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BRIEF COMMUNICATION

Extending the Table of Stages of Normal Development of the Axolotl: Limb Development

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The existing table of stages of the normal development of the axolotl (*Ambystoma mexicanum*) ends just after hatching. At this time, the forelimbs are small buds. In this study, we extend the staging series through completion of development of the forelimbs and hindlimbs. *Developmental Dynamics 226:555–560, 2003.*

Key words: axolotl; *Ambystoma mexicanum*; urodele; development; limb

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INTRODUCTION

Eggs, embryos, and larvae of the axolotl, *Ambystoma mexicanum*, have been used in studies of vertebrate development and regeneration for an extensive period of time. Axolotls and other urodele amphibians are unique: as adults, they perfectly regenerate lost limbs and other structures. It has been suggested that mechanisms involved in limb regeneration are similar to those controlling larval limb development (Muneoka and Bryant, 1982; Bryant and Gardiner, 1992; Khan et al., 1999). Fewer studies have been performed on the development of the urodele limb compared with those done on amniote limbs or regenerating urodele limbs. For example, several fibroblast growth factors have been cloned in urodeles, including axolotls. These include Fgf-1 and -2 (Boilly et al., 1991; Mullen et al., 1996; Dungan et al., 2002); and Fgf-4, -8, and -10 (Han et al., 2001; Christensen et al., 2002). All have been found in developing urodele limb buds, although their patterns of expression do not always match those of amniotes. The roles of these genes in urodeles may be different from those of higher vertebrates. It would be profitable to expand investigations on axolotl limb bud development to better understand the relationships between amniote and anamniote limb development, and the relationships between processes of limb development and regeneration. Precise communication of the results of limb development experiments requires the existence of a standard description of the appearance and characteristics of the growing limbs.

Normal stages of axolotl development from egg fertilization to the emergence of forelimb buds and hatching have previously been described (Schreckenberg and Jacobson, 1975; Bordzilovskaya and Detlaff, 1979; Armstrong and Malacinski, 1989). To briefly recapitulate the information concerning early limb development: at the late tail bud stages, the prospective forelimb mesoderm is located inferior to somites 3–5 (ventral to the pronephros just posterior to the gill primordium). By stage 36, the forelimbs are visible as faint swellings in ventral views, and are discrete protuberances by stage 40 (just before hatching). Hatching begins at stage 41. During the time that the larval mouth breaks open and the gills elongate (stages 42–

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43), the forelimb buds form triangular shapes that point dorsoposteriorly (Bordzilovskaya and Detlaff, 1979). Here, we extend that series through completion of the development of the forelimb and hindlimb, when all skeletal elements are present.

RESULTS AND DISCUSSION

Shubin and Alberch (1986) describe limb skeletal development of nonurodele tetrapods as a series of cartilage condensations, branching events, and segmentation events. First, de novo cartilage condensation forms the humerus (femur), followed by a branching event that produces the radius (ulna) and the tibia (fibula). The radius and tibia are the preaxial (anterior) elements. These do not branch; they segment to form carpal or tarsal elements (e.g., the radiale, element Y, and the prepollux). The ulna and fibula, the postaxial (posterior) elements, do undergo branching events. These produce carpal and tarsal elements, as well as the digits (metacarpals and metatarsals). The first metacarpal (metatarsal) formed is the penultimate digit: for example, the fourth (IV) of five toes, in the case of anurans. It is produced as one of a pair; the other element is a distal carpal (tarsal). The rest of the digits are produced by the sequential branching of this latter element. Each branching produces a daughter distal carpal (tarsal) and a metacarpal or metatarsal.

The urodele limb does not adhere strictly to this proximal-to-distal sequence in skeletal element formation. After the radius (tibia) and ulna (fibula) are in place, and before these elements undergo segmentation and branching, distal structures form: a carpal or tarsal-like structure called the basale commune condenses de novo and branches to form digit II metacarpal (metatarsal), then digit I metacarpal (metatarsal). Then, the radius (tibia) and ulna (fibula) undergo branching and segmentation events and the basale commune segments to produce the third distal carpal (tarsal). This structure branches to form the digit III metacarpal (metatarsal) and a daughter distal carpal (tarsal). In the urodele forelimb, the fourth distal carpal segments to form digit IV metacarpal; in the hindlimb, one more branching and a subsequent segmentation event produce digits IV and V metatarsals. A second difference between the urodele limb and that of most other vertebrates is that anterior structures form first. In the hindlimb, the order of digit production is (II, I), III, IV, V. In anurans and other five-toed vertebrates, the order of hindlimb digit formation is (IV, V), III, II, I, that is, posterior skeletal elements form first (Shubin and Alberch, 1986).

To extend the stages of axolotl limb development beyond those of Bordzilovskaya and Detlaff (1979), we used photographs of unstained and stained limbs. Experiments performed on the developing limb of urodeles rely on the ability to stage anesthetized larvae under the binocular microscope using reference images. External descriptions of these limb stages were based on those for the *Ambystoma punctatum* (*maculatum*) forelimb described by Harrison (1969) and the descriptions of developmental stages of *Xenopus laevis* externally visualized hindlimbs (Nieuwkoop and Faber, 1967). It is important to note that the cartilage and bone staining protocols common for visualization of vertebrate whole limb skeletal elements show the appearance of the larger skeletal elements, but usually not those of smaller transiently present ones, such as the distal carpal or tarsal elements. Therefore, our descriptions are less detailed but are not in conflict with those of Shubin and Alberch (1986). Larval length is measured in millimeters from snout to tail tip. The external appearance of the limb for each stage is described first, followed by the description of the Victoria Blue cartilage stained limb and the Alizarin Red bone stained limb, if applicable. Figure 1 shows stages 44–52, and Figure 2 shows stages 53–57.

Stage 44 (age: hatching to 5–6 days posthatching (dph)): (Fig. 1B) hatching is complete. Larvae are 11–13 mm in length.

Stage 45 (age: 6–8 dph): (Fig. 1A,B) the forelimb continues to be triangular to conical in shape. Cartilage staining may show the location of the shoulder girdle. Length: 12–15 mm.

Stage 46 (age: 6–10 dph): (Fig. 1C,D) the forelimb is twice as long as it is wide, with a rounded or cone-shaped tip. Cartilage staining shows the shoulder girdle, and faintly, the humerus. Length: 14–17 mm.

Stage 47 (age: 6–10 dph): (Fig. 1E,F) the distal tip of the forelimb is flattened. It is beginning to undergo a torsion that will orient the radial–ulnar plane 30 degrees to the vertical in the fully formed limb. As yet, no interdigital notch is present. The humerus is readily visible in cartilage staining. Length: 14–17 mm.

Stage 48 (age: 7–10 dph): (Fig. 1G,H) the forelimb has a small interdigital notch. Digits I and II are visible as metacarpal condensation rays. A small ulnar bulge is present on the posterior distal part of the forelimb. The radius, and sometimes also the ulna, is visible in cartilage staining. Length: 15–17 mm.

Stage 49 (age: 7–10 dph): (Fig. 1I,J) the interdigital notch is more prominent, and the ulnar bulge is larger. The forelimb is approximately twice as long as it is wide. In cartilage staining, the radius and ulna are visible, and carpals are faintly visible. Length: 15–18 mm.

Stage 50 (age: 7–12 dph): (Fig. 1K–N) forelimb: under the binocular microscope, digits III and IV are visible as nodules inside the ulnar bulge. The forelimb is slightly bent at the

Fig. 1. Axolotl limb stages 44–52. **A:** A left lateral view drawing of a stage 45 larva. **B:** Freshly killed larval stages 44 and 45, lateral view of the left forelimbs (fl; enclosed in the red boxes). **C,E,G,I,K,O,S:** Photographs of freshly killed stages 46, 47, 48, 49, 50, 51, and 52 left forelimbs, respectively. (For contrast, limbs are supported by a piece of black plastic.) **N,R,V:** Photographs of freshly killed stages 50, 51, and 52 hindlimbs (hl), respectively. **D,F,H,J,L,P,T:** Victoria Blue (v.b.) -stained forelimbs at stages 46, 47, 48, 49, 50, 51, and 52, respectively. **M,Q,U:** Alizarin Red (a.r.) -stained forelimbs at stages 50, 51, and 52, respectively. Scale bars = 0.5 mm in A, 0.05 mm in B–V where shown.

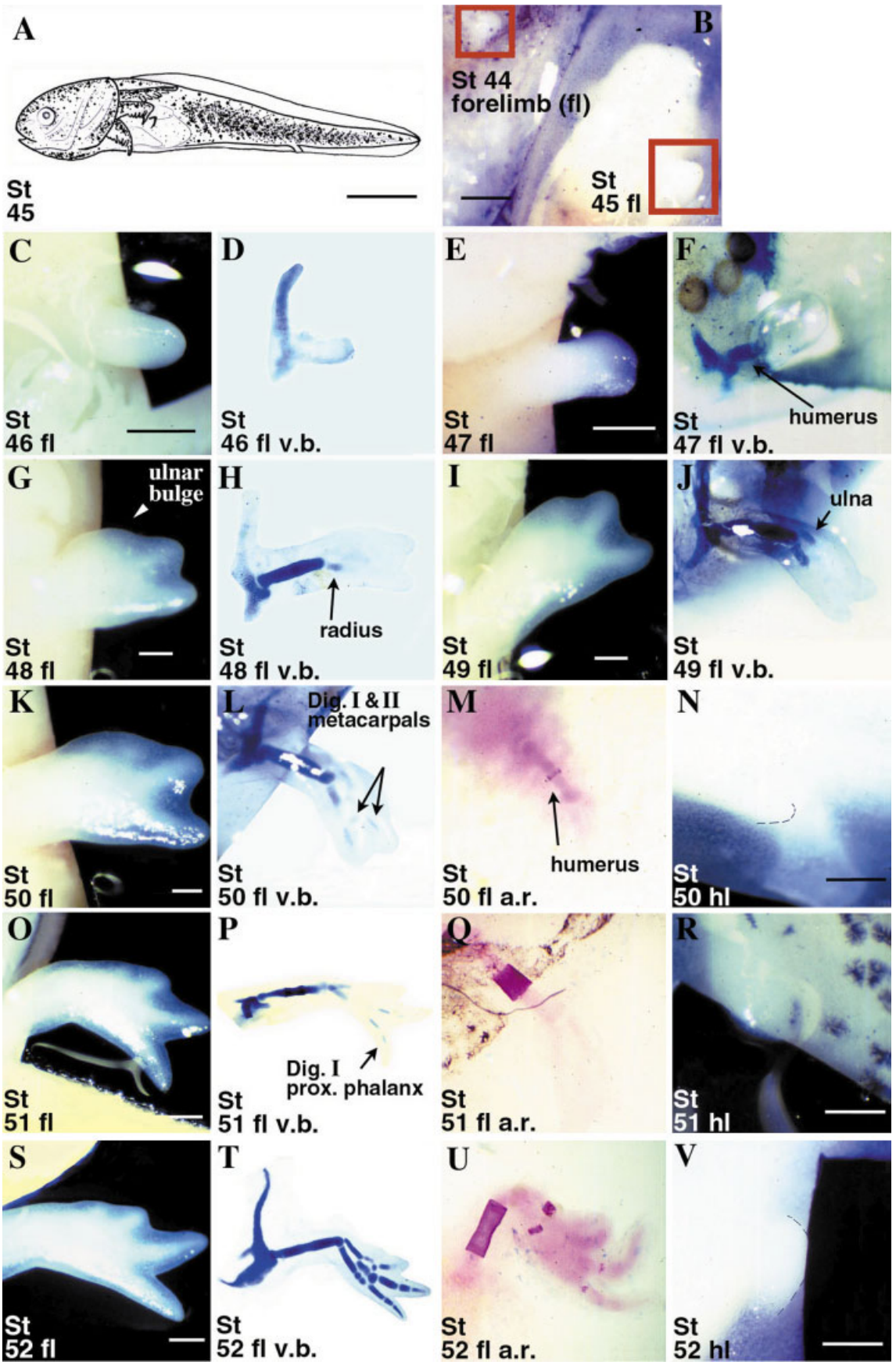

Fig. 1.

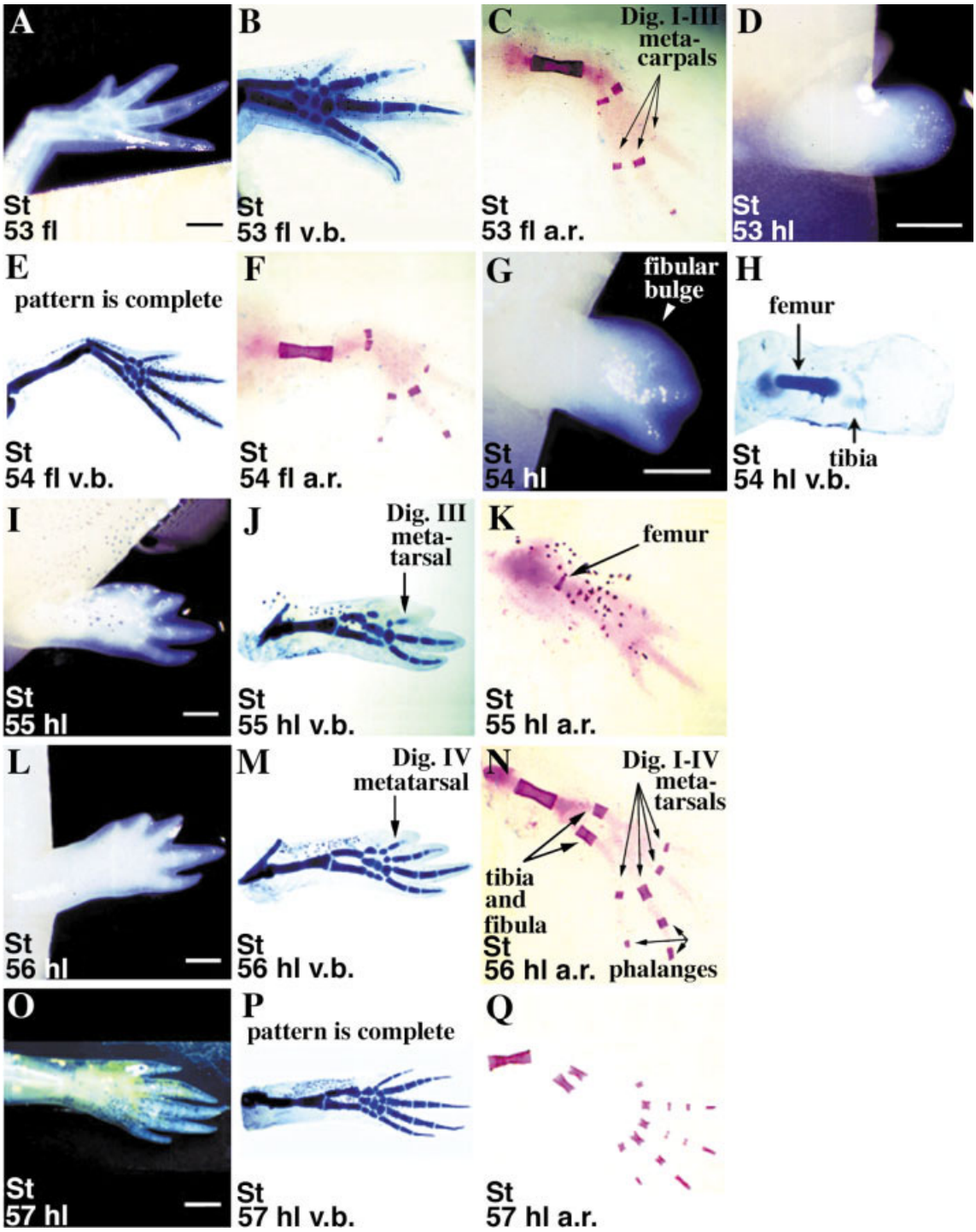

Fig. 2. Axolotl limb stages 53–57. **A:** Photograph of a freshly killed stage 53 forelimb (fl). **B,E:** Victoria Blue (v.b.) -stained forelimbs at stages 53 and 54, respectively. **C,F:** Alizarin Red (a.r.) stained forelimbs at stages 53 and 54, respectively. **D,G,I,L,O:** Photographs of freshly killed left hindlimbs at stages 53, 54, 55, 56, and 57, respectively. **H,J,M,P:** Victoria Blue stained hindlimbs at stages 54, 55, 56, and 57, respectively. **K,N,Q:** Alizarin Red stained hindlimbs at stages 55, 56, and 57, respectively. Scale bars = 0.05 mm in A–Q where shown.

elbow. Digit I and II metacarpals are faintly visible in cartilage staining. The first indication of bone staining is present in the diaphysis of the humerus. Hindlimb: Under the microscope, the hindlimb is barely visible as a small round opaque area underneath the skin, anterodorsal to the anal canal. The hindlimb does not yet protrude from the body wall. Length: 16–18 mm.

Stage 51 (age: 17–19 dph): (Fig.

1O–R) forelimb: the larva is able to move the shoulder joint. The interdigital notch between digits I and I is deep, and there may be a small indentation between digits II and III. In cartilage staining, digits I and II are visible as metacarpals. Digits I and II may possess faintly stained proximal phalanges. In bone staining, the diaphysis of the humerus is strongly stained. Hindlimb: the hindlimb protrudes slightly from the body wall. Length: 19–21 mm.

Stage 52 (age: 18–21 dph): (Fig. 1S–V) forelimb: the interdigital notch between digits II and III is a right angle or slightly acute. A small indentation may be present between digits III and IV. In cartilage staining, digits I and II are complete (both possess two phalanges), and digit III metacarpal is usually visible. In bone staining, the diaphyses of the radius and ulna are visible, and the metacarpals of digits I and II are faintly stained. Hindlimb: the tip is rounded, but the limb is still wider than it is long. Length: 21–23 mm.

Stage 53 (age: 20–25 dph): (Fig. 2A–D) forelimb: digit III is now half as long as digit II. The interdigital notch between digit III and IV is a right angle or slightly acute. In cartilage staining, digit III is complete, with three phalanges. Digit IV possesses the metacarpal and the first phalanx but may not yet have formed the second phalanx in some individuals. In other individuals, the forelimb pattern (referring to the number of phalanges on each digit, listed in anterior-to-posterior sequence) is complete (2:2:3:2). Bone staining is present in metacarpals I and II and is faintly present in metacarpal III. Hindlimb: the hindlimb is two to three times longer than it is wide. Its tip is flattened, and it is beginning to undergo the torsion described previously for the forelimb (see the description for stage 47). Length: 25–28 mm.

Stage 54 (age: for this stage and the rest of the stages, the larvae are 25 days posthatching or older; the stage is determined primarily by the morphology of the hindlimb): (Fig. 2E–H) forelimb: the forelimb is complete in all individuals. Bone staining is present in the phalanges of digits I and II. Hindlimb: digits I and II are visible as metatarsal rays, with a small interdigital notch. There is a small posterior (fibular) bulge in the distal portion. The first evidence of cartilage staining is present: the femur stains strongly, and the tibia may stain faintly. In contrast to the forelimb, the pelvic structures do not appear in cartilage staining until after the femur is present. Length: 25–30 mm.

Stage 55: (Fig. 2I–K) forelimb: bone staining includes all four metacarpals and most of the phalanges (not shown). Hindlimb: Digit II is distinctly longer than digit I, and their interdigital notch is deep. The posterior digits are visible in the fibular bulge under the microscope as rays or nodules. A small notch is present between digits II and III; its shape may be a right angle or slightly acute. Cartilage staining shows a small pelvic bone, the femur, the tibia and fibula, metatarsals, and phalanges for digits I and II, and sometimes, faintly, digit III's metatarsal. The first evidence of bone staining is present in the diaphysis of the femur. Length: 25–35 mm.

Stage 56: (Fig. 2L–N) hindlimb: all posterior digits are visible under the microscope as metatarsal rays. The interdigital notch between digits II and III is deep. There is a small notch present between digits III and IV; its shape may be a right angle or slightly acute. In cartilage staining, digits III and IV metatarsals are present, although digit IV may not yet possess visible phalanges. Bone staining includes the femur, tibia, fibula, metatarsals for digits I–III (and sometimes IV) and a few phalanges on digits I and II. Length: 27–40 mm.

Stage 57: (Fig. 2O–Q) hindlimb: the hindlimb is complete (pattern: 2:2:3:3:2 or 2:2:3:4:2). Bone staining includes all five metatarsals and most of the phalanges. Length: 40–50 mm.

EXPERIMENTAL PROCEDURES

Axolotl Larvae Visualization

Axolotl larvae were obtained from the Indiana University Axolotl Colony in Bloomington, Indiana. They were maintained in accordance with the colony's husbandry procedures and fed with brine shrimp. Freshly killed (0.4% MS222) larvae were photographed with a Nikon Optiphot camera mounted on a binocular microscope. A strip of black plastic was inserted between the nonstained limbs and the bodies of the larvae to ensure that the outline of the limbs could easily be seen. Stained limbs were placed on microscopes slides under coverslips before photography. Image processing was performed with Adobe Photoshop software. A pen and ink drawing of the lateral view of a stage 45 axolotl larva was prepared by H.L.D.N.

Cartilage and Bone Staining

Cartilage staining was based on Bryant and Iten (1974). Formaldehyde-fixed limbs were dehydrated to 70% ethanol with 1% hydrochloric acid (acid alcohol) then stained for 2 hr with 1% Victoria Blue B in acid alcohol. Destaining was performed for 1 hr each in 70%, 95%, and 100% ethanol. Soft tissues were cleared with methyl salicylate, which was also the storage solution.

Bone staining was based on Kimmel and Trammell (1981). Freshly killed larval limbs were soaked in 0.05% Alizarin Red in 2% potassium hydroxide for 2 hr, hardened and cleared in 50% glycerol for 2 hr, and stored in 100% glycerol.

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