Limb development and evolution: a frog embryo with no apical ectodermal ridge (AER)

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ABSTRACT

The treefrog *Eleutherodactylus coqui* is a direct developer—it has no tadpole stage. The limb buds develop earlier than in metamorphosing species (indirect developers, such as *Xenopus laevis*). Previous molecular studies suggest that at least some mechanisms of limb development in *E. coqui* are similar to those of other vertebrates and we wished to see how limb morphogenesis in this species compares with that in other vertebrates. We found that the hind limb buds are larger and more advanced than the forelimbs at all stages examined, thus differing from the typical amniote pattern. The limb buds were also small compared to those in the chick. Scanning and transmission electron microscopy showed that although the apical ectoderm is thickened, there was no apical ectodermal ridge (AER). In addition, the limb buds lacked the dorsoventral flattening seen in many amniotes. These findings could suggest a mechanical function for the AER in maintaining dorsoventral flattening, although not all data are consistent with this view. Removal of distal ectoderm from *E. coqui* hindlimb buds does not stop outgrowth, although it does produce anterior defects in the skeletal pattern. The defects are less severe when the excisions are performed earlier. These results contrast with the chick, in which AER excision leads to loss of distal structures. We suggest that an AER was present in the common ancestor of anurans and amniotes and has been lost in at least some direct developers including *E. coqui*.

Key words: *Eleutherodactylus*; limb bud; anurans; direct development; heterochrony.

INTRODUCTION

The frogs and toads most often studied by developmental biologists, including *Xenopus laevis*, show indirect development. They have a biphasic life history, which includes a free-living, aquatic larval stage (tadpole). Adult characters develop at metamorphosis. By contrast, direct developers hatch as froglets without going through a discrete tadpole stage or metamorphosis. They produce a few large-yolked eggs (Elinson, 1987; Duellman & Trueb, 1994). Direct development has evolved independently in several amphibian clades from the primitive condition of indirect development (Wake, 1989; Hanken et al. 1997*a*). Indirect development may in turn have evolved from direct development by the insertion of larval characters into the frog life history (Elinson, 1990).

Direct development has been studied in the large genus of neotropical treefrogs *Eleutherodactylus* (Lynn, 1942; Gitlin, 1944; Adamson et al. 1960; Chibon, 1960). Some features of embryonic development in this group are quite different from other anurans. *Eleutherodactylus coqui* shows an evolutionary reduction or loss of some larval cranial cartilages, lateral lines, external gills, keratinised mouthparts and the cement gland (Hanken et al. 1992; Moury & Hanken, 1995; Fang & Elinson, 1996). There are many shifts in developmental timing (heterochrony; Richardson, 1995) relative to indirect developing species. Adult skull bones appear early (Hanken et al. 1992) and patterns of jaw muscle development are

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greatly modified (Hanken et al. 1997*b*). The limb buds appear shortly after neurulation.

Other aspects of embryonic development remain conserved among direct and indirect developers. For example, the general features of cranial neural crest migration in *E. coqui* are similar to those in other anurans (Moury & Hanken, 1995; Fang & Elinson, 1996). Furthermore the expression of genes related to *Drosophila distal-less* in the developing head and limbs of *E. coqui* are similar to those in *Xenopus laevis* (Fang & Elinson, 1996). Nevertheless, even conserved homeobox genes show some differences in expression patterns. For example *Dlx* transcripts are detected in mandibular crest cells in *E. coqui* at an earlier stage than in *Xenopus laevis* (Fang & Elinson, 1996).

We know relatively little about limb development in direct developers (Elinson, 1994; Fang & Elinson, 1996). Some basic questions remain unanswered, such as whether *E. coqui* limb buds have an apical ectodermal ridge (AER). The AER, a projecting ridge of stratified or pseudostratified columnar epithelium, has essential signalling functions in the chicken embryo (Laufer et al. 1997; Rodriguez-Esteban et al. 1997). It maintains outgrowth and polarising activity (Niswander et al. 1993; Vogel & Tickle, 1993; Summerbell, 1974), an action mediated by FGF-4 (Niswander et al. 1993).

Evolutionary reduction of the AER is often associated with limb loss. For example, some limbless reptiles have a poorly developed AER which undergoes regression before the limb bud has fully developed (Raynaud, 1985). An AER has been found in all species of anuran in which it has been sought (Hanken, 1986), in all amniote classes, and at the tip of the paired fin buds of some teleosts (Geraudie, 1978; Wood, 1982; Thorogood, 1991). An AER has not been found in urodeles where it has been specifically sought (Hanken, 1986).

Dlx genes are strongly expressed in the AER of the limb in many vertebrates (Dolle et al. 1992; Ferrari et al. 1995; Morasso et al. 1995; Mullen et al. 1996). These genes appear to be expressed generally in outgrowing appendages including insect legs and wings and crustacean limb branches (Averof & Cohen, 1997; Panganiban et al. 1997; Shubin et al. 1997). The expression of *Dlx* genes in the apical limb ectoderm in *E. coqui* (Fang & Elinson, 1996) suggests that the mechanisms of limb development are similar to those in other vertebrates. We have examined this question using histology and electron microscopy to determine the patterns of limb morphogenesis in *E. coqui*. The structure of the apical ectoderm has been characterised. Epithelial-mesenchymal interactions have been examined by ablating the apical ectoderm at different stages and looking at the effects on skeletal patterning.

MATERIALS AND METHODS

Staging

Embryos were staged according to the Townsend & Stewart (1985) series, which defines 15 embryonic stages from fertilisation (1) to hatching (15).

Animal care

Adult *Eleutherodactylus coqui* (Thomas, 1966) were collected in the wild in Puerto Rico, where they are abundant. Collection was licensed by the Department of Natural Resources, Puerto Rico. Laboratory colonies were established and embryos obtained following spontaneous natural breeding (Elinson et al. 1990; Elinson, 1994). The majority of embryos were from a colony maintained in Toronto (R. P. Elinson); others, including all embryos used for manipulations, were from a colony maintained at Boulder (J. Hanken). Eggs were removed from the attending male and rinsed in Holtfreter's antibiotic saline (80 mg/l gentamicin sulphate in 10% Holtfreter's solution). They were then placed on moist filter paper in a Petri dish, covered and kept in the dark at 24 °C.

Preparation for histology and electron microscopy

Embryos were de-jellied either chemically (2% cysteine, buffered to pH 7.8–8 with 5 N NaOH) or manually with watchmakers' forceps. Most were fixed in half-strength Karnovsky's fixative (Karnovsky, 1965), at 4 °C for 18 h, and shipped to London in sterile phosphate-buffered saline (PBS) or in fixative. In a few cases, 4% paraformaldehyde, or Bouin's fluid (Sanderson, 1994) was used as the primary fixative. Embryos were rinsed in PBS, then run stepwise to 70% ethanol for long-term storage. Where Bouin's fixative had been used, residual picric acid was washed out by repeatedly changing the ethanol over many weeks. Embryos were then processed in one of the following ways.

Plastic sections

Specimens were run to 0.1 M cacodylate buffer, osmicated in 1% osmium tetroxide in buffer (4 °C, 2–18 h) and rinsed in PBS. They were dehydrated in

graded ethanols to 100% and embedded in Spurr's resin (Spurr, 1969) using propylene oxide as the intermediate reagent. Sections for light microscopy were cut at 1–2 μm and stained with crystal violet in borax. Ultrathin sections for transmission electron microscopy (TEM) were mounted on grids and stained with uranyl acetate and lead citrate. They were examined at 60 kV on a Phillips 300 electron microscope.

Scanning electron microscopy (*SEM*)

Embryos were osmicated and dehydrated with ethanol, dried at the critical point of carbon dioxide, and mounted on aluminium stubs. Sputter coating with gold was followed by examination in a Zeiss DSM940 microscope.

Paraffin histology

Specimens were taken to 100% ethanol and cleared in methyl salicylate (3 changes of at least 8 h each). The friability of the yolk was counteracted by infiltrating the embryos with low viscosity nitrocellulose (Merck) in methyl salicylate (1%, 18 h; then 2%, 18 h). The embryos were rinsed in toluene (5 min), hardened in chloroform (30 min) and transferred to 3 changes (2 h each) of histological paraffin wax (Merck, 60 °C, under vacuum). Serial sections were cut at 3–6 μm and stained with safranin, methyl blue and Orange G (Sentein, 1976).

Wholemounts

For examination of external morphology, embryos were stained for 18 h with 0.03% Alcian blue in acid alcohol (70% ethanol with 1% concentrated hydrochloric acid). They were then differentiated for 48 h in acid alcohol and stored in 70% alcohol. Viewing through a stereo dissecting microscope, fitted with an orange G filter, showed the pale cellular tissue of the embryo against a dark background of yolky tissue. Measurements (to the nearest 10 μm) were made with an eyepiece graticule fitted to a stereo dissecting microscope.

Ectoderm excision

All procedures were conducted in accordance with local regulations (University of Colorado). Viable embryos were de-jellied and placed in Petri dishes with a 2% agar bed, covered with 10% Holtfreter's antibiotic saline. Spontaneous movements were reduced by immersion for up to 15 min in 0.03% aqueous ethyl m-aminobenzoate tricaine methanesulphonate. Distal hind limb bud ectoderm was teased away from the underlying mesenchyme of stage 4–6 embryos using watchmakers' forceps, hairloops, and scalpels. Embryos were then transferred to 10% Holtfreter's antibiotic saline and maintained at 24 °C in the dark. At stage 13, when the cartilaginous skeleton is well developed, embryos were fixed and stained for cartilage with Alcian blue (Klymkowsky & Hanken, 1991).

RESULTS

Normal limb bud development

The number of embryos examined at each stage is given in Table 1.

Stage 3. The hind limb fields are first seen, in stained wholemounts, as diffuse patches of lateral plate mesoderm on the surface of the yolk sac (Fig. 1*a*). They lie to each side of the blastopore, and become slightly raised above the yolk surface near the end of this stage. A broad, shallow depression is thereby formed between the hind limb area and the trunk. In paraffin sections, many nuclei can be seen among the yolk platelets in the hind limb region. Very few nuclei can be seen in the yolk-laden mesoderm of the prospective forelimb region. The forelimb region is either absent or only faintly indicated in wholemounts at this stage.

Stage 4. The hind limb buds are craniocaudally elongated ridges (Figs 1*b*, *c*). Dimensions of the limb

Table 1. *Numbers of embryos used to study normal stages of limb morphogenesis in* E. coqui, *with method of processing**

	Number of embryos				
Stage	SEM	Semithin	TEM	Paraffin	Wholemount
3	3	7	0	4	10
4	4	4	1	5	12
5	2	9	3	3	9
6	2	5	2	0	7
6/7†	1	0	0	2	3
7	2	2	2	0	2
8	1	1	0	0	9

* SEM, scanning electron microscopy; semithin, plastic sections for light microscopy; TEM, transmission electron microscopy; wholemount, embryo stained with Alcian blue and examined under the stereo dissecting microscope. All embryos used for TEM were examined by light microscopy and are therefore listed twice.
† 6/7 indicates embryos intermediate between stages 6 and 7.

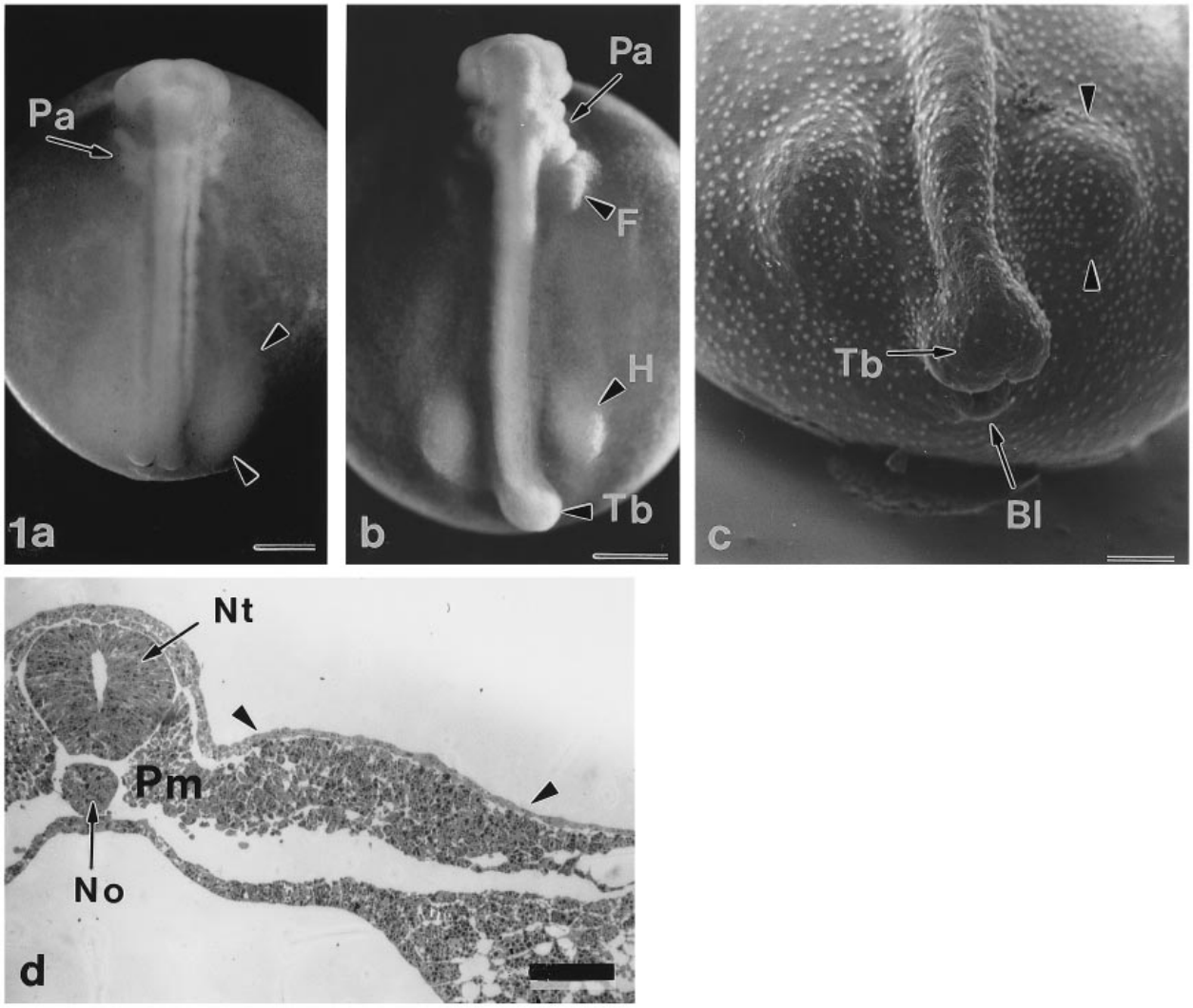

Fig. 1. (*a*) Dorsal view of stained, stage 3 wholemount, viewed with an orange filter. The hind limb fields (right field marked with arrowheads) have developed as condensed lateral plate mesoderm on the surface of the yolk sac. The pharyngeal arches (Pa) are also visible. Anterior is to the top. Bar, 500 μm. (*b*) Dorsal view of stained, stage 4 wholemount, viewed with an orange filter. Hind limbs (H) are now distinct buds. Forelimb buds (F) are developing immediately caudal to the pharyngeal arches (Pa). Tb, tailbud. Anterior is to the top. Bar, 500 μm. (*c*) SEM of stage 4 hind limb buds (right bud between arrowheads). There is no apical ridge. Tb, tailbud; Bl, blastopore. Dorsal view; anterior is to the top. Bar, 200 μm. (*d*) Transverse plastic section through the hind limb field (between arrowheads) of an early stage 4 embryo. Dorsal is to the top, lateral is to the right. The lateral plate is continuous with the paraxial mesoderm (Pm), although in wholemounts, the limb buds appear to be detached from the trunk. The large cavity in the lower left corner of the micrograph is the archenteron. No, notochord; Nt, neural tube. Crystal violet stain. Bar, 200 μm.

buds are given in Table 2. The buds project well above the yolk surface, leaving a deep gutter between the hind limb bud and the trunk. Because the yolk sac in *E. coqui* is so large, the somatopleure spreads away from the embryonic axis almost horizontally. For this reason, the limbs buds appear to project vertically. In wholemounts, the hind limb buds appear to be detached from the trunk. However in sections the limb mesoderm is seen to be continuous with the paraxial mesoderm (Fig. 1*d*). It is also continuous laterally with a very thin layer of mesoderm spreading over the yolk sac. The hind limb bud is covered by simple squamous ectoderm and periderm layers.

In the youngest embryos of stage 4, the forelimb fields are indistinct. Later in this stage (Fig. 1*b*) they start to project slightly above the yolk surface. They develop immediately caudal to the branchial region.

Stage 5. In early stage 5 the hind limb buds are hemispherical masses covered with ectoderm and periderm (Fig. 2*a–c*). The mesenchyme is more densely packed around the periphery than it is in the core of the limb bud. By late stage 5 the buds have elongated so that they are about as long as they are wide (Table 2). The ectoderm at the limb tip has become cuboidal, while that at the base is still squamous. As a consequence, the apical ectoderm is now thicker (30.7 μm including periderm; S.D. = 4.62) than the more proximal ectoderm (18 μm; S.D. =

Table 2. *Dimensions of limb buds at different stages, measured from wholemounts**

		Forelimb				Hind limb			
		Width (μm)		Length (μm)		Width (μm)		Length (μm)	
Stage	n	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
4	8	—	—	—	—	830	127	187	38.2
5	5	411	20.6	409	30.9	491	18.4	583	51
6	5	399	33.3	536	50.6	462	37.3	703	41.5
6/7	3	387	25.8	550	45.2	422	25.8	775	73.9
7	2	358	7.69	588	14.7	450	7.69	904	29.1
8	8	458	21.2	697	51.8	550	34.4	1104	87.3

* The forelimb buds are too indistinct at stage 4 to be measured accurately. n, number of embryos. S.D., standard deviation. All 4 limbs, when present, were measured in each embryo. Length, proximodistal axis of limb (in the case of stage 4 hind limbs, this is the height the bud projects above the yolk sac). Width, anteroposterior axis of limb, measured at the widest point. In later stages this was across the digital plate. In the case of stage 4 hind limbs it is useful to note the additional parameter of mediolateral dimension (at the widest point); this averaged 481 μm (S.D. = 54.4; n = 8).

4.89). The transition between squamous and cuboidal ectoderm is a gradual one. The appearance in sections is of a thickened cap of ectoderm over the tip of the limb bud (Fig. 2*c*).

The lateral plate extends between fore and hind limbs as a thin sheet, 2–3 cells thick. The forelimb buds are similar in shape to the hind limbs, but smaller (Table 2). They are also histologically less differentiated. Yolk platelets predominate in the mesenchyme of the forelimb, whereas cell nuclei predominate in the hind limb (data not shown).

Stage 6. The hind limbs are now longer than they are wide (Table 2; Fig. 3*a*). A faint constriction in midpoint of the long axis marks the beginning of digital plate formation. Sections show a marginal sinus in both pairs of limbs, subjacent to the thickened apical ectodermal cap (Fig. 3*b*). Premuscle and precartilage condensations resembling those seen in amniotes (Ede, 1976) are developing in the hind limb, but not yet in the forelimb.

The forelimbs are conical projections, longer than they are wide (Table 2). Cells in the ectodermal cap covering the limb tip are becoming columnar (Fig. 3*c*). The periderm is a yolky squamous layer with occasional rounded, ciliated, yolk-filled cells protruding above the surface (Fig. 3*c*). The cap of ectoderm over the tip of the limb is a columnar or pseudostratified epithelium (Fig. 3*c*). The forelimb cap is 22.26 μm thick (S.D. = 1.90) including periderm, compared to 10.33 μm (S.D. = 2.42) for ectoderm at the base of the limb. However, there is no evidence from histological sections or EM of any ridge-like organisation in the ectoderm.

Stage 6/7. These embryos are intermediate in character between stages 6 and 7. Both pairs of limbs show a shallow notch on the anterior margin, although the one on the forelimb is indistinct (Fig. 3*d*).

Stage 7. The anterior borders of both fore and hind limbs are distinctly notched (Fig. 4*a*). The hind limb digital plate is pointed because of growth of the digit IV anlage. The forelimb digital plate is rounded in dorsal view. The apical ectoderm is cuboidal. Nerves are growing into the base of the limbs.

Stage 8. The digital plates show strong dorsoventral flattening (Fig. 4*b*). A small projection on the posterior border of the hind limb digital plate indicates the future position of digit V (Fig. 4*c*). The apical ectoderm is cuboidal or squamous and there is no longer a distinct apical cap at the tip of the limbs (Fig. 4*d*). Dorsal and ventral ectoderm shows yolk-laden peridermal cells, but the apical ectoderm is free of such cells. The amount of yolk in the mesenchyme is greatly reduced and confined largely to the proximal chondrogenic cells. Nerves have grown into both pairs of limbs (Fig. 4*d*). In the hind limbs they run between the premuscle masses at the periphery of the limb, and the more central precartilage condensations. The latter are visible in the digital plate and in the core of more proximal areas of the hind limb.

The forelimb also shows cellular condensations but these are not yet as discrete as those in the hind limb (Fig. 4*d*). Therefore, as at all other stages examined, the forelimb is slightly less advanced in development than the hind limb.

Excision of apical ectoderm

The effects of removing distal hind limb ectoderm at stages 4–6 are summarised in Table 3. The normal

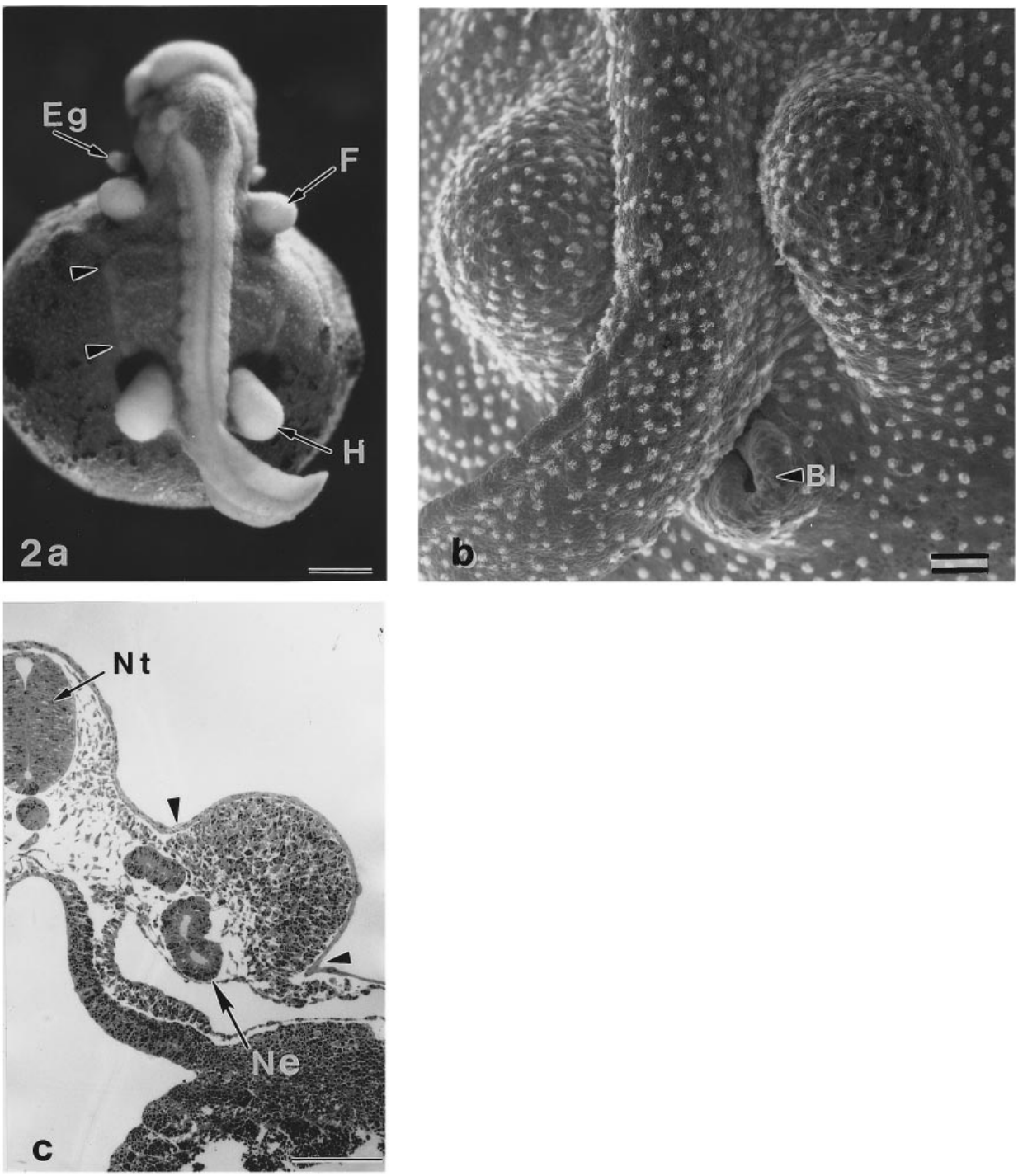

Fig. 2. (*a*) Dorsal view of a stained, stage 5 wholemount, viewed with an orange filter. Anterior is to the top. Forelimbs (F) and hind limbs (H) are prominent buds, as long as they are wide or slightly longer. Arrowheads indicate the margin of the thin lateral plate which extends between the limbs. Eg, external gill. Bar, 500 μm. (*b*) SEM of early stage 5 hind limb buds, dorsal view. There is no evidence of an AER. The white spots covering the embryo are ciliated periderm cells. In contrast to A, the tail curls to the left in this specimen. Bl, blastopore. Anterior is to the top. Bar, 100 μm. (*c*) Transverse plastic section through a stage 5 embryo. The forelimb bud (between the arrowheads) projects from the somatopleure, in close relation to the nephric tubules (Ne). Nt, neural tube. The archenteron is the space in the bottom left corner of the micrograph. Dorsal is to the top, lateral is to the right. Crystal violet stain. Bar, 200 μm.

skeletal pattern of the *E. coqui* hind limb is shown in Figure 5*a*, *b*. Of the 6 cases obtained from stage 4 excisions, and analysed at stage 13, 2 had normal limbs. In the other 4 embryos, the proximal ends of metatarsals I and II were fused (Fig. 5*c*). Of the 5 embryos operated on at stage 5, all survived (Fig. 5*d*). All showed loss of tarsal elements I and II, and all had an abnormally small prehallux (about 2/3 normal

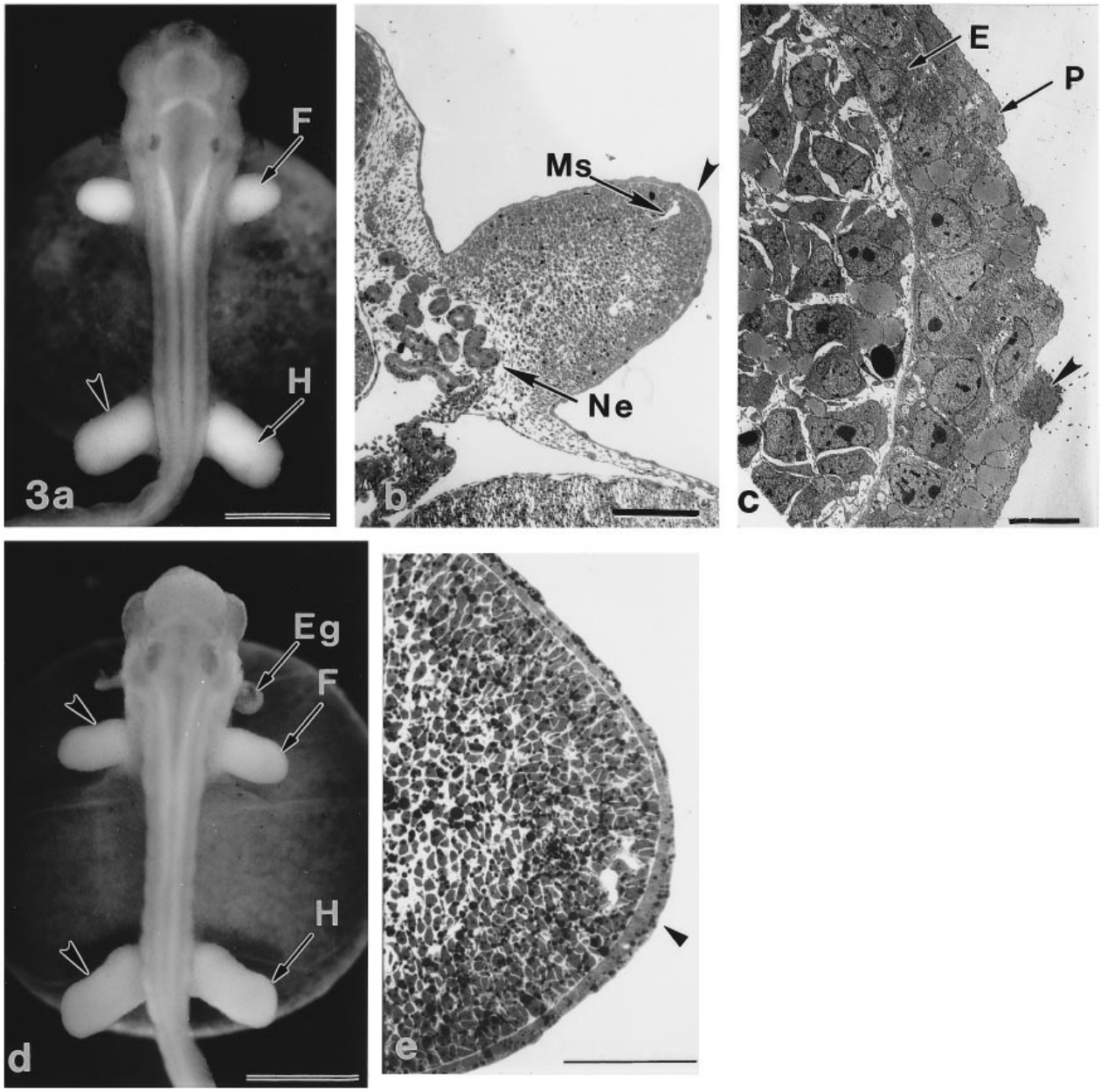

Fig. 3. (*a*) Dorsal view of a stained, stage 6 wholemount, viewed with an orange filter. Forelimb (F) and hind limb (H) buds are longer than wide. A faint constriction (arrowhead) marks the beginning of digital plate formation in the hind limb. Anterior is to the top. Bar, 1 mm. (*b*) Transverse plastic section through a stage 6 embryo. The forelimb bud shows a marginal sinus (Ms) and a slightly thickened apical ectodermal cap (arrowhead). Ne, nephric tubules. Dorsal is to the top, lateral is to the right. Crystal violet stain. Bar, 200 μm. (*c*) Ultrathin section taken from the tip of the limb as indicated by arrowhead in *b*. The limb mesenchyme (on the left) is covered by a cuboidal ectoderm layer (E) and a simple squamous periderm (P). Arrowhead indicates a ciliated periderm cell. Bar, 10 μm. (*d*) Dorsal view of a stained, stage 6/7 wholemount, viewed with an orange filter. Both the fore (F) and hind limbs (H) show a notch (arrowheads) on the anterior margin, although the forelimb notch is less distinct. The notch marks off the digital plate from the proximal part of the limb. Eg, external gill. Anterior is to the top. Bar, 1 mm. (*e*) Plastic section through stage 6 forelimb. Anterior is to the top. The ectoderm is thicker at the apex of the limb bud (arrowhead) than more proximally. Bar, 100 μm.

size). Three showed fusion between the proximal ends of metatarsals I and II. One further specimen was lacking metatarsal I. The phalanges for digit I in this embryo extended from the tip of metatarsal II.

Two cases were obtained from stage 6 excisions. Both lacked tarsal elements 1 and 2 and showed an abnormally small tarsal element 3 and prehallux (about 1/2 normal size; Fig. 5*e*). Both cases also showed complete lack of the metatarsal and phalanges of digit 1, and loss of all phalanges of digits II and III. The metatarsal elements of digits II and III were abnormally small—around 1/3 normal size—and tapered distally to a point. Digits IV and V appeared normal in both specimens.

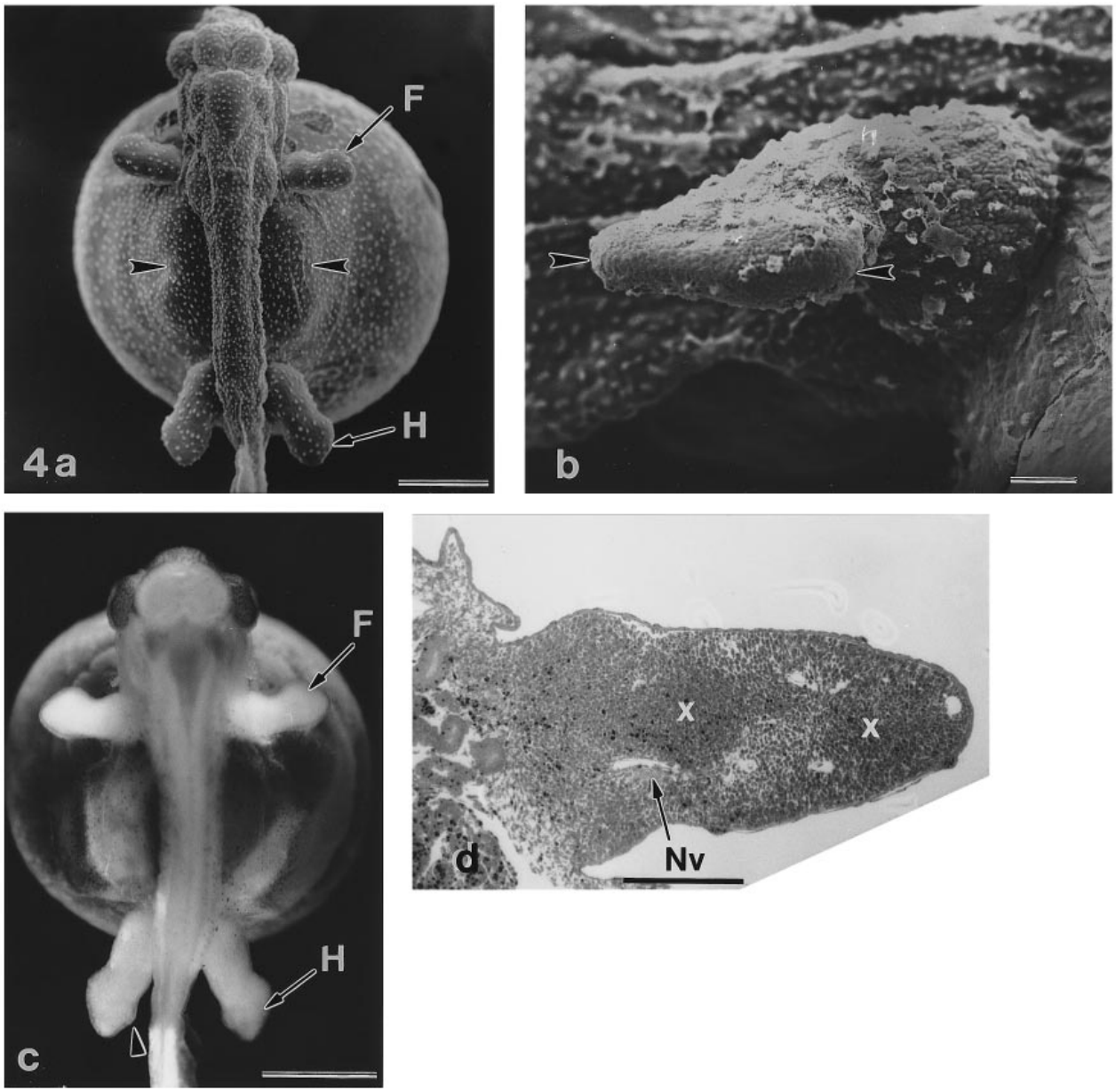

Fig. 4. (*a*) SEM of stage 7 embryo, dorsal view. Both fore (F) and hind limbs (H) have a distinct notch on the anterior margin, demarcating the digital plate. The digital plate on the hind limb has a pointed outline, while that on the forelimb is more rounded. The surface of the yolk sac between the 2 pairs of limbs is raised (arrowheads), due to the accumulation of subblastodermal fluid. This is a normal feature of development in *E.coqui*. Anterior is to the top. Bar, 500 μm. (*b*) Right hind limb of stage 8 embryo, lateral view. The digital plate (between arrowheads) is flattened dorsoventrally, while the proximal part of the limb has a circular cross section. There is no evidence of an apical ridge at the margin of the digital plate. Anterior is to the right, dorsal to the top. Bar, 100 μm. (*c*) Dorsal view of a stained, stage 8 wholemount, viewed with an orange filter. Both the fore (F) and hind (H) limbs have a distinctly pointed digital plate demarcated from the rest of the limb by a notch on the anterior margin. The digit V anlage is visible on the posterior margin of the hind limb digital plate (arrowhead on left hind limb). Anterior is to the top. Bar, 1 mm. (*d*) Transverse plastic section through a stage 8 embryo to show the forelimb. Cellular condensations (x) in the mesenchyme represent early chondrogenic cells. Nv, neurovascular bundle. Dorsal is to the top, proximal is to the left. Crystal violet stain. Bar, 200 μm.

DISCUSSION

We have examined limb development in the direct-developing frog *E. coqui* from stages 3–8. We have also examined the hind limb skeleton at stage 13. The hind limb fields first appear as condensed mesenchyme when the cranial neural folds are still open. This is far earlier than in indirect developers such as *Xenopus laevis* and shows convergence on the amniote pattern of developmental timing (Richardson, 1995). The hind limbs are larger than the forelimbs and are slightly more advanced in development at all stages

Table 3. *Summary of excision experiments**

Stage at operation	No. operated	Result (number of cases)
4	8	Normal (2)
		Metatarsals I and II fused proximally (4)
5	5	Tarsals I and II absent (1)
		Tarsals I and II absent; metatarsals I and II fused proximally (3)
		Tarsals I and II absent; metatarsal I absent (1)
6	4	Metatarsal and phalanges of digit I absent; all phalanges of digits II and III absent; tarsals I an II absent (2)

* The number of embryos operated at different stages is indicated. All survivors reaching stage 13 are listed under 'Result'. The effects on limb morphology, as assessed in cleared wholemounts stained for cartilage, are summarised. Some cartilage elements were abnormally small; see text for description.

examined. Hind limbs also appear earlier than forelimbs in *X. laevis*, but in some urodeles and amniotes this pattern is reversed (Richardson, 1995).

The distal ectoderm forms a broad, thickened cap. It becomes cuboidal at stage 5 and then columnar or pseudostratified at stage 6. However, there was no ridge-like organisation in the apical ectoderm at any stage examined. This is particularly obvious when *E. coqui* limb buds are compared with those of the chick by SEM (Fig. 6*a*, *b*). The surface of the limb tip appears smooth in *E. coqui* with no sign of a ridge. In contrast the chick ectoderm is organised into a prominent ridge which projects above the surface. In lacking an apical ridge, *E. coqui* differs from all other anurans in which a ridge has been sought (reviewed by Hanken, 1986). The AER in *X. laevis* consists of a 3-layered ectodermal thickening clearly visible by SEM (Tarin & Sturdee, 1971). Nothing comparable is seen in *E. coqui*.

The condition of the apical ectoderm in *E. coqui* appears to be similar to that in urodeles. Although a ridge is lacking in those species of urodele where it has been sought, the epithelial-mesenchymal interactions involved in limb morphogenesis appear to be similar to those in amniotes (Sturdee & Connock, 1975; Mullen et al. 1996). In the axolotl there is a thickened cap of ectoderm at the apex of the developing limb bud (and the regenerating limb). The cap cells express a gene related to *distal-less*, as does the distal ectoderm of *E. coqui* (Fang & Elinson, 1996). Thus it is likely that the apical ectoderm of urodeles and *E. coqui*, and the AER cells of other vertebrates, are homologous signalling populations.

Why are the signalling cells of the ectoderm organised into a ridge in some vertebrates but not others? One clue comes from work on the chick limb bud, which normally shows strong dorsoventral flattening (Fig. 6*a*). If the AER is removed, and outgrowth maintained by fibroblast growth factor, dorsoventral flattening is reduced and the buds become more cylindrical; this suggests that the ridge might have mechanical functions (Vogel & Tickle, 1993; Niswander et al. 1994). This is supported by the findings reported here in *E. coqui*. Also consistent with this view is the observation that in some teleosts, the AER is very prominent (Wood, 1982) and the fins extremely flattened. However, ridge development does not always correlate with dorsoventral flattening. In the trout pelvic fin, the ridge is not prominent yet the fin bud is flattened (Geraudie, 1978). Furthermore the limbs of *E. coqui* show dorsoventral flattening at the digital plate stage, even though no ridge is present (Fig 4*b*).

We propose the following hypothesis of AER evolution. The AER was present in the common ancestor of anurans and amniotes. It is retained in amniotes and in indirect-developing frogs (e.g. *X. laevis*), but has been lost in at least some direct developers (e.g. *E. coqui*). It is unknown whether the lack of an AER in urodeles represents the retention of a primitive condition, or an evolutionary loss. In species lacking an AER, signalling functions are carried out by distal ectoderm cells and the limb may lack dorsoventral flattening. This condition is approached in *X. laevis*, in which limb buds lack dorsoventral flattening and have an AER which is described as 'modest' compared with amniotes (Tarin & Sturdee, 1971). In the chick and other species with a well-developed AER, the ridge may have 2 quite different functions: cell-cell signalling, and maintenance of limb bud shape.

The ectoderm excisions reported here must be interepreted with caution. When the apical ectodermal ridge (AER) is removed from a developing chick limb bud, there is progressive loss of distal elements in a stage-dependent manner (Saunders, 1948; Summerbell, 1974). Experiments of this type are more problematic in amphibians because the apical ec-

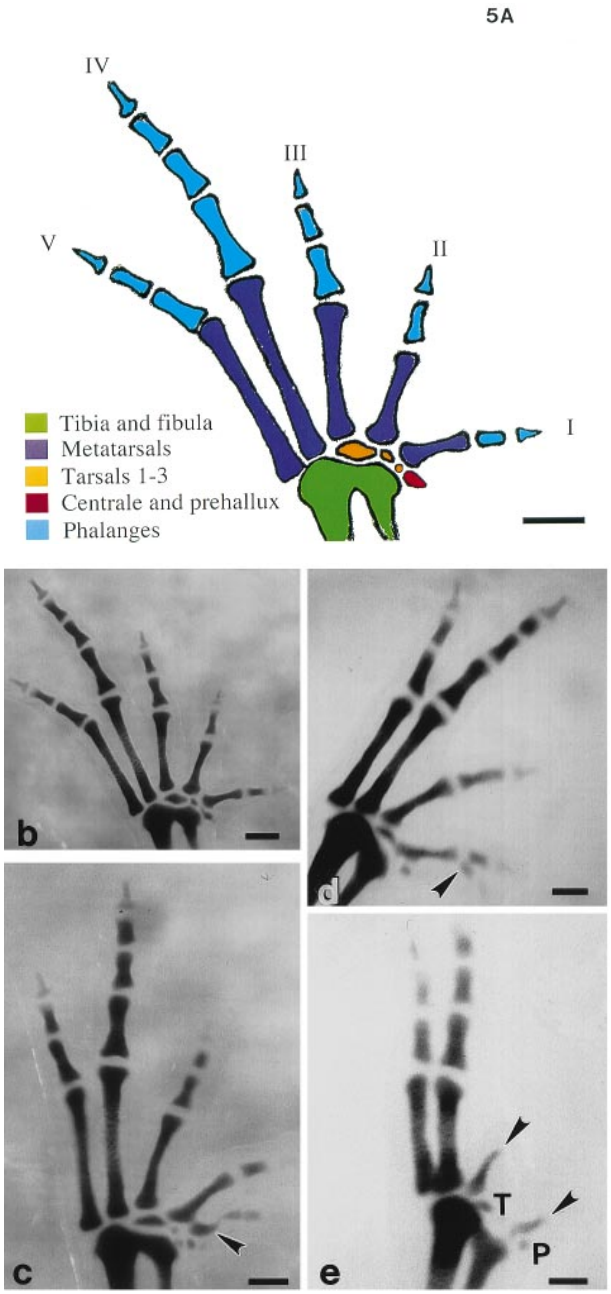

Fig. 5. Hind limbs at stage 13. Bars, 200 μm. All are left limbs, dorsal view, with anterior to the right. (*a*) Diagram showing the arrangement of skeletal elements in the normal limb. Digits numbered I–V. (*b*) Normal hind limb, cleared and stained. (*c*) Hind limb of specimen that had the distal ectoderm removed at stage 4. Arrowhead points to base of metatarsal I, which is fused to metatarsal II. (*d*) Hind limb of specimen that had the distal ectoderm removed at stage 5. Arrowhead points to proximal phalanx of digit I. Metatarsal I has been lost, yet all phalanges are present for each digit. In addition, there has been a loss of tarsal elements 1 and 2. (*e*) Hind limb of specimen that has had its distal ectoderm removed at stage 6. The arrowheads point to metatarsals II and III. Digit I has been lost completely but digits IV and V are normal. T, tarsal III; P, prehallux. Though not easy to see, digit IV does have its most distal phalanx present.

toderm may regenerate following excision (reviewed by Hinchliffe & Johnson, 1980). Our data on *E. coqui* should therefore be considered preliminary. We found

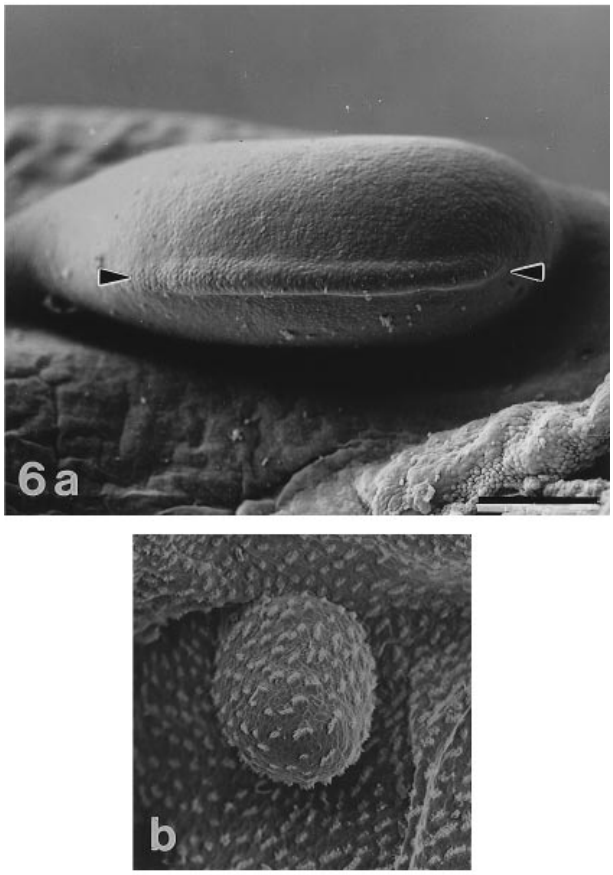

Fig. 6. Comparison between the embryonic forelimb buds of a chicken and *E. coqui* at the same scale. Both views show the distal aspect of the limb bud, with anterior to the left and dorsal to the top. Bar in *a*, 200 μm. (*a*) Chicken (Ross White) embryo, late Hamburger & Hamilton (1951) stage 20 (from an unpublished study by Richardson and Cope). Note the large size, the prominent apical ectodermal ridge and the conspicuous dorsoventral flattening (even though this is much earlier than the digital plate stage). (*b*) *E. coqui* forelimb bud, stage 6/7, oblique dorsolateral view. Compared with that in the chick, the limb bud in *E. coqui* is much smaller, has a cylindrical profile and lacks dorsoventral flattening before the digital plate stage.

that excision of distal ectoderm does not produce truncation of the limb skeleton, although defects in anterior parts of the skeletal pattern were noted.

Excisions caused more extensive defects in *E. coqui* when they were done at later stages. This is in contrast to the chick, where excising the AER at early stages causes more extensive defects (Saunders, 1948). Tschumi (1957) obtained results comparable to those from the chick in his experiments on limb development in *X. laevis*. When he stripped limb buds of their ectoderm and grafted them into the flank, they showed distal truncation. The level of truncation depended on the stage of the limb when grafted. This indicates that in *X. laevis*, as in the chick, limb structures are laid down in proximodistal sequence, and ectoderm is required for this process.

Different results were obtained by Lauthier (1985) with the urodele *Pleurodeles waltl*. If the limb bud was stripped of ectoderm and grafted into the flank, a complete set of limb structures developed. The same was true even if the limb bud was stripped and capped with heterologous ectoderm. Further work is required to look at the possiblity of regeneration of apical ectoderm in *E. coqui*. However, in principle, our initial findings show more resemblance between direct-developing *E. coqui* and urodeles than to amniotes and the metamorphosing frog *X. laevis*.

Our study indicates that although patterns of developmental gene expression are known to be conserved among vertebrates, at least some features of early limb development show evolutionary modification. The AER does not develop until relatively late in mice compared with the chick (Wanek et al. 1989). This suggests that the temporal deployment of conserved mechanisms can be changed during evolution (Richardson, 1995). There are differences in the effects of apical ectoderm excision, although further work is required to assess the possibility of regeneration of apical ectoderm following excision in *E. coqui*. Limb buds in *E. coqui* are smaller than those in amniotes, they lack an AER and are not dorsoventrally flattened until the digital plate forms. These differences are consistent with growing evidence of evolutionary lability of vertebrate development (Richardson et al. 1997).

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