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DO LARVAL TRAITS RE-EVOLVE? EVIDENCE FROM THE EMBRYOGENESIS OF A DIRECT-DEVELOPING SALAMANDER, *PLETHODON CINEREUS*

Ryan R. Kerney,[1,2,3] David C. Blackburn,[3,4] Hendrik Müller,[5,6] and James Hanken[6]

[1]*Biology Department, Dalhousie University, 1355 Oxford St., Halifax, Nova Scotia B3H 4J1, Canada*

[2]*E-mail: ryankerney@gmail.com*

[4]*Natural History Museum and Biodiversity Institute, University of Kansas, Lawrence, Kansas 66045*

[5]*Institut für Spezielle Zoologie und Evolutionsbiologie mit Phyletischem Museum, Friedrich-Schiller-Universität Jena, Erbertstr. 1, 07743 Jena, Germany*

[6]*Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138*

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Recent molecular phylogenies suggest the surprising reacquisition of posthatching metamorphosis within an otherwise direct-developing clade of lungless salamanders (family Plethodontidae). Metamorphosis was long regarded as plesiomorphic for plethodontids, yet the genus *Desmognathus*, which primarily includes metamorphosing species, is now nested within a much larger clade of direct-developing species. The extent to which the putative reacquisition of metamorphosis in *Desmognathus* represents a true evolutionary reversal is contingent upon the extent to which both larva-specific features and metamorphosis were actually lost during the evolution of direct development. In this study we analyze development of the hyobranchial skeleton, which is dramatically remodeled during salamander metamorphosis, in the direct-developing red-backed salamander, *Plethodon cinereus*. We find dramatic remodeling of the hyobranchial skeleton during embryogenesis in *P. cinereus* and the transient appearance of larva-specific cartilages. Hyobranchial development in this direct-developing plethodontid is highly similar to that in metamorphosing plethodontids (e.g., *Desmognathus*). The proposed reacquisition of hyobranchial metamorphosis within *Desmognathus* does not represent the "re-evolution" of a lost phenotype, but instead the elaboration of an existing developmental sequence.

KEY WORDS: **Development, direct development, Dollo's Law, evolutionary developmental biology, life history evolution, Plethodontidae.**

"Dollo's Law" posits that complex phenotypes are unlikely to be regained once they are lost (Dollo 1893; Gould 1970). Recent studies of trait evolution in a phylogenetic context challenge this claim by providing examples of complex phenotypes, including entire life-history stages, that are allegedly lost and then regained in particular lineages (e.g., Whiting et al. 2003; Collin 2004, but see Collin and Miglietta 2008; Goldberg and Igić 2008). One particularly striking case is the proposed reacquisition of an aquatic larval stage in plethodontid salamanders of the genus *Desmognathus*, which is nested within a larger clade of direct-developing species (Chippindale et al. 2004; Mueller et al. 2004; Macey 2005; Kozak et al. 2005, 2009; Vieites et al. 2007,

[3]These authors made equal contributions to this work

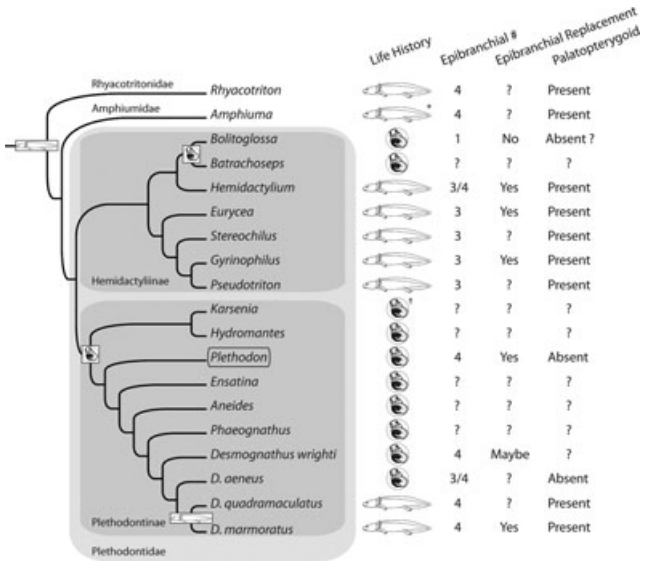

Figure 1. Distribution of larval skeletal characters in the family Plethodontidae. Recent phylogenetic studies posit the reacquisition of free-living larvae within *Desmognathus*. Our study reports larval hyobranchial features (four epibranchials and the developmental replacement of the first epibranchial) in embryos of direct-developing *Plethodon cinereus*, but there remains no evidence for a larval palatopterygoid cranial bone in this species (Dent, 1942; R. Kerney, unpubl. data). Distributions of larval skeletal structures in other plethodontids are based on the following: number of epibranchials—Alberch (1987), Dent (1942), Hilton (1947), Houghton (1903), Marks (2000), Rose (1995a, 2003), Smith (1920), Wake and Hanken (1996), Worthington and Wake (1971); epibranchial replacement—Alberch (1987, 1989), Rose (2009), Smith (1920), Wake and Hanken (1996); palatopterygoid bone—Dent (1942), Ehmcke and Cleven (2000), Marks (2000), Kinglsey (1892), Larsen (1963), Wake (1966), Worthington and Wake (1971). Phylogenetic relationships and higher level taxonomy based on Vieites et al. (2011); details on relationships within *Desmognathus* based on Kozak et al. (2005); and mapping of life-history evolution based on Chippindale et al. (2004). **Amphiuma* occasionally hatch after gills are resorbed (Gunzburger 2003); †*Karsenia* is presumed to exhibit direct development, although no clutches have been formally described.

2011; Fig. 1). The free-living larvae of metamorphosing plethodontid salamanders have several unique morphological features that are lost or modified during metamorphosis (Wilder 1925). In contrast, direct-developing amphibians lack a free-living aquatic larval stage; hatched young are morphologically similar to adults, albeit miniature (Hanken 1999). Historically, researchers have regarded amphibian larvae as sufficiently complex that they cannot re-evolve in a lineage after their evolutionary loss through direct development (Duellman and Hillis 1987; Bruce 2005; Wiens et al. 2007). This belief hinges, in part, on the assumption that larva-specific structures are not retained in the ontogeny of direct-developing species. There are, however, few detailed studies of the embryogenesis of direct-developing amphibians to justify this assumption.

Early assessments of phylogenetic relationships, based on morphological and developmental characters, consistently found the previously recognized subfamily Desmognathinae to be a sister clade to all other lungless salamanders (family Plethodontidae; reviewed in Lombard and Wake 1986). Subsequent analyses, based on extensive taxonomic sampling and numerous genetic loci, revealed that the two direct-developing species of *Desmognathus* (*D. wrighti* and *D. aeneus*) are the earliest branching lineages of this genus, whereas the direct-developing *Phaeognathus hubrichti* is the immediate outgroup (Titus and Larson 1996). This result, combined with recent findings that the genus *Desmognathus* is deeply nested within a larger direct-developing plethodontid clade, suggests that direct development is the ancestral life history of this clade and that a biphasic life history was reacquired within *Desmognathus* (Chippindale et al. 2004).

This proposed evolutionary reversal of life history within plethodontid salamanders is controversial among amphibian biologists (Bonnet et al. 2005; Bruce 2005; Chippindale and Wiens 2005). Whereas the corresponding phylogenetic analyses are compelling, there is little understanding of the developmental changes that would be required to regain an aquatic larva. Hence, much of the controversy surrounding the re-evolution of larvae remains difficult to evaluate and the associated disagreements are yet to be resolved. If, for example, direct development is ancestral for *Desmognathus*, then several larval plethodontid characters presumed absent from direct-developing species have reappeared in the ontogeny of metamorphosing *Desmognathus* (Mueller et al. 2004). These presumed absences, however, have not been investigated in detail. The likelihood that larval characters have been regained is in part contingent on whether these traits are absent in the ontogeny of direct-developing plethodontids.

Dent (1942) provides a partial embryonic staging table of the red-backed salamander, *Plethodon cinereus*, and limited morphological descriptions. According to Dent, larval features that are absent in *P. cinereus* include lateral line neuromasts, Leydig cells, the palatopterygoid bone, and features of the hyobranchial skeleton (for distribution of larval skeletal features, see Fig. 1). In the context of Dent's morphological data on the hyobranchial skeleton, the proposed reacquisition of larvae in *Desmognathus* requires the reappearance of a fourth larval epibranchial cartilage (also see Hilton 1947; "epibranchial" termed "ceratobranchial" in Reilly and Lauder 1988; Trueb 1993; Rose 2003). Dent does not evaluate other metamorphic changes to the hyobranchial skeleton, such as replacement of the larval first epibranchial with a separate adult cartilage, separation of the first ceratobranchial and second basibranchial from the branchial plate, or formation of the radial cartilages and anterior blades of the ceratohyal (Smith 1920; Alberch et al. 1985; Alberch and Gale 1986). If embryonic development

of *P. cinereus* recapitulates these metamorphic changes, then reacquisition of a free-living larval stage in *Desmognathus* might simply be an elaboration of an existing developmental sequence.

In this study, we analyze embryonic development of the hyobranchial skeleton in the direct-developing salamander *P. cinereus* by using a combination of sectioned material and whole-mount clearing and staining. Our primary goal is to determine the extent to which ancestral larval traits are expressed during embryonic development. Based on the extent of metamorphic change observed in *Plethodon* embryos, we evaluate the proposed regain of a larval trait in metamorphosing *Desmognathus*. This analysis allows us to assess whether larval characters "re-evolved" in *Desmognathus* or if, instead, larval reacquisition represents an elaboration of developmental morphologies already expressed during the ontogeny of direct-developing plethodontid salamanders.

Table 1. Number of specimens examined for each developmental stage and type of preparation.

Stage	Serial sections	Cleared and stained whole mounts
15	5	0
16	2	0
17	1	0
18	1	0
19	3	1
20	1	4
21	1	2
22	2	3
23	1	2
24	2	1
Juvenile	2	1

Materials and Methods

SPECIMEN COLLECTION

Clutches of embryos were collected from Concord, Massachusetts, USA, and the Halifax Regional Municipality, Nova Scotia, Canada. Specimens were collected under permits from the Massachusetts Department of Fish and Game (to the Museum of Comparative Zoology, Harvard University) and the Nova Scotia Department of Natural Resources (to R. Kerney). Embryos were staged according to Dent (1942), who does not describe stage 15. We determined this stage to be intermediate between Dent stages 14 and 16, with a diagnostic feature of the tailbud covering the blastopore. This stage is similar to the "5.5 mm embryo" described by Piersol (1910). We sampled stages 15 through 24 (hatchling) along with three posthatching juveniles (snout–vent lengths 15, 17, and 17 mm; Table 1). All embryos were removed from the egg capsule with watchmaker forceps. Younger embryos were treated with 2.5% cysteine (pH 8.5) for 10 min and then rinsed in sterile phosphate-buffered saline (PBS, pH 7.6) prior to removal from their egg capsules. Embryos were fixed in either MEMFA (MOPS, EGTA, magnesium sulfate, formaldehyde) or 4% paraformaldehyde (both pH 7.6) for 1.5 h. Embryos were then rinsed in PBS and stored in 70% methanol at −20°C.

HISTOLOGY

Whole-mount clearing and staining followed Klymkowsky and Hanken (1991). Cleared-and-stained specimens were stored in a 1:1 ethanol:glycerol solution at room temperature. Hyobranchial skeletons were then dissected with fine scissors and watchmaker forceps, photographed using a digital camera mounted on a dissecting microscope, and illustrated.

Nineteen embryos and two juvenile salamanders were processed for histological sectioning (Table 1). Individual specimens were separated from their yolk and transferred through a Citrisolv series into Paraplast X-tra blocks. Blocks were sectioned at a thickness of 7 μm with a Leitz (1512) rotary microtome and stained with either Masson or Mallory triple (trichrome) connective tissue stain (Presnell and Schreibman 1997). The hyobranchial skeleton of a single stage-19 embryo was reconstructed in three dimensions (3D). Condensations of individual hyobranchial cartilages were identified through their distinct cellular morphology. These were outlined in serial images, aligned with AutoAligner (Bitplane Inc., Zurich), and reconstructed in 3D with Imaris software (Bitplane Inc.).

Results

EARLY HISTOGENESIS OF THE HYOBRANCHIAL SKELETON

The visceral arches are visible in histological sections by stage 15 (forelimb bud). The hyoid arch and three branchial arches occur posterior to the mandibular arch (Fig. 2A). By stage 17 (hind limb bud) external gills begin to branch off each branchial arch (Fig. 2B). These gills expand by stage 19, when the opercular fold overlies the hyoid arch. Condensations of hyobranchial cartilages also form during stage 19. Three epibranchial condensations are present, one at the base of each external gill (Fig. 2C).

Three-dimensional reconstruction of stage-19 hyobranchial condensations reveals that the cartilage anlagen initially are largely continuous with one another (Fig. 2D–F). Segmentation into distinct elements occurs during later stages. The most anterior portion of what becomes the first basibranchial projects slightly dorsally near its junction with the paired ceratohyals. Each second ceratobranchial condensation also projects dorsally, above the branchial plate (Smith 1920). The condensation that eventually segments to become the second basibranchial projects ventrally and posteriorly from this plate. The posterior end of

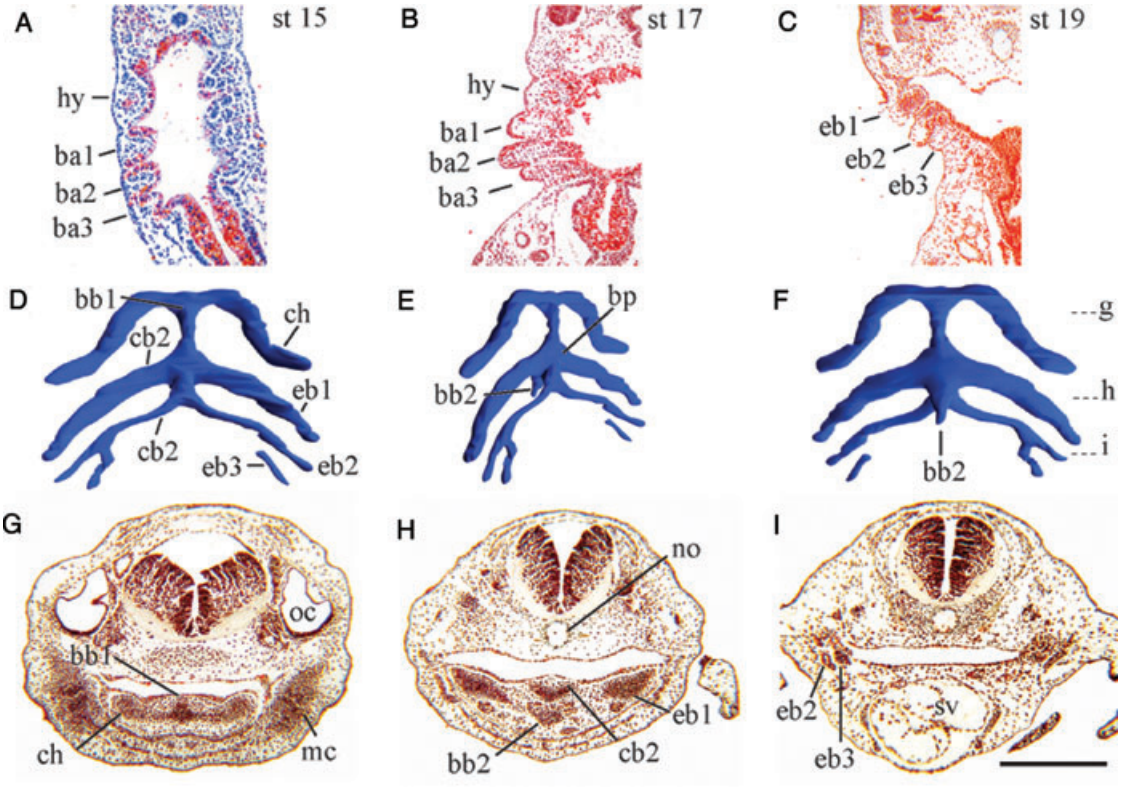

Figure 2. **Histological sections of visceral arches and hyobranchial condensations in *P. cinereus*. (A–C) Coronal sections of the pharyngeal region showing the visceral arches (A), their budding external gills (B), and the initiation of epibranchial condensation at the base of the gills (C) during stages 15, 17, and 19, respectively. Anterior at the top. (D–F) Histological reconstruction of condensations of an early stage-19 hyobranchial skeleton, depicted in dorsal, dorsolateral, and ventral views, respectively. (G–I) Selected transverse sections used to create the above reconstruction (levels indicated by dashed lines in F). Abbreviations: ba = branchial arches 1, 2, and 3; bb = basibranchial cartilages 1 and 2; bp = branchial plate; cb = ceratobranchial cartilages 1 and 2; ch = ceratohyal cartilage; eb = epibranchial cartilages 1, 2, and 3; hy = hyoid arch; mc = Meckel's cartilage; no = notochord; oc = otic capsule; sv = sinus venosus. (A) Mallory-stained, and (B, C, and G–I) Masson-stained sections. Scale bar, 0.5 mm.**

the second basibranchial condensation terminates ventral to other elements of the hyobranchial skeleton and anteroventral to the bulbus arteriosus of the heart.

Initial condensations of the first and second epibranchials are continuous with the condensations of the first and second ceratobranchials, respectively. The third epibranchial condensation is detached on one side but connected to the second epibranchial condensation on the other (Fig. 2D). Subsequent development of the hyobranchial skeleton is most readily described from cleared-and-stained whole mounts.

ANALYSES OF CARTILAGE DEVELOPMENT AND METAMORPHOSIS

The first indication of Alcian blue-positive cartilages in the embryonic hyobranchial skeleton occurs late in stage 19, after the formation of cellular condensations (Fig. 3). The hyobranchial skeleton is comprised initially of three paired, posterolaterally directed cartilaginous rods: the ceratohyal and two rods that later constitute the first and second ceratobranchials and epibranchials. As with the preceding cellular condensations, the early ceratobranchials are continuous with the median (unpaired) basibranchial elements. Alcian staining is lighter between the first ceratobranchial and the first epibranchial, which separate by stage 20. Despite the presence of a third epibranchial condensation by early stage 19 (Fig. 2C), the corresponding third epibranchial cartilage is not detectable with Alcian blue in whole mounts until stage 20 (Fig. 3).

During stage 20, the hyobranchial skeleton transforms from largely planar to concave, which causes it to narrow in ventral and dorsal views. Early in this stage, the first ceratobranchial and second basibranchial are not distinct from the branchial plate. The second ceratobranchial and the first epibranchial, however, have already segmented. The second and third epibranchials, which remain fused anteriorly at the beginning of stage 20, are completely separate in later stage-20 individuals. A small nodule of cartilage is present at the distal end of the third epibranchial during early stage 20. This nodule is the attachment site for fibers of the posterior-most levator arcuum muscle (Fig. 4B), which

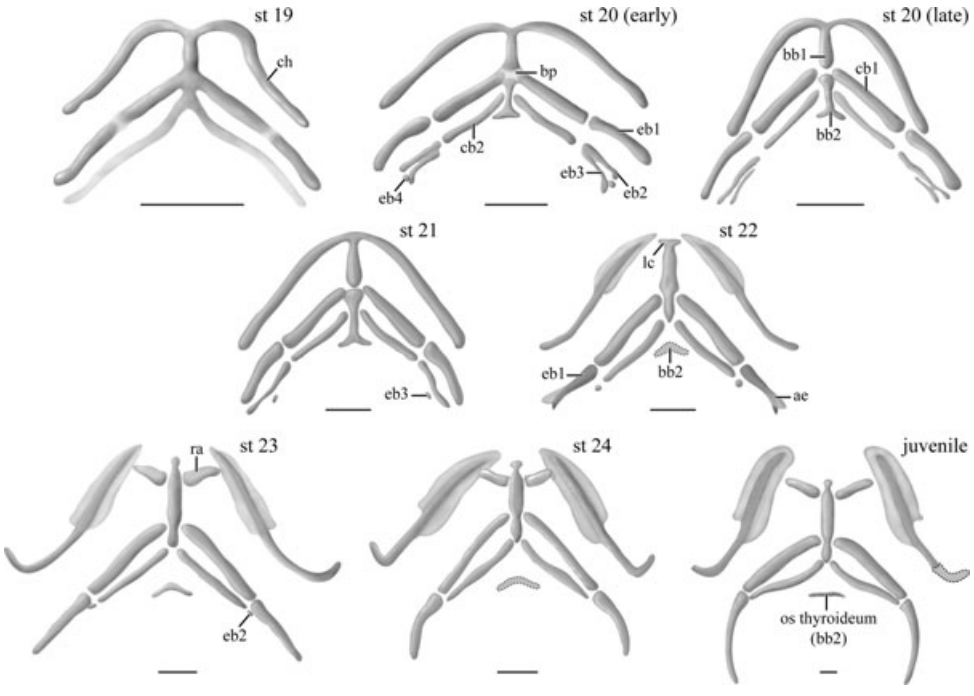

Figure 3. **Development of the cartilaginous hyobranchial skeleton over seven developmental stages (19–24 and juvenile; illustrations are drawn from cleared-and-stained specimens). Based on data from serial sections (Fig. 4), we interpret the nodule distal to the posterior-most third epibranchial cartilage early in stage 20 as a rudimentary fourth larval epibranchial (eb4). During stage 22, the adult epibranchial (ae) is apparent as a bifurcation of the first larval epibranchial. The *os thyroideum* of the juvenile is derived from the posterior end of the second basibranchial of stages 20–21. Additional abbreviations: lc = lingual cartilage; ra = radii. Scale bars, 0.5 mm.**

supports our identification of this cartilage as a fourth larval epibranchial (Rose 1995a). This nodule is quickly resorbed and is not visible in later stage-20 preparations or, indeed, at any other stage.

Several changes occur between stages 21 and 22. The third epibranchial begins to regress during stage 21 and disappears by stage 22. Each ceratohyal separates from the first basibranchial and forms blades—thin sheets of matrix that extend along the medial and lateral surfaces of their anterior halves. Paired lingual cartilages form as lateral projections, which are continuous with the anterior tip of the first basibranchial (Hilton 1947).

The adult epibranchial emerges from the lateral edge of the initial first epibranchial during stage 22, resulting in a bifurcated structure. A similar developmental sequence has been described for metamorphosing plethodontid salamanders (Smith 1920; Alberch et al. 1985; Alberch and Gale 1986; Rose 1995a). For consistency with these earlier reports, we refer to the initial epibranchial as the "larval" epibranchial, despite the fact that *P. cinereus* is a direct-developing species. Fibers of the first subarcualis rectus muscle (SA1) envelop the posterior end of the adult epibranchial (Fig. 4E). Anteriorly, adult and larval epibranchials are fused; SA1 fibers border the lateral side of the combined element (Fig. 4F,G). There is no indication of a perichondral origin of the adult epibranchial as described for *Eurycea bislineata* (Smith 1920; Alberch et al. 1985; Alberch and Gale 1986). Instead, chondrocytes from the two cartilages are in direct apposition with one another along the anteromedial end of the combined element (Fig. 4E–G).

The anterior projection of the second basibranchial is no longer present by stage 22 (Fig. 3). The posterior end of the second basibranchial persists through metamorphosis; it will ossify in juveniles as the *os thyroideum* (Smith 1920; or "urohyal" of Lombard and Wake 1986). The geniohyoideus muscle is attached to the anterior margin of the second basibranchial, whereas the abdominohyoideus muscle is attached posteriorly. These muscular attachments persist through metamorphic changes in the embryonic second basibranchial and remain attached in the juvenile and adult via the *os thyroideum*.

By stage 23, the hyobranchial skeleton has nearly attained the definitive morphology characteristic of juveniles and adults. Paired radial cartilages (after Wake and Deban 2000; or "cornua" of Piatt 1935; Hilton 1947; Wake 1966) occur on either side of the first basibranchial cartilage. Lingual cartilages are no longer distinct as lateral processes from the anterior first basibranchial. The posterior end of the ceratohyal is elongate and forms a dorsolateral bend, which becomes a hook in subsequent stages. Anterior ceratohyal blades are well defined. The first larval epibranchial is no longer visible in either sections or whole mounts, whereas

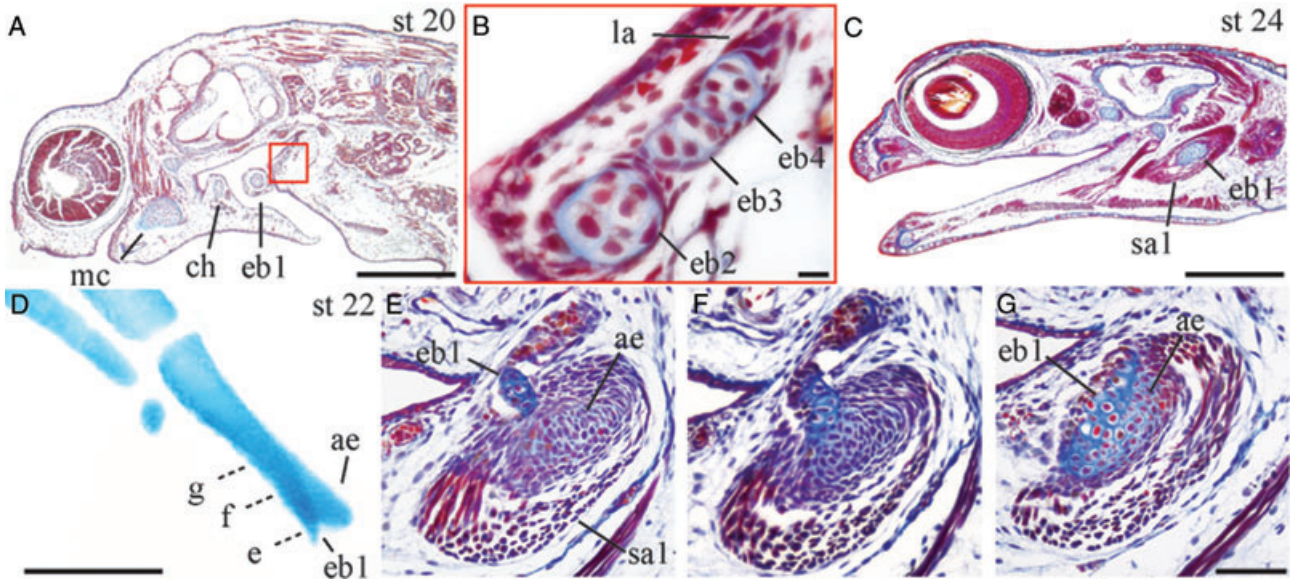

Figure 4. Mallory-stained sections revealing the fourth larval epibranchial cartilage (A and B) and formation of the adult epibranchial (C–G). (A–C) Sagittal sections; (E–G) transverse sections. (B) Higher magnification of the boxed region (red) in (A). (D) Cleared-and-stained preparation with the levels of sections (E–G) indicated as dashed lines. Additional abbreviations: la = levator arcuum muscle; sa1 = first subarcualis rectus muscle. Scale bars, 0.5 mm in A, C, and D; 10 μm in B; and 50 μm in E–G.

the second epibranchial persists as a small nodule distal to the posterior tip of the second ceratobranchial.

Stage 24 is characterized by complete resorption of the external gills (Dent 1942). The second epibranchial also has resorbed, whereas the adult epibranchial continues its transformation into an elongate, curved rod. Ceratohyal blades are slightly broader and have extended posteriorly along the central ceratohyal shaft. The medial blade extends farther than the lateral blade, as in adult *Plethodon* sp. (Hilton 1947).

Shortly after hatching, the adult epibranchial extends further posteriorly and the ceratohyal blades expand anteriorly. The medial portion of the second basibranchial/*os thyroideum* has begun to ossify. The hyobranchial skeleton has largely assumed its adult morphology.

Discussion

The recent and unexpected placement of metamorphosing species of *Desmognathus* within a more inclusive direct-developing clade suggests that patterns of life-history evolution are more labile than previously believed (Chippindale et al. 2004; Mueller et al. 2004; Macey 2005; Kozak et al. 2005, 2009; Vieites et al. 2007, 2011). Several larval features that initially assigned *Desmognathus* to a basal position within the family are instead considered examples of homoplasy in the light of recent phylogenetic studies (Wake 1966; Wake et al. 2011). We have used the resulting controversy regarding the evolution of complex larval phenotypes to guide our study of embryogenesis in a direct-developing salamander. Comparable studies of phylogeny and life-history evolution in other amphibian clades demonstrate that free-living larvae are often not regained following the evolution of direct development (e.g., Arthroleptidae, Blackburn 2008; Brevicipitidae, Müller et al. 2007; Rhacophorinae, Meegaskumbura et al. 2002 and Bahir et al. 2005; Terrarana, Heinicke et al. 2009). However, patterns of life-history evolution within extant amphibians can be variable, as suggested by the regain of larvae within hemiphractid (marsupial) frogs (Mendelson et al. 2000; Wiens et al. 2007; Wiens 2011) and the regain of the postmetamorphic stage within neotenic ambystomatid salamander lineages (Shaffer 1984; Wiens et al. 2005). Further studies of life-history evolution will benefit from analyzing the ontogeny of specific traits without assuming that evolution of direct development entails complete loss of the larval stage, as we have attempted in our study of *Plethodon*.

The initial patterning and subsequent modifications to the hyobranchial skeleton of embryonic *P. cinereus* are similar to hyobranchial development and metamorphosis in plethodontid species with free-living larvae (Smith 1920; Piatt 1935; Hilton 1947; Wake 1966; Alberch and Gale 1986; Rose 1995b). In direct-developing frogs and caecilians, consequences of the evolution of direct development on cranial ontogeny are often complex. Loss of a free-living larval stage does not invariably involve the complete loss of specific larval structures (e.g., Blackburn 2004; Müller 2006; Kerney et al. 2007). Retention of components of the metamorphic ontogeny in *P. cinereus* could be attributable to selection for maintaining larval developmental programs or to a lack of selection against them. In either case, retention of

larval features (e.g., four epibranchials and epibranchial replacement; Fig. 1) and their subsequent metamorphic transformation in embryos of direct-developing *P. cinereus* suggests that a larval hyobranchial skeleton and its metamorphosis have not been evolutionarily "regained" in *Desmognathus*. Instead, larval hyobranchial features in *Desmognathus* represent an elaboration of the ontogeny and developmental morphology of their allegedly direct-developing ancestors.

THE FOURTH LARVAL EPIBRANCHIAL CARTILAGE

In *P. cinereus* we find a greatly reduced, transient epibranchial IV (Fig. 4B). This cartilage is the insertion for the fourth levator arcuum muscle, which confirms the homology of this transient element with the fourth larval epibranchial cartilage of other salamander families (e.g., Ambystomatidae, Piatt 1939) and the variable fourth epibranchial of the plethodontid *Hemidactylium scutatum* (Rose 1995a). There is, however, no sign of additional depressor arcuum or constrictor arcuum muscle fibers attached to this transient cartilage in *P. cinereus*. This cartilage is not reported in previous studies of *P. cinereus* (Dent 1942), but we have verified its presence in both cleared-and-stained whole mounts and serial sections of stage-matched individuals.

At one time the presence of a fourth epibranchial cartilage in larvae of metamorphosing species of *Desmognathus* was regarded as a plesiomorphic trait, reflecting the basal position of this taxon within Plethodontidae (Wake 1966). Indeed, absence of this cartilage was proposed as a synapomorphy for all nondesmognathine plethodontids (Wake 1966; Lombard and Wake 1986). Two earlier studies, however, report its presence in plethodontid taxa other than *Desmognathus*. Houghton (1903) describes a distinct fourth epibranchial in *E. longicauda* ($n = 3$), although subsequent studies fail to find this cartilage in this or any other species of *Eurycea* (Wake 1966: $n = 13$ for *E. longicauda*, $n = 50$ for all *Eurycea* sp.; Rose 1995b: $n = 11$ for *E. longicauda*, $n = 295$ for all *Eurycea* sp.). This discrepancy is likely due to Houghton's taxonomic misidentification of his material. He depicts, for example, connections between the distal ends of the epibranchial cartilages, a feature of ambystomatid larvae (Hörstadius and Sellman 1946) that is unknown in plethodontids (Hilton 1947; Wake 1966). In a separate study, Rose (1995a) describes paired fourth epibranchials (ceratobranchials) in 45 of 49 larval *H. scutatum*. These elements condense separately from the third epibranchials but later fuse to them posteriorly. Additionally these fourth epibranchials receive fibers of the levator, depressor, and constrictor arcuum muscles (the latter described here as the "subarcualis rectus" and by Piatt 1939 as the "omo-arcualis").

We propose that the presence of epibranchial IV in larvae of metamorphosing species of *Desmognathus* may be an elaboration of a transient epibranchial IV that persisted in the ontogeny of their direct-developing ancestors. Rose (1995a) examined two direct-developing species of *Desmognathus*. He describes an unpaired cartilage at the distal end of the third epibranchial in a single *D. wrighti* ($n = 4$) but found no indication of a vestigial fourth epibranchial in *D. aeneus* ($n = 16$). Marks (1994, 2000), however, reports four epibranchials in embryos of *D. aeneus*. Given that the fourth epibranchial of *P. cinereus* is very transient (restricted to only a portion of stage 20), it may have been missed in some previous studies of direct-developing *Desmognathus* due to limited ontogenetic sampling.

The relative timing of hyobranchial element formation and metamorphic transformation is conserved between *P. cinereus* and metamorphosing plethodontids (e.g., *Eurycea*; Rose 1995b). However, some larval cartilages, such as the fourth epibranchial, are smaller in *P. cinereus* and appear for a short developmental period in comparison to metamorphosing plethodontids. This might be attributable to changes in patterning or developmental timing associated with direct development. Perhaps the corresponding prechondrogenic condensation has been reduced, or the period of elongation shortened due to rapid metamorphic remodeling of the cartilage during a brief embryonic period. This raises the question of why a vestigial fourth epibranchial is formed at all. Retention of pleiotropic gene regulatory networks that are involved in fourth epibranchial patterning may explain its transient appearance in *P. cinereus*. Alternatively, the vestigial fourth epibranchial may have an unknown role in developmental morphogenesis. Vestigial structures are of increasing interest in evolutionary developmental biology. Understanding their formation and possible developmental loss will require integration of morphological, developmental, and genomic perspectives.

FORMATION OF THE ADULT EPIBRANCHIAL CARTILAGE

Smith (1920) first described the metamorphic replacement of larval first epibranchial cartilages with adult epibranchial cartilages in her study of the plethodontid salamander *E. bislineata*, a metamorphic change that does not occur in any other salamander family. Subsequent studies by Alberch and colleagues further characterized the morphological and endocrine basis of this dramatic transformation (Alberch et al. 1985; Alberch and Gale 1986). During metamorphosis in *Eurycea* chondrocytes of the larval first epibranchial undergo cell death and the cartilage is resorbed. Simultaneously, the adult epibranchial forms de novo from cells derived from the ventral perichondrium of the larval epibranchial, which proliferate and form a mesenchymal condensation within which the adult epibranchial chondrifies. Alberch (1987, 1989) interpreted this as a process of developmental compartmentalization, in which larval and adult structures have distinct cellular origins.

Remodeling of the first epibranchial cartilage during stage 22 (Fig. 4D–G) offers a further example of metamorphic changes

during embryogenesis in direct-developing *P. cinereus*. During this stage, the posterior larval first epibranchial regresses whereas the adult first epibranchial forms as its replacement, sheathed in fibers of the subarcualis rectus 1 muscle (Fig. 4E). In contrast to *Eurycea*, larval and adult epibranchials are not completely separate; the two cartilages retain an anterior cartilaginous connection in all specimens examined in this study. Moreover, there is no indication that the adult epibranchial forms as a separate condensation that is not connected to its larval counterpart. Instead, formation of the adult epibranchial in *P. cinereus* may be attributable to appositional growth of the larval cartilage rather than de novo formation from a compartmentalized cell source.

Recently, Rose (2009) reported separate larval and adult first epibranchial cartilages in three other metamorphosing plethodontid species (*Gyrinophilus porphyriticus, D. marmoratus*, and *H. scutatum*). Although adult epibranchial formation in *G. porphyriticus* seems to be similar to that in the closely related *E. bislineata*, none of the other species exhibits a separate origin as dramatic as that reported for *Eurycea* (Alberch and Gale 1986, their Fig. 13). Instead, the anterior ends of the larval and adult elements are often fused (Rose 2009, Fig. 4), which also is apparent in our material, indicating that the anterior end of the larval element is retained in and contributes to the adult element (see also Rose 1995c). Consistent with this, both Piatt (1935) and Smith (1920) describe only a partial replacement of the larval element by the adult epibranchial in *E. bislineata*. However, these earlier works were not as extensive as later investigations (Alberch et al. 1985; Alberch and Gale 1986; Rose 1995b) in terms of numbers of specimens or use of multiple skeletal stains and sectioning techniques.

There is little research on the developmental compartmentalization of larval versus adult epibranchial cartilages in direct-developing plethodontid salamanders. In his observations of the epibranchial skeleton during embryonic development of *P. cinereus*, Dent (1942) does not report changes that are suggestive of compartmentalization. Alberch (1989) describes a cluster of cells adjacent to the larval first epibranchial in *D. wrighti,* which he interprets as indicating a separate origin of the adult cartilage. The same study, however, does not find distinct larval epibranchials in the direct-developing *Bolitoglossa subpalmata* (also described in Wake 1966; Alberch 1987; Wake and Hanken 1996). Loss of these cartilages may indicate the release from a larval developmental constraint in *Bolitoglossa* (Alberch 1989), which facilitated the evolution of ballistic feeding in this and related genera (Wake 1982; Roth and Wake 1985; Lombard and Wake 1986). However, this is an initial account, supported by data from a single specimen, which remains to be verified by more thorough descriptions of hyobranchial development in *Bolitoglossa*. More data on hyobranchial ontogeny in genera with ballistic feeding (including the direct-developing genera *Bolitoglossa* and *Hydromantes*; Deban et al. 2007) are needed to more fully evaluate this correlation.

Conclusion

Dollo's Law posits that complex traits cannot re-evolve. Yet, recent phylogenetic studies (Chippindale et al. 2004; Mueller et al. 2004; Macey 2005; Kozak et al. 2005, 2009; Vieites et al. 2007, 2011) overturn long-standing models of plethodontid evolution (reviewed in Lombard and Wake 1986) and suggest that the placement of metamorphosing *Desmognathus* within a larger clade of direct-developers violates Dollo's Law. This conclusion is supported by likelihood-based ancestral state reconstructions (Chippindale et al. 2004) and is independent of net diversification rate (Wiens 2011). However, our developmental data suggest that interpretations of life-history evolution based solely on ancestral character state reconstructions may provide a misleading view of trait evolution and subsequent rejection of Dollo's Law. This is primarily due to an oversimplification of life-history stages themselves. Larvae are characterized by a set of traits (e.g., fourth larval epibranchial, Leydig cells), each of which should be studied and evaluated individually. Simply stating that a taxon exhibits direct development provides no information as to the presence or absence of specific phenotypes that are of interest in the evolution of morphology and ecology of larvae.

Controversial interpretations of ancestral character state reconstructions are not limited to plethodontid life-history evolution. Such reconstructions can be problematic under a range of conditions (Cunningham et al. 1998; Webster and Purvis 2002), including incomplete phylogenies (Ronquist 2004), high rates of transitions between character states (e.g., Schluter et al. 1997), correlation of character state change with diversification (e.g., Maddison et al. 2007; Goldberg and Igić 2008; FitzJohn et al. 2009; Wiens 2011), or directional trends in character change (Oakley and Cunningham 2000; Webster and Purvis 2002). Experimental studies in which phylogeny and ancestral states are known reveal ancestral character state reconstructions to be inaccurate except under limited conditions, such as low rates of character change (Oakley and Cunningham 2000). A range of phenomena can mislead studies that evaluate support for Dollo's Law based on ancestral state reconstructions or models of trait evolution in a phylogenetic context. However, regardless of the ancestral state reconstruction methodology employed, or whether Dollo's Law is accepted or rejected in a phylogenetic analysis, we stress that evaluations of life-history evolution require extensive understanding of the specific traits that characterize different life history stages. The substantial metamorphic changes in direct-developing *P. cinereus* offer a cautionary tale for interpreting character evolution that is allegedly linked to specific life histories without a detailed understanding of developmental morphology.

Indeed, analyses of trait evolution in a phylogenetic context are emerging as important guides for evaluating Dollo's Law using developmental data (Galis et al. 2010; Kohlsdorf et al. 2010).

Why have some direct-developing lineages apparently reacquired the ancestral metamorphic life history but not others? By examining the occurrence of larval features in diverse direct-developing amphibians, we may determine the extent to which ontogeny has been developmentally repatterned (Hanken 1992; Kerney et al. 2010). The degree of developmental repatterning, in turn, may determine whether metamorphosis is regained in certain lineages. Such an approach requires detailed analyses of individual anatomical regions. Our study of developmental morphology suggests that the anatomy of the hyobranchial skeleton in larval *Desmognathus* does not represent a "re-evolution" of larval traits. Other direct-developing plethodontids may have more extensive developmental repatterning and loss of larval structures (e.g., *Bolitoglossa* spp., Alberch 1989), which may account for the apparent lack of metamorphic life histories in their evolutionary lineage. However, more comparative developmental data are required to determine the extent to which losses of larval structures are irreversible. Our results underscore the need for additional detailed studies of embryonic development in direct-developing plethodontid salamanders, especially in the genus *Desmognathus* in which the free-living larval stage is believed to have reappeared. Future study of the ontogenies of amphibians with diverse life histories promises to facilitate our understanding of the evolvability of life histories and complement results obtained by the use of phylogenetic comparative methods.

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