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Jaw muscle development as evidence for embryonic repatterning in direct-developing frogs

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SUMMARY

The Puerto Rican direct-developing frog *Eleutherodactylus coqui* (Leptodactylidae) displays a novel mode of jaw muscle development for anuran amphibians. Unlike metamorphosing species, several larval-specific features never form in *E. coqui*; embryonic muscle primordia initially assume an abbreviated, mid-metamorphic configuration that is soon remodelled to form the adult morphology before hatching. Also lacking are both the distinct population of larval myofibres and the conspicuous, larval-to-adult myofibre turnover that are characteristic of muscle development in metamorphosing species. These modifications are part of a comprehensive alteration in embryonic cranial patterning that has accompanied life history evolution in this highly speciose lineage. Embryonic 'repatterning' in *Eleutherodactylus* may reflect underlying developmental mechanisms that mediate the integrated evolution of complex structures. Such mechanisms may also facilitate, in organisms with a primitively complex life cycle, the evolutionary dissociation of embryonic, larval, and adult features.

1. INTRODUCTION

Direct development is a novel reproductive mode in Recent amphibians. It is characterized by evolutionary loss of the free-living, aquatic larval stage typical of metamorphosing species; embryogenesis culminates in the birth of a fully formed, juvenile frog, salamander, or caecilian. Direct development has evolved independently in each of the three living orders and is characteristic of many hundreds of extant species (Wake 1989). Indeed, it is the predominant reproductive mode in some large clades, e.g. plethodontid salamanders (Wake & Hanken 1996), and probably evolved at least ten times in anurans alone (Duellman & Trueb 1986). Direct development can have important ecological and evolutionary consequences. These range from loss of the need for aquatic breeding sites (McDiarmid 1978), to release from larval constraints on adult morphology associated with the ancestral, metamorphic ontogeny (Alberch 1987, 1989; Wake & Larson 1987; Wake & Roth 1989). Yet, consequences of the evolution of direct development for the developmental biology of individual taxa are poorly known (Elinson 1990; Elinson *et al.* 1990; Wake & Marks 1993). This is especially true for those features whose

modification may affect the evolutionary success and diversification of particular lineages (Alberch 1987, 1989).

We examined embryonic development of the jaw musculature in the Puerto Rican frog *Eleutherodactylus coqui* (Leptodactylidae; figure 1) to assess the consequences of direct development for these and other cranial tissues. We focused on the muscles responsible for mouth opening and related movements in frogs. There are five such muscles in the larvae of most families, including the Leptodactylidae (Starrett 1973; figure 2*a,b*). All five muscles are derived from the second embryonic (hyoid) arch and are innervated by the seventh cranial (facial) nerve (Edgeworth 1935). At metamorphosis these larval muscles coalesce to form the depressor mandibulae, the single, complex jaw-opening muscle found in all adult frogs (De Jongh 1968). This reorganization entails shifts in muscle origin to as many as three sites within the head and insertion to the posterior tip of the mandible. Accompanying these changes in muscle number, size, orientation and attachment is myofibre turnover: larval myofibres degenerate and are replaced by adult myofibres recruited from satellite cells within the larval muscles (De Jongh 1968). These conspicuous, qualitative changes in muscle morphology, which accompany metamorphosis in species displaying the

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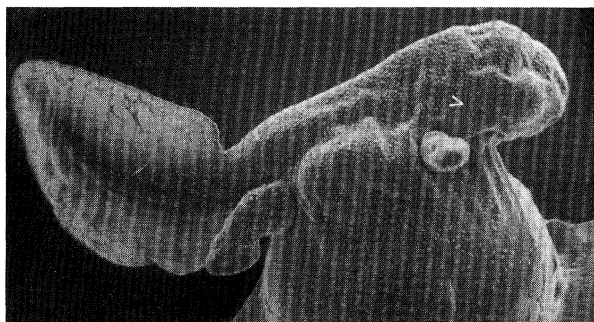


Figure 1. Scanning electron micrograph of an embryo of *Eleutherodactylus coqui*, Townsend & Stewart (T-S) stage 7. Several unusual features of the embryos of direct-developing frogs are visible in this lateral view. These include precocious development of both fore- and hindlimbs, and the enlarged tail. (Limbs do not form until well after hatching in metamorphosing frogs.) The embryo sits atop an enormous yolk mass. The white arrowhead points to the location of the jaw muscles depicted in figures 2–4. The total length of the embryo is 7–8 mm.

ancestral ontogeny, provide an excellent opportunity to assess the fates of larval versus adult features during the evolution of direct development.

2. MATERIALS AND METHODS

Embryonic and early post-hatching specimens of direct-developing *E. coqui* were obtained from spontaneous matings among wild-caught adults maintained as a breeding colony at the University of Colorado. Comparable data regarding the presumed ancestral, metamorphic ontogeny were derived for the metamorphosing anuran *Leptodactylus albilabris* (Leptodactylidae). This species has a morphologically generalized tadpole, and is among the most appropriate outgroups for evaluating derived features associated with direct development in *Eleutherodactylus* and other closely related genera (Heyer 1975; Lynch 1971; Wassersug & Heyer 1988). Larval specimens of *L. albilabris* were collected from temporary breeding sites and preserved in the field. Both species were collected in the vicinity of El Verde Field Station, Luquillo Experimental Forest, Puerto Rico. Specimens of *E. coqui* were staged according to the table of Townsend & Stewart (1985), which defines 15 embryonic stages for this species between fertilization (0) and hatching (15). Larval *Leptodactylus* were staged according to the table of Gosner (1960), which defines 46 stages between fertilization (0) and the completion of metamorphosis (46); hatching typically occurs during stages 22–23. Animal collection, care, and maintenance followed standard procedures (Elinson *et al.* 1990; Hanken *et al.* 1992; Moury & Hanken 1995) in accordance with the regulations of the Puerto Rican Department of Natural Resources and CU Boulder.

Immunostained whole mounts (Dent *et al.* 1989; Klymkowsky & Hanken 1992) were prepared by using monoclonal antibodies for chicken fast-myosin heavy chain (F59; Miller *et al.* 1985) or newt skeletal muscle (12/101; Kintner & Brockes 1985) as the primary antibody, supplemented with 5% dimethyl sulphoxide. Serial plastic sections (5 µm) were stained with Gill haematoxylin and eosin (Alley 1989; Humason 1979). Paraplast sections (8–10 µm) were stained for connective tissues (Hall 1985; Humason 1979). Scanning electron microscopy followed the procedures of Moury & Hanken (1995).

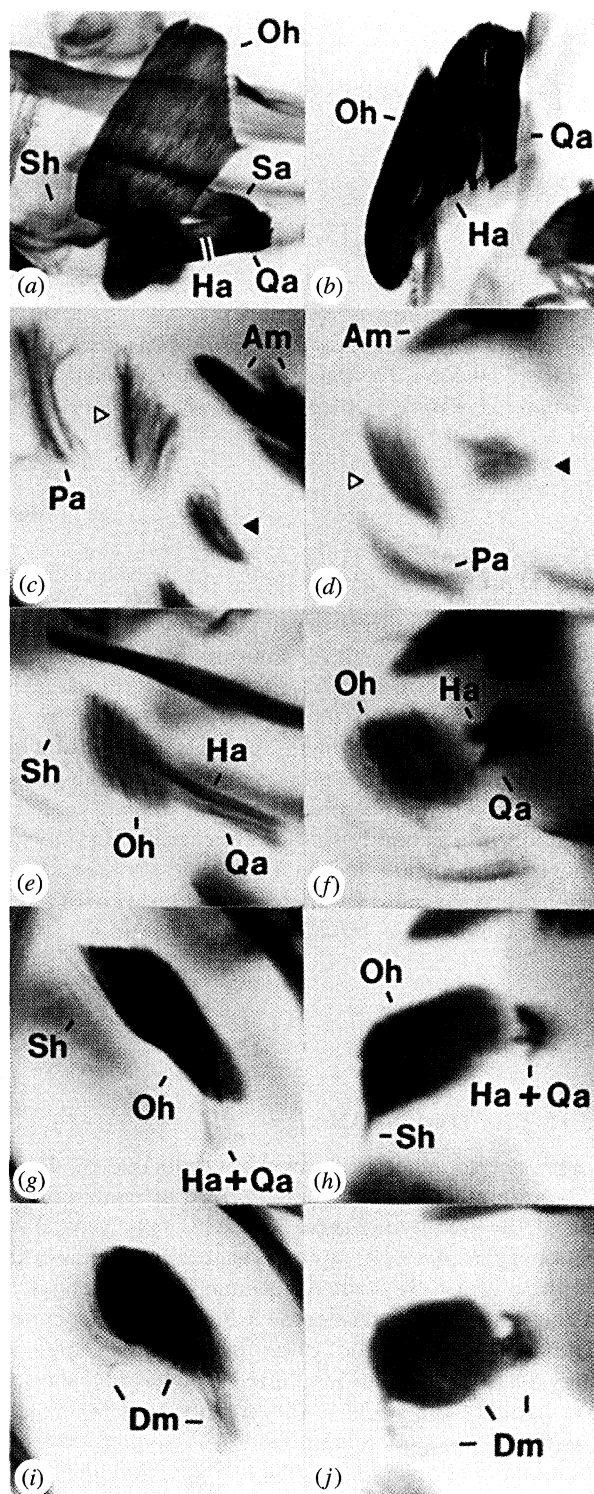


Figure 2. Jaw-opening and related musculature in immunostained whole mounts. Left-hand panel: lateral views, anterior is to the right; right-hand panel: ventral views, anterior is to the top. (a, b) Tadpole of the metamorphosing frog *Leptodactylus albilabris* (Leptodactylidae), Gosner stage 28, showing the five larval muscles characteristic of most families of living anurans: Oh, orbithyoideus; Sh, suspensoriohyoideus; Sa, suspensorioangularis; Ha, hyoangularis; Qa, quadratoangularis. The suspensoriohyoideus is very thin and comprises a small number of myofibres. (c, d) Embryos of direct-developing *E. coqui*, T-S stages 7 and 9, respectively. Superficial (open arrows) and deep (closed arrows) myogenic condensations are well-separated. (e, f) *E. coqui*, stage 11. Embryonic homologues of four larval muscles can be distinguished by relative position, origin,

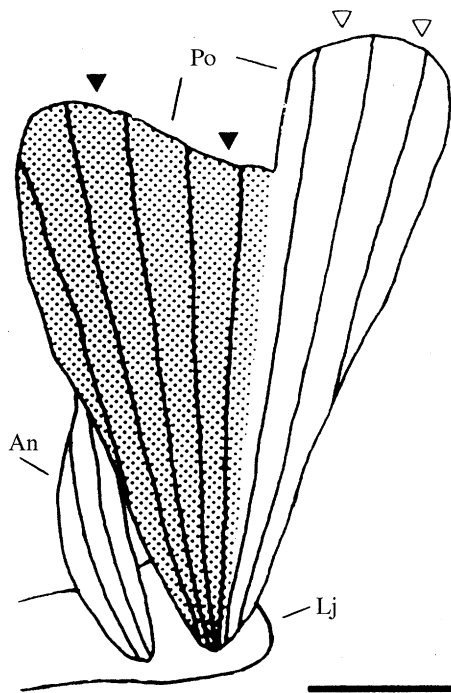


Figure 3. Derivation of the depressor mandibulae muscle in *E. coqui* at hatching (T-S stage 15); lateral view, anterior is to the left. The shaded portion of the large, posterior slip (Po) is derived from the embryonic orbitohyoideus and originates from the squamosal bone (closed arrows); the unshaded portion is derived from the embryonic suspensoriohyoideus and originates from dorsal fascia (open arrows). The small, anterior slip (An) is derived from the fused embryonic hyoangularis and quadratoangularis. At hatching, it originates from the anterior edge of the posterior slip, but it will eventually originate from the margin of the eardrum, which is not yet formed. Both slips insert on the lower jaw (Lj). Scale bar, 0.5 mm.

3. RESULTS

The primitive pattern of jaw muscle ontogeny that is characteristic of metamorphosing frogs is not seen in *E. coqui*, which instead conspicuously lacks many larval features (figure 2*c–j*). One of the five larval-specific muscles (suspensorioangularis) is completely absent. There are counterparts of the four other muscles, but these display a transient existence that spans at most six of the 15 embryonic stages. Moreover, all four muscles initially assume an abbreviated, mid-metamorphic morphology, and are subsequently remodelled to form the adult depressor mandibulae before hatching. The orbitohyoideus and suspensoriohyoideus form from a superficial myogenic condensation that develops ventral to the otic vesicle (figure 2*c–f*). Initially (stages 7–9), the origin of each muscle lies within mesenchyme; neither forms an attachment with the palatoquadrate cartilage, as seen in larval anurans (Starrett 1973). Both insert on the ceratohyal cartilage. The hyoangularis

and insertion. (*g, h*) *E. coqui*, stage 12. The four muscle rudiments are beginning to coalesce. (*i, j*) *E. coqui*, stage 13. The four muscle rudiments have fused to form the adult depressor mandibulae (Dm) present at hatching (figure 3). Additional abbreviations: Am, adductor (jaw-closing) musculature; Pa, posterior visceral arch musculature.

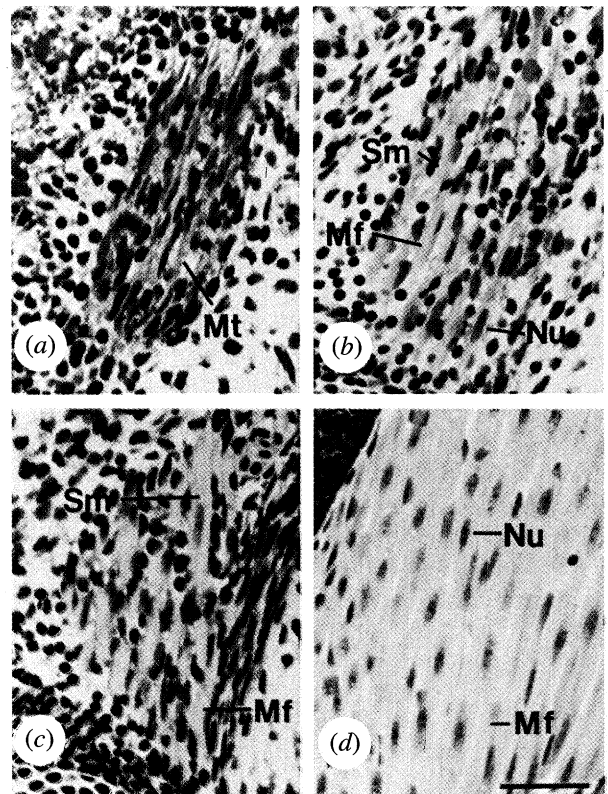


Figure 4. Jaw myofibre development in *E. coqui*. Each image represents a histological (plastic) section through the jaw closing musculature at one of four different embryonic stages. Together they depict the smooth progression through the processes of cellular differentiation, maturation, and growth that is typical of muscle development in this species. All muscles are oriented lengthwise, with the origin at the top-right and the insertion bottom-left. (*a*) Elongate myotubes, which stain a faint pink, with multiple dark nuclei, appear initially at T-S stage 9. This is preceded by muscle cell condensations, which are first visible at stage 7 (figure 2*c*). Muscle cells are spindle-shaped by stage 8 (not depicted). (*b*) Myotubes are maturing into myofibres, which form sarcomeres (muscle striations) at stage 11. (*c*) Myofibre diameter is gradually increasing at stage 12. (*d*) At hatching (stage 15), all myofibres are mature, with laterally placed nuclei. At no stage is there any indication of either a distinct population of larval myofibres or larval-to-adult myofibre turnover. Both features are characteristic of muscle development in metamorphosing anurans (see text). Abbreviations: Mf, myofibre; Mt, myotube; Nu, nucleus; Sm, sarcomeres. Scale bar, 50 μ m.

and quadratoangularis form from a second, deeper myogenic condensation (figure 2*c–f*). They initially (stages 8–9) originate from the ceratohyal and palatoquadrate cartilages, respectively; both insert on Meckel's cartilage.

Events corresponding to later stages of the ancestral (post-hatching) metamorphosis begin at stage 12 (figure 2*g–j*). The orbitohyoideus and suspensoriohyoideus merge and establish an origin from the squamosal bone and cartilaginous otic capsule (anteriorly) and dorsal fascia (posteriorly); their insertion shifts to Meckel's cartilage. The two deep muscles (the hyoangularis and the quadratoangularis) also coalesce.

Their origin shifts from the ceratohyal and palatoquadrate cartilages to fascia along the anterior edge of the fused superficial muscles; they retain their insertion on Meckel's cartilage. At hatching (stage 15), the depressor mandibulae has largely assumed its adult form and patterning (Lynch 1993; figure 3).

Absence of larval-type muscle morphology is associated with the lack of both larval myofibres and myofibre turnover. Jaw muscles exhibit a smooth continuum in their processes of cellular differentiation, maturation, and growth from the onset of myogenesis (figure 4). There is no indication of the massive destruction of early myofibres and subsequent repopulation of existing muscle beds by new myoblasts, which define jaw muscle development in metamorphosing anurans (De Jongh 1968). For example, diagnostic features of myofibre turnover include an obvious contrast between the large, degenerating larval myofibres and the small, newly formed replacement myotubes that develop into adult myofibres (Takisawa & Sunaga 1951; Takisawa *et al.* 1976). Typically, the larval myofibres fragment into small, globular units that are easily distinguished from the elongate cellular profiles of myogenesis (Alley 1989). None of these features is observed during jaw-muscle development in *E. coqui*.

4. DISCUSSION

Jaw muscle development in direct-developing *Eleutherodactylus coqui* differs considerably from that seen in metamorphosing anurans. Many larval-specific features are absent, and embryonic muscle primordia initially assume an abbreviated, mid-metamorphic configuration that is soon remodelled to form the adult morphology before hatching. Also lacking are larval myofibres and myofibre turnover, two conspicuous and characteristic features of muscle development in metamorphosing species. Comparable studies of many additional, phylogenetically diverse, direct-developing anurans are needed to establish whether such changes in jaw development are a frequent or even an inevitable consequence of the evolution of this derived life history, or if instead they are unique to this lineage.

The unusual mode of jaw muscle development in *E. coqui* is significant in several respects. First, it is part of a comprehensive evolutionary modification of embryonic cranial patterning in the genus *Eleutherodactylus*, which comprises more than 500 species of direct-developing frogs (Duellman 1993). Two prominent features—absence of larval characters and precocious (embryonic) formation of adult structures—are characteristic of the development of many other cranial components, including the skull, brain, cranial nerves, external gills, and oral integument (Elinson 1990; Elinson *et al.* 1990; Fang & Elinson 1996; Hanken *et al.* 1992; Hughes 1959; Lynn 1942; Schlosser & Roth 1997). The developmental basis for this coevolution of cranial patterning is unknown, but it probably involves the embryonic neural crest (Hanken & Thorogood 1993). Recent studies of amniotes have documented a prominent role for the neural crest in cranial development, including pattern formation (Couly *et al.* 1992; Krumlauf 1993; Noden 1991). Results of earlier and contemporary studies of

both metamorphosing (Hall 1950; Hörstadius 1950; Olsson & Hanken 1996; Wagner 1949) and direct-developing (Fang & Elinson 1996; Hanken *et al.* 1997; Moury & Hanken 1995) amphibians are consistent with a similar role in these vertebrates.

Comparable, pervasive changes to many features of embryonic development also have accompanied the independent evolution of direct development in lungless salamanders (Plethodontidae). Termed 'ontogenetic repatterning' (Roth & Wake 1985; Wake & Roth 1989), these modifications in morphogenesis and developmental timing are correlated—as in *E. coqui*—with the evolutionary loss of larval features and precocious (embryonic) formation of adult components. Unlike *Eleutherodactylus*, however (see below), ontogenetic repatterning in lungless salamanders underlies the appearance of many novel systems of morphological and functional integration (e.g. Roth & Schmidt 1993; Wake 1982). These novel features have contributed importantly to the extraordinary evolutionary success of this highly speciose, geographically widespread, and morphologically and ecologically diverse taxon (Wake & Hanken 1996).

Second, muscle development in *E. coqui* includes several instances of the retention of an ancestral feature following evolutionary loss of function. For example, insertion of the orbitohyoideus muscle on the ceratohyal cartilage is a unique adaptation in larval anurans, associated with their specialized mode of aquatic feeding and respiration (Wassersug & Hoff 1979). It represents a primitive trait for living frogs and is present in the larvae of all species that display the ancestral, metamorphic ontogeny. At metamorphosis, coincident with the shift to terrestrial feeding and respiration, this insertion is lost and a new one is established on the lower jaw, as occurs in all other vertebrates. In *E. coqui*, insertion of the orbitohyoideus on the ceratohyal is retained as a transient embryonic feature, even though the primitive mode of aquatic feeding and respiration has been lost in this exclusively terrestrial species. Instead, the developing embryo is sustained on substantial yolk reserves provisioned in the large egg (Elinson 1987) and gas exchange is probably mediated by the enlarged and highly vascularized tail, which is resorbed at or soon after hatching (Lynn 1942; Townsend & Stewart 1985; figure 1). Retention of the initial insertion of the orbitohyoideus on the ceratohyal, as well as other primitive anuran features, contrasts with many other aspects of musculoskeletal development in *E. coqui*. The cranial ossification sequence, for example, differs substantially from that characteristic of metamorphosing anurans (Hanken *et al.* 1992). Reasons for the evolutionary conservation of these ancestral features following loss of primary function—be they additional, secondary functions that are still retained, or genetic and developmental constraints—are unknown.

Finally, extensive modifications to the primitive, biphasic pattern of cranial ontogeny have had no obvious consequences for adult morphology. Cranial morphology in *Eleutherodactylus* generally is typical of leptodactylid frogs (Lynch 1971, 1993). The genus especially lacks the conspicuous morphological novelty whose appearance is correlated with the evolution of

direct development in many urodeles (Wake & Larson 1987; Wake & Marks 1993; Wake & Roth 1989; see above). Large-scale changes to early development with little or no effect on adult morphology also is characteristic of the evolution of direct development in many invertebrates (Raff 1996; Wray & Raff 1991). It may represent a common evolutionary phenomenon in taxa with a primitively complex life history. This, in turn, may reflect the existence of similar developmental mechanisms that underlie metamorphosis in phylogenetically distant lineages, and which facilitate the evolutionary dissociation of embryonic, larval, and adult features (Alberch 1987).

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