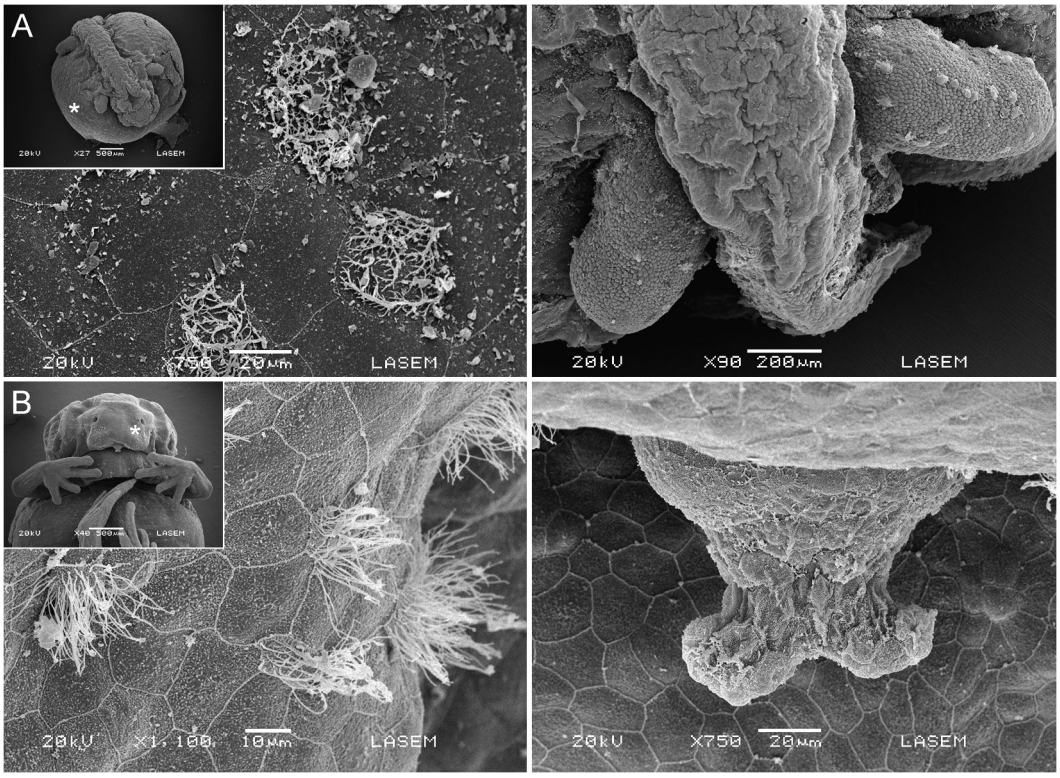

Figure 4. Scanning electron micrographs of specimens of *Oreobates barituensis.* (A) TS6, ciliated cells from yolk mass (left, as indicated by the asterisk) and detail of the ciliated hind limbs and vent tube (right). (B) TS12, ciliated cells from the nostril region (left, as indicated by the asterisk) and detail of the egg tooth (right).

port) is common in terraranan species and among other taxa with endotrophic development (Wells, 2007).

Some data on the number of oocytes in female oviducts is available for few *Oreobates* species (Crump, 1974; Lynch, 1975; Padial and De la Riva, 2005). The number of eggs in the clutches we collected was similar to the number of post-vitellogenic oocytes present in the oviducts of dissected females, which suggests that a female of *O. barituensis* spends all her reproductive output for a given season in a single oviposition event.

The development period that we estimated for *Oreobates barituensis* (35 to 40 days in natural conditions) represents one of the longest known periods among terraranans. Pombal (1999) reported a period of 64 days for *Brachycephalus ephippium*. Even when the time from cleavage to hatching is species-specific and the information available is quite scarce, it is interesting to note that in terminal groups, the whole development is longer than in the most basal family within Terrarana (Eleutherodactylidae, about 15-25 days; Gitlin, 1944; Lynn and Lutz, 1946; Jameson, 1950; Nina and Del Pino, 1977; Townsend and Stewart, 1985). Figure 5A depicts the delayed development of *O. barituensis* in comparison with that of *Eleutherodactylus coqui*.

Townsend and Stewart (1985) proposed a staging table based on *Eleutherodactylus coqui* embryos as a useful tool for a general staging for the genus. In general terms, we could also recognize these stages in *Oreobates barituensis*, but several differences that we observed point out that comparative studies in several taxa are necessary to understand morphological and developmental diversity. Most relevant variations are discussed as follows.

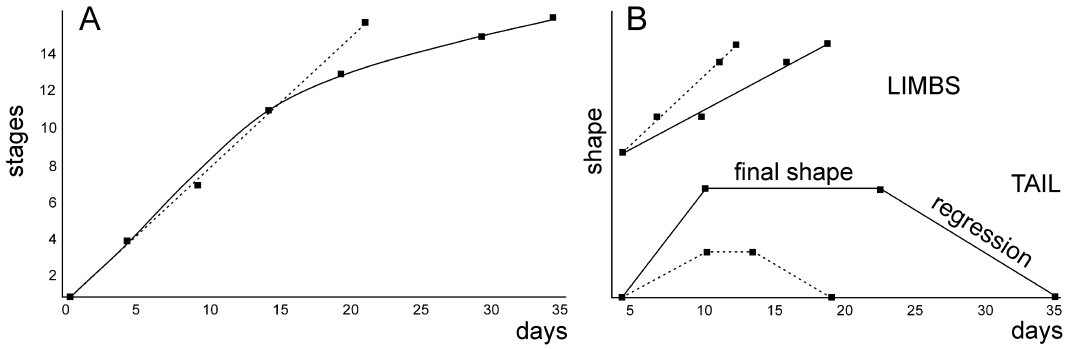

Figure 5. Plots comparing embryonic development of *Eleutherodactylus coqui* (dotted lines) and *Oreobates barituensis* (solid lines). (A) Rates of time development per stage of Townsend and Stewart (1985). Timing in *E. coqui* was taken from the original paper, and an average of the described developmental period (17-26 days) was used. Embryos were raised at comparable temperatures (20-25°C). Note that embryonic development in *O. barituensis* takes longer mainly because of an arrested rate at later stages. (B) Limbs and tail developmental events. The Y-axis represents shape stages in limb and tail development, described in two separate ordinal scales. Limb development is defined as the sequential differentiation of limb buds, elbows and knees, and toes, and finally toe elongation; note the similar onset and the isomorphic limb final shape achieved after a slower rate and a later offset in *O. barituensis.* Tail development is summarized as tail bud stage, full tail 70% the snout-vent length, and full enveloping tail; in *O. barituensis*, a peramorphic final shape results from an accelerated rate.

Limbs, gills, and surface ciliation

Limb development is slower in *Oreobates barituensis* than in *Eleutherodactylus coqui*, with a similar final shape achieved through a slower developmental rate combined with a later offset time (fig. 5B). Also, fore- and hindlimb buds appear approximately in synchrony. Limb synchrony, predisplaced (sensu Reilly et al., 1997) hind limbs, and predisplaced forelimbs have been described in metamorphosing and direct-developing frogs (e.g., Richardson et al., 1998; Thibaudeau and Altig, 1999; Fabrezi et al., 2009; Goldberg, 2009). All these variations indicate that limb development in anurans is more variable than suspected (Bininda-Emonds et al., 2007). As regard to the external gills, while they can be present or absent in *Eleutherodactylus* (Townsend and Stewart, 1985), they are absent in *O. barituensis* and in related *Craugastor augusti* (Jameson, 1950) and *Ischnocnema guentheri* (Lynn and Lutz, 1946). In *O. barituensis*, the profuse, persistent ciliation similar to that described for the closely related *Pristimanis urichi* (Nokhbatolfoghahai et al., 2005, 2006; Nokhbatolfoghahai, 2006), together with the large, vascularized tail, would replace gills in the respiratory function.

Vent tube

A dextral vent tube that occurs in *Oreobates barituensis* from TS6 to TS14 has not been reported in other direct-developing frogs. The vent tube represents a typical tadpole structure which opens to the exterior from the ventral body wall in the sagittal plane or toward the right or left side of the body, either free or attached to the ventral fin (Thibaudeau and Altig, 1999). Its presence in a direct-developing frog deserves an exhaustive sampling as it represents new data for the discussion of the resultant deletion of the tadpole or "larval cassette" from the anuran ancestral life-history in direct developers (Callery and Elinson, 2000; Anstis et al., 2011), and reinforces the idea that each direct-developing lineage possesses a particular suite of ancestral (larval) and derived traits.

Tail

The tail in direct-developing frogs is one of the most striking organs because of its pecu-

liar function and structure. In most taxa, the tail has two regions: an axial core formed of the notochord, spinal cord and associated skeletal muscle, and fins modified as vascular extensions positioned dorsally and ventrally to the axial core, just like in tadpoles (Nokhbatolfoghahai et al., 2010). During development, general features are also similar within terraranans: the tail appears at TS4, then it extends and bends to one side, achieves its maximum development around TS12, and finally it regresses and disappears at hatching (e.g., Gitlin, 1944; Lynn and Lutz, 1946; Townsend and Stewart, 1985). In *Oreobates barituensis*, the tail extensions show an extraordinary growth and as early as TS6 already enclose the entire embryo. Specimens of *O.* cf. *discoidalis* (FML 02342; around TS5) have a short, apparently *Eleutherodactylus*-like tail, but more advanced embryos would be needed to assess if this is not a previous stage of tail development characteristic of this lineage. An enveloping tail was described in *Craugastor augusti* (Jameson, 1950; Valett and Jameson, 1961), but in this case tail expansions grow one over the other, resulting in external and internal sides; also, because the earliest stage examined was about TS11, we are not certain about the stage that the tail acquires its maximum development in that species. Among the Brachycephalidae, tail extensions may be short (e.g., *Brachycephalus ephippium*; Pombal Jr., 1999) or well-developed enough to cover the forelimbs (e.g., *Ischnocnema guentheri*; Lynn and Lutz, 1946). Considering time data, we interpret that heterochronic changes have occurred during tail ontogeny in *O. barituensis.* A comparison with the tail development of *E. coqui* suggests that in *O. barituensis*, a peramorphic pattern results from an accelerated growth rate (i.e., a final shape exceeding the ancestral shape is achieved after a similar time period; Reilly et al., 1997) (fig. 5B).

Recently, Nokhbatolfoghahai et al. (2010) explored tail morphology in the closely related species *Pristimantis urichi* and showed that the expanded part is not produced by the growth of dorsal or ventral fins (like in *Eleutherodactylus coqui* and other direct developers), but rather, is an extension of the lateral sides and tip of the tail around the yolk mass. Conversely, Valett and Jameson (1961) interpret tail expansions of the enveloping tail of *Craugastor augusti* as fin elongation; they described that posterior to the point where the spinal cord disappears, the notochord and the tail rotate to the right, thus implying that the dorsal extension becomes the tail right side and the ventral the left side (also see their fig. 2). *Oreobates barituensis* shows the same structural pattern than that of *P. urichi*. Based on the location of neural tube, notochord and muscles, the dorsolateral skin of the tail expands laterally and posteriorly; like regular fins, these lateral extensions are composed of thin epidermal layers separated by connective tissue and blood vessels (fig. 3). We are not certain about histological details of the ontogeny of the tail, and we lack early specimens to trace the first indications of tail extension formation as to discard an initial dorsal/ventral pattern. Nevertheless, this novel laterally expanded tail configuration appears to be so far exclusive to *Pristimantis* and *Oreobates*.

According to Hanken (2003), the evolution of direct development is correlated with the origin of novel morphological and functional configurations that are absent in metamorphosing species. In this context, the distinctive morphology of the tail extensions in *Oreobates barituensis* could have arisen from a reorganization or repatterning during embryonic development in which a spatial change could be involved. This kind of structural change represents another source of change other than the timing changes studied in direct development (i.e., morphological modification of larval structures). Heterotopy is defined as an evolutionary modification of the location of a particular feature (Webster and Zelditch, 2005), or as an evolutionary change in the relative arrangement in space of developmental events (Arthur,

2011), so that novel proximity relationships are established between features. It has been recognized as an important source in the generation of morphological novelty and it implies that the ontogeny is redirected, so that evolutionary change occurs along a novel direction, not that of the ancestral ontogeny (Hall, 1999; Webster and Zelditch, 2005). In this context, lateral extensions of the tail of *O. barituensis* and *Pristimantis urichi* (at least) might be interpreted as an evolutionary novelty resulting from heterotopic change of tail fins. Descriptions of tail extension development in more terraranan species, including several approaches from structural to molecular levels, are necessary to verify the distribution of this novel structure throughout the clade and to assess the nature of the evolutionary origin of this change.

Our findings indicate that, within current knowledge of developmental variation among direct-developing frogs, the ontogenetic trajectory of *Oreobates barituensis* exhibits a suite of embryonic characteristics that result from structural and temporal changes relative to those of related taxa. These unusual traits are exclusive to embryonic stages and reveal a pattern of dissociation of developmental events between the embryonic and postembryonic stages with no consequences for adult morphology. Finally, we propose that the distinctive tail in *O. barituensis* is derived from both heterochronic (the whole tail) and heterotopic (tail extensions) shifts during the ontogeny of this structure.

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