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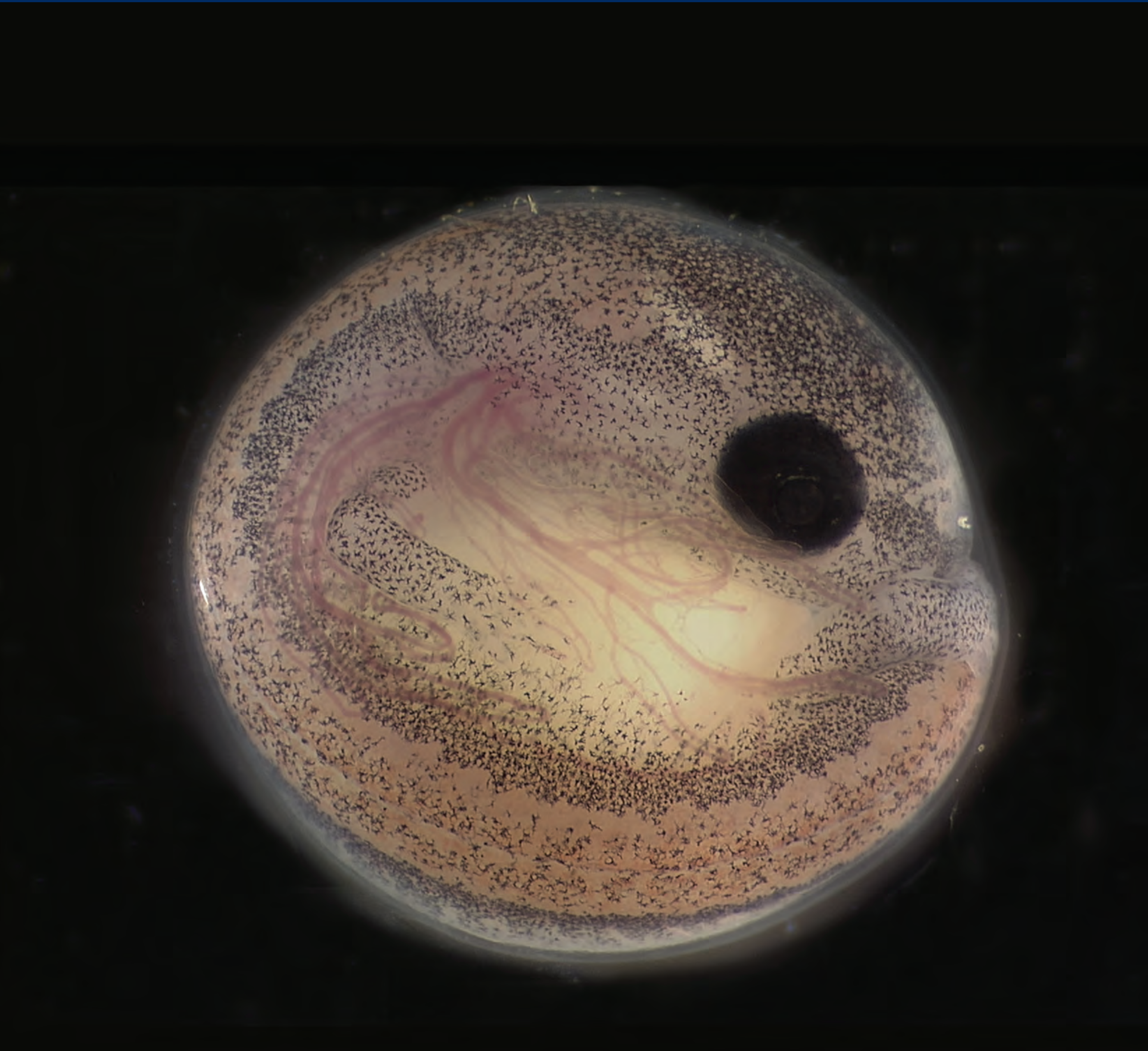

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RESEARCH

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Early limb patterning in the direct-developing salamander *Plethodon cinereus* revealed by *sox9* and *col2a1*

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Direct-developing amphibians form limbs during early embryonic stages, as opposed to the later, often postembryonic limb formation of metamorphosing species. Limb patterning is dramatically altered in direct-developing frogs, but little attention has been given to direct-developing salamanders. We use expression patterns of two genes, *sox9* and *col2a1,* to assess skeletal patterning during embryonic limb development in the direct-developing salamander *Plethodon cinereus*. Limb patterning in *P. cinereus* partially resembles that described in other urodele species, with early formation of digit II and a generally anterior-to-posterior formation of preaxial digits. Unlike other salamanders described to date, differentiation of preaxial zeugopodial cartilages (radius/tibia) is not accelerated in relation to the postaxial cartilages, and there is no early differentiation of autopodial elements in relation to more proximal cartilages. Instead, digit II forms in continuity with the ulnar/fibular arch. This amniote-like connectivity to the first digit that forms may be a consequence of the embryonic formation of limbs in this direct-developing species. Additionally, and contrary to recent models of amphibian digit identity, there is no evidence of vestigial digits. This is the first account of gene expression in a plethodontid salamander and only the second published account of embryonic limb patterning in a direct-developing salamander species.

1 | INTRODUCTION

Salamander limb development is unique among extant tetrapods. The limb buds lack a morphologically distinct apical ectodermal ridge (Tank, Carlson, & Connelly, 1977; Torok, Gardiner, Izpisúa Belmonte, & Bryant, 1999), rarely assume a distinct paddle shape during digit formation (Franssen, Marks, Wake, & Shubin, 2005; Holmgren, 1933; Shubin & Alberch, 1986) and express the novel three-finger-protein-family regulator prod1, which is required for digit development and regeneration (Kumar, Gates, Czarkwiani, & Brockes, 2015). The novelty of salamander limb development is most prominent in the sequence of skeletal patterning. All other extant tetrapods have *postaxial* patterning, in which limb homologs of digit IV form first in both the manus and pes followed by a general posterior-to-anterior order of digit formation (Hinchliffe & Griffiths, 1982; Hinchliffe & Johnson, 1980; Holmgren, 1933; Shubin & Alberch, 1986). In salamanders, however, formation of the digits follows a *preaxial* pattern of development (reviewed in Shubin & Wake, 2003; Fröbisch, 2008). Digit II forms first, often in synchrony with digit I and the basale commune (a derived mesopodial element), followed by a general anterior-to-posterior order of digit formation. In most salamanders, this coincides with earlier differentiation of the *preaxial* radius/tibia in comparison to the *postaxial* ulna/fibula. The "*preaxial polarity*" of salamanders is also found in fossil temnospondyls and

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could possibly be the ancestral condition for tetrapods (Fröbisch, Bickelmann, Olori, & Witzmann, 2015).

Salamanders also are unique in forming distal mesopodial and autopodial elements without a continuous condensation linking them to more proximal cartilages. In particular, the basale commune and metacarpals I and II tend to form before more proximal cartilages of the mesopodium and zeugopod (Blanco & Alberch, 1992; Fröbisch, 2008; Shubin & Alberch, 1986; Shubin & Wake, 2003; Vorobyeva & Hinchliffe, 1996; Wake & Shubin, 1998). This constitutes an exception to the general proximal-to-distal order of skeletal differentiation found in all non-salamander tetrapod limbs (Blanco & Alberch, 1992; Shubin & Alberch, 1986).

Within salamanders, unique patterns of limb development are associated with different life history strategies and taxonomic groups (Blanco & Alberch, 1992; Franssen et al., 2005; Wake & Shubin, 1998; Shubin & Wake, 2003). Whereas most research into salamander limb development has focused on the neotenic axolotl (*Ambystoma mexicanum*; Guimond et al., 2010) and metamorphosing newts (*Cynops* and *Notophthalmus*; Kumar et al., 2015), terrestrial direct development is the most common life history mode among living urodeles (Wake & Hanken, 1998; Smirnov, 2008). Terrestrial direct development in amphibians is associated with the "pre-displacement" of limb formation into embryonic stages (Franssen et al., 2005; Gross, Kerney, Hanken, & Tabin, 2011; Kerney & Hanken, 2008; Richardson et al., 1998). Limbs begin to form early during embryogenesis, coincident with neural tube closure, and are largely complete prior to hatching (Collazo & Marks, 1989; Kerney, 2011; Marks & Collazo, 1998; Richardson et al., 1998). This earlier onset of limb formation is more similar to that found in amniotes than in metamorphosing amphibians (Bininda-Emonds et al., 2007; Galis, Wagner, & Jockusch, 2003; Young & Tabin, 2016).

There are only two published accounts of limb development in direct-developing salamanders (Franssen et al., 2005; Shubin & Wake, 1991). All direct-developing salamanders belong to the Plethodontidae, which is the most speciose family of salamanders. Moreover, the fact that most plethodontids exhibit terrestrial direct development means that more than half of all living salamander species have this developmental mode (Hanken, 2003). Similar to amniotes, digits in direct-developing *Desmognathus aeneus* form within a limb "paddle" instead of extending from a conical limb bud "palette" as occurs in most metamorphosing species (Franssen et al., 2005; Nye, Cameron, Chernoff, & Stocum, 2003). A paddle, however, is also seen during limb development in the free-living larva of the stream-dwelling *D. quadramaculatus* (Marks, 1995), indicating that a paddle is not strictly associated with loss of the larval stage. Direct developers also exhibit other features of limb development that are regarded as "amniote-like" (Shubin & Wake, 1991; Wake & Shubin, 1998). The "digital arch" (distal mesopodial elements and condensations of digits I–III; Shubin & Wake, 2003) forms early and, in *Bolitoglossa subpalmata,* is continuous with condensations of the ulnare/fibulare and forms in a "weak pre- to post-axial sequence" (Shubin & Wake, 1991). Early formation of the digital arch is less apparent in type-II-collagen-stained *D. aeneus,* which form metacarpal II and a digit II phalanx before the basale commune and with no apparent connection to the ulnare/fibulare (Franssen et al., 2005). Additionally, preaxial dominance and early distal condensations in the autopod are less pronounced in both of these direct-developing species than they are in metamorphosing salamanders.

More comparative data for direct-developing species are needed to determine the extent to which limb patterning in salamanders may be altered during life history evolution. In aquatic larvae of metamorphosing species, limbs develop after hatching and thus may experience specific selective pressures related to growing a limb while moving about in pond or stream environments. These pressures are less relevant to the embryos of direct-developing species, which are enclosed within a protective egg capsule.

Here we describe the molecular morphology of limb-skeletal patterning in direct-developing *Plethodon cinereus* by analyzing expression of mRNA for the genes *sox9* and *col2a1* visualized through whole-mount in situ hybridization. Sox9 is an HMG-box-containing transcription factor that can reveal the initial differentiation of pre-chondrocytes before they form extracellular matrix (Chimal-Monroy et al., 2003; Welten, Verbeek, Meijer, & Richardson, 2005; Kerney & Hanken, 2008; Kerney, Gross, & Hanken, 2010; Lorda-Diez, Montero, Diaz-Mendoza, Garcia-Porrera, & Hurle, 2011; Hayashi et al., 2015). In situ hybridizations detecting *sox9* mRNA are a consistently useful technique for analyzing early skeletal patterning (de Bakker et al., 2013; Guimond et al., 2010; Kerney & Hanken, 2008; Kerney et al., 2010; Kumar et al., 2015; Montero et al., 2017). The gene for type-II collagen, *col2a1*, is a direct target of Sox9 transcriptional activation in the chondrocyte lineage of amniotes and amphibians (Bell et al., 1997; Kerney, Hall, & Hanken, 2009; Lefebvre, Huang, Harley, Goodfellow, & de Crombrugghe, 1997; Ng et al., 1997). In the direct-developing frog *Eleutherodactylus coqui,* expression of *sox9* reveals novel early distal patterning of the autopod, while expression of *col2a1* (Kerney et al., 2010) is identical to the subsequent distribution of type-II collagen revealed by immunohistochemistry (Hanken et al., 2001). These two transcripts are used as proxies for the specification and differentiation of chondrocytes, respectively (Montero et al., 2017; de Bakker et al., 2013). Finally, we evaluate early limb patterning in *P. cinereus* revealed by gene expression in the context of previous work on skeletal patterning during salamander limb development and evolution.

2 | METHODS

We collected embryos of *Plethodon cinereus* in Halifax, Nova Scotia, Canada (Nova Scotia Department of Natural

Resources permits to RK), and Michaux State Forest in Adams County, Pennsylvania (Pennsylvania Fish and Boat permit 727-type 1 to RK). Jelly capsules were removed from early embryos after a 10-min immersion in 0.5% cysteine (pH 8.5) followed by multiple washes in phosphate-buffered saline (PBS; pH 7.6) and manual extraction with watchmaker forceps in PBS (Kerney, 2011; Kerney, Blackburn, Müller, & Hanken, 2012). Embryos were fixed for 2 hr in MEMFA, rinsed twice (5 min each) in DEPC-treated PBS, and stored in 70% methanol at −20 °C (Kerney & Hanken, 2008). Embryos were staged according to the preliminary table proposed by Dent (1942) and expanded by Kerney (2011).

Cloning of *sox9* and *col2a1* followed the protocol of Kerney and Hanken (2008). Embryonic RNA was extracted from a single individual with the Trizol Reagent (Life Sciences) following the manufacturer's protocol. Superscript II (Invitrogen) reverse transcriptase was used to make a cDNA template using random hexamers. Orthologies of *col2a1* (GenBank MG515834) and *sox9* (GenBank MG515833) sequences from *Plethodon cinereus* were verified via phylogenetic analyses of the protein-coding sequences (Figure 1). We obtained amino acid sequences of *P. cinereus* Col2a1, Sox9 and closely related orthologs from GenBank for selected model organisms. We created multiple alignments using MAFFT v.7.0 (Katoh & Standley, 2013), which were trimmed to the length of our sequences from *P. cinereus*. We used the best-fit model of sequence evolution (Col2a1: LG+G; Sox9: JTT+I+G+F) selected via ProtTest v.2.4 server (Darriba, Taboada, Doallo, & Posada, 2011; http://darwin.uvigo.es/software/prottest2_server.html) in a maximum-likelihood analysis conducted using PhyML v.3.0 with 100 non-parametric bootstrap replicates (Guindon et al., 2010; Figure 1).

Whole-mount in situ hybridizations followed established *Xenopus* protocols (Sive, Grainger, & Harland, 2000) with slight modifications for the larger embryos of direct-developing amphibians (Kerney & Hanken, 2008). Whole-mount clearing and cartilage staining with Alcian blue followed Hanken and Wassersug (1981).

TABLE 1 Stage-specific sample sizes for *sox9* and *col2a1* in situ hybridizations

Stage	*Sox9* in situ	*Col2a1* in situ
15	3	3
16	5	1
17	2	4
18	1	2
19	1	3
20	1	4
21	1	1

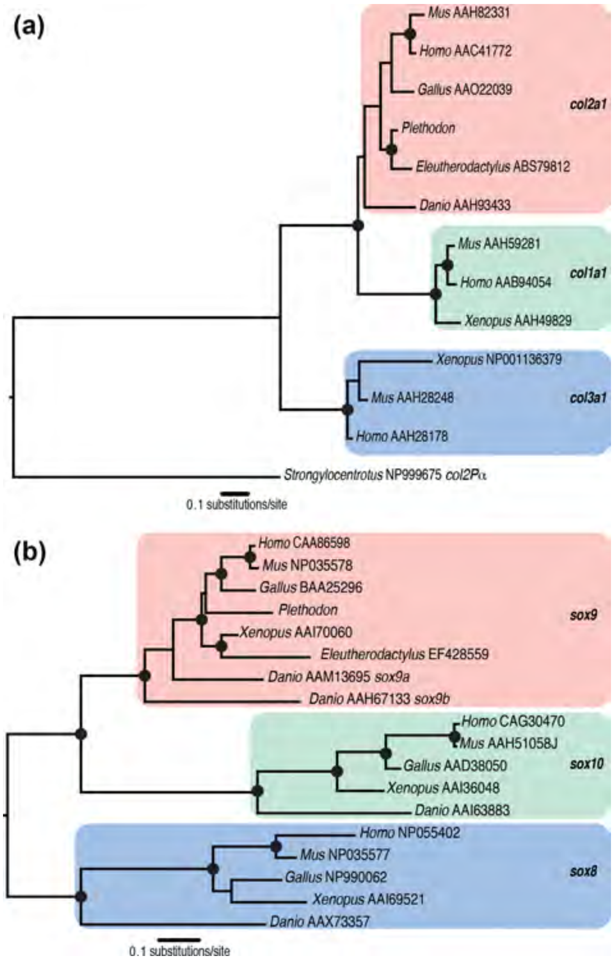

FIGURE 1 Maximum-likelihood phylogeny for fibrillar collagen (a) and soxE class (b) homologs from selected model organisms. GenBank accessions for each amino acid sequence follow the taxon name

3 | RESULTS

Sox9 expression precedes *col2a1* expression during the differentiation of limb cartilages (Figure 2). *Sox9* is first detected in the forelimb during Stage 17, at which point it is restricted to the presumptive proximal humerus. By Stage 19, *sox9* expression has expanded to include the ulnar column of the forelimb, the radius and manual digits II and III. There is also distinct expression in the “median column” (Vorobyeva & Hinchliffe, 1996) of the presumptive intermedium and element C of the mesopodium, which lies between the expression domains of the radiale anteriorly and the ulnare posteriorly. By Stage 20, expression of *sox9* in the forelimb expands to include digit I and the median column is more clearly defined. In the Stage-21 forelimb, digit IV is more elongate, though *sox9* expression is no longer detectable in this digit or in the rest of the forelimb.

Expression of *sox9* in the hind limb begins by Stage 19 in the presumptive femur, the fibular column, pedal digit II and a

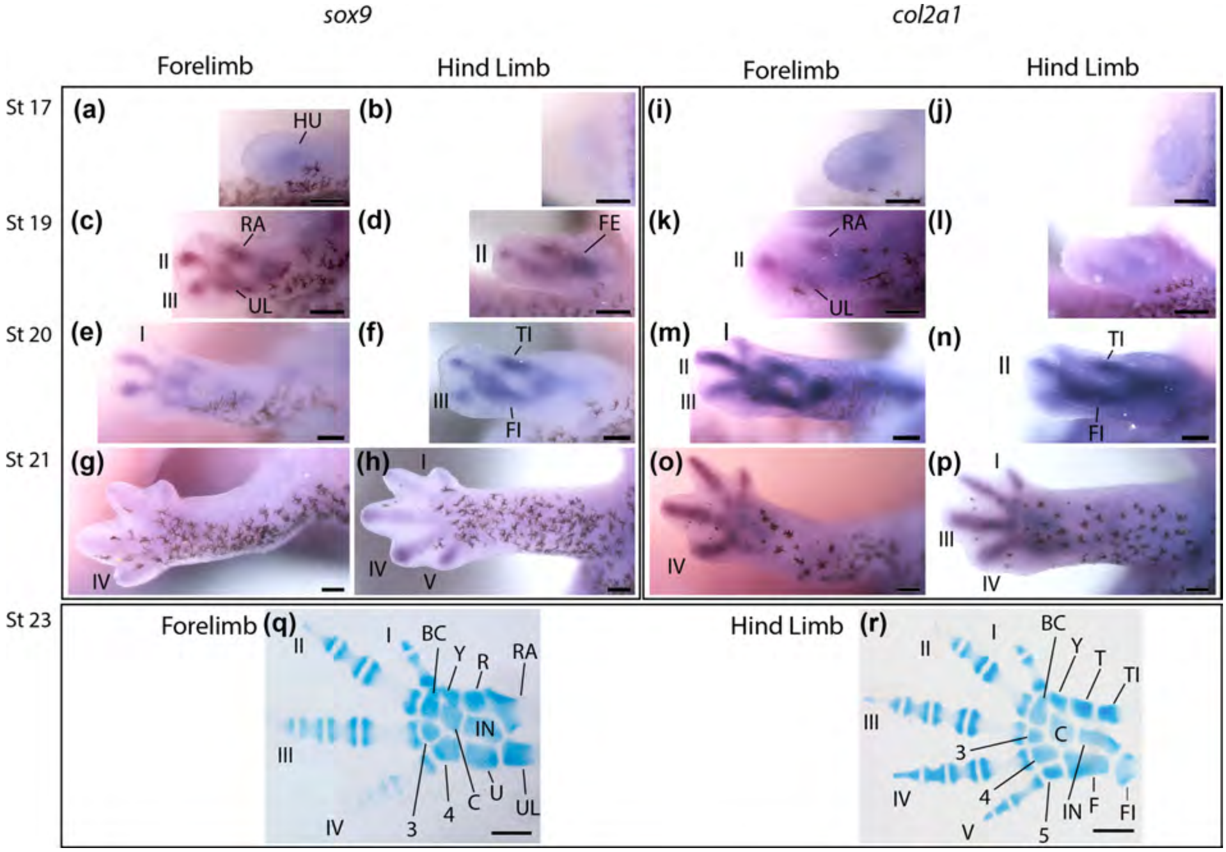

FIGURE 2 Developmental expression of *sox9* and *col2a1* in embryonic fore- and hind limbs. All panels depict left limbs in dorsal view; anterior is to the top. Arabic numerals denote distal carpals; digits are indicated by Roman numerals. BC, basale commune (fused 1st and 2nd distal carpals/tarsals); C, centrale; F, fibulare; FE, femur; FI, fibula; HU, humerus; IN, intermedium; R, radiale; RA, radius; T, tibiale; TI, tibia; U, ulnare; UL, ulna; Y, element Y. Scale bars equal 0.2 mm

proximal portion of the presumptive tibia. In the Stage-20 hind limb, *sox9* expression includes digit III, but expression in the median column is not distinct from the presumptive fibulare. In the Stage-21 hind limb, *sox9* expression only reveals the distal phalanges of all five digits. Proximal expression is no longer detectable.

Col2a1 expression in the limbs begins slightly later than *sox9* expression but otherwise follows similar spatial and temporal patterns. At Stage 17, there is faint and indistinct *col2a1* expression in the forelimb but none in the hind limb. During Stage 19, expression of *col2a1* in the forelimb includes the ulnar column, manual digit II and a proximal portion of the radius. By Stage 20, forelimb expression expands to include digits I and II. Expression in the median column of the mesopodials is discernable and continuous with the presumptive ulnare (similar to *Salamandrella keyserlingii*; Vorobyeva & Hinchliffe, 1996). In Stage 21, *col2a1* is strongly expressed in all four digits of the forelimb and reveals some differentiation of the phalanges, metacarpals, and carpals.

Col2a1 expression begins in the hind limb by Stage 19, when there is a faint and indistinct proximal region of expression. In the Stage-20 hind limb, *col2a1* is expressed in the presumptive fibular column and a portion of the tibia, but it is most strongly expressed in digit II with only faint expression in digits I and III. By Stage 21, *col2a1* is expressed in all digits except digit V. Hind limb mesopodial elements are well defined, but the tibial column and more proximal limb cartilages are not discernable in *col2a1* whole-mount in situ hybridizations during Stage 21 (Table 1).

4 | DISCUSSION

4.1 | Patterns of differentiation

A cascade of molecular events and physical interactions specify and commit mesenchymal cells to become pre-cartilaginous condensations in the limb (Cooper, 2015). Various condensation markers include Aniken patterns of peripheral cells (Shubin & Alberch, 1986), type-II collagen immunoreactivity (Franssen et al., 2005), peanut agglutinin reactivity (Larsson & Wagner, 2002) and the expression of *col2a1* mRNA (Kerney & Hanken, 2008). Earlier markers include expression of *noggin:lacz* or *sox9:lacz* transgenes (Zhu et al., 2008), *galectins CG-1A* and *CG-8* (Bhat et al., 2011) and *sox9* mRNA (de Bakker et al., 2013). Expression of *sox9* mRNA was found to be the earliest marker of differentiating chondrocytes in a comparative study of induced ectopic cartilage in the embryonic chicken wing (Lorda-Diez et al., 2011). Given the apparent conservation of the role of Sox9 in chondrocyte differentiation, whole-mount in situ hybridization with *sox9* has become a standard for detecting early skeletal patterning (Montero et al., 2017).

Interestingly, the early patterning detectable through *sox9* mRNA distribution or reporters does not always parallel the subsequent patterning of fully differentiated condensations (de Bakker et al., 2013; Kerney & Hanken, 2008; Zhu et al., 2008). For instance, Zhu et al. (2008) discovered novel patterns of mouse digit loss through a conditional Shh deletion study that mirrored the specification patterns revealed by *sox9:lacz* and *noggin:lacz*. Contrary to these observations, our data for *Plethodon cinereus* do not reveal differences between the early patterning revealed by *sox9* and the subsequent formation of a committed condensation as revealed by *col2a1*. This consistency reveals that the earlier patterning mechanisms, which result in the specification of these chondrocytes based on *sox9*, is reflected in the timing of their subsequent differentiation, as revealed by *col2a1*. Thus, early patterning revealed by *sox9* is not always different from that revealed by later markers. Subsequent studies examining the molecular patterning of tetrapod limbs would benefit from including markers of multiple stages of chondrocyte differentiation.

4.2 | Preaxial autopod patterning is continuous with the postaxial Ulna/Fibula

Early patterning of the limb skeleton in *Plethodon cinereus,* while consistent in many respects with that described for other salamander species, is exceptional in several important ways that may be a consequence of direct development. Similar to other urodeles described to date, preaxial dominance of digit II is apparent in both *sox9* and *col2a1* expression in the fore- and hind limbs. However, this digital expression is continuous with ulnar/fibular expression and displays neither a distinct distal *sox9* expression domain nor a separate condensation of *col2a1*-expressing cells. In metamorphosing salamanders (reviewed in Shubin and Wake, 2003) and in *Desmognathus aeneus,* the one direct developer that has been analyzed in detail (Franssen et al., 2005), distal and independent condensations of the basale commune and metacarpals/metacarpals I and II form separately from the more proximal zeugopodial cartilages. The apparently continuous patterning of the *sox9*- and *col2a1*-expressing digits with more proximal condensations may be linked to the more rapid specification of the limb in *P. cinereus*. However, such continuous patterning is not observed in direct-developing *E. coqui*, where there is early specification of an unconnected *sox9* domain in digit IV (Kerney & Hanken 2008).

4.3 | No preaxial dominance in the zeugopod

Preaxial dominance of zeugopodial elements, in which the radial/tibial column of differentiating cells is advanced in comparison to the ulnar/fibular column, is a common phenomenon in metamorphosing salamanders (Fröbisch, 2008). The additional presence of advanced radial/tibial development in some fossil temnospondyls suggests that preaxial dominance may have been widespread among early tetrapods (Fröbisch et al., 2015). Preaxial dominance in the zeugopod also occurs in direct-developing *Desmognathus aeneus* (Franssen et al., 2005). However, differentiation of chondrocytes is nearly synchronous between the two zeugopodial columns within the *P. cinereus* limb bud.

4.4 | Early differentiation of axial digit II

The sequence of autopodial *sox9* and *col2a1* expression in *P. cinereus* is similar to that of type-II collagen antibody reactivity in direct-developing *D. aeneus* (Franssen et al., 2005), as is the order of digit formation in both species: II-III-I-IV-V (in the hind limb). However, in *Bolitoglossa subpalmata,* another direct-developing plethodontid salamander, digits I and II form simultaneously with the basale commune prior to digit III (Shubin & Wake, 1991). Simultaneous formation of digits I and II also is common in metamorphosing salamanders (e.g., *Desmognathus quadramaculatus*—Marks, 1995; *Salamandrella keyserlingii*—Vorobyeva & Hinchliffe, 1996; *Dicamptodon tenebrosus*—Wake & Shubin, 1998; *Triturus marmoratus*—Blanco & Alberch, 1992; *Ambystoma mexicanum*—Shubin & Alberch, 1986). Delayed differentiation of digit I in *P. cinereus* and *D. aeneus* remains unique to these species and may be attributable to direct development within the subfamily Plethodontinae, despite the presence of the more common pattern in direct-developing *B. subpalmata* (subfamily Hemidactyliinae). The one other known exception among urodeles to the synchronous formation of digits I and II is in the highly reduced hand of the neotenic *Proteus anguinus* in which digit I forms first (Shubin & Alberch, 1986). The few direct-developing salamander species studied to date offer an inconsistent picture regarding the correlation between derived features of limb formation and life history. However, it should be emphasized that a sample size of three species is an extreme under-representation of direct development, the most common developmental mode of extant salamander species.

4.5 | No evidence of vestigial digits

Wagner, Khan, Blanco, and Misof (1999) propose that the preaxial polarity characteristic of salamanders can be attributed to their digits I and II being homologous with digits III and IV of amniotes. According to this hypothesis, a common ancestor of salamanders (or possibly temnospondyls; Fröbisch et al., 2015) lost digits I, II, and V, leaving only digits III and IV. Subsequently, new posterior digits III–IV (III–V in the hind limb) were "reinvented," making the retained original digits III and IV assume the positional identity of I and II in salamanders. Unique late-stage expression of *hoxa-11* in digit III of the eastern newt, *Notophthalmus viridescens,* is cited in support of this model and as evidence that the urodele digits III–IV (V) are developmental novelties (Wagner et al., 1999). A similar frame-shift hypothesis proposed to account for the identity of bird digits (Wagner & Gauthier, 1999; Wang, Young, Xue, & Wagner, 2011). This avian model was recently supported by patterns of *sox9* expression, which reveal vestigial digits in the developing chicken wing (de Bakker et al., 2013).

Similar vestigial digits have been proposed for amphibians. Steiner (1921) describes a vestigial prehallux along with a vestigial sixth digit in the foot in the tiger salamander, *Ambystoma tigrinum* (cited by Galis, van Alphen, & Metz, 2001), although neither structure is seen with *sox9* staining in the axolotl or newts (Guimond et al., 2010; Kumar et al., 2015). More recently, Hayashi et al. (2015) provide evidence that the prehallux in *Xenopus tropicalis* may constitute a sixth digit, supporting the notion that amphibians are not constrained to a maximum of five digits (Galis et al., 2001). Evolutionary retention of vestigial anterior digits in salamanders might be anticipated from Wagner et al.'s (1999) frame-shift hypothesis, especially since such vestiges have been reported in birds (e.g., de Bakker et al., 2013). However, retention of *sox9*-expressing vestiges may be less likely in salamanders than in birds given that the divergence times of temnospondyls from early tetrapods are far longer than those of Aves from terrestrial theropods. Our study revealed no evidence of transient vestiges of digits that would be consistent with these hypotheses of "extra" salamander digits.

4.6 | Limb development and regeneration in plethodon cinereus

Data regarding limb development in direct-developing species are relevant to recent proposals concerning the origin of salamander limb regeneration. Galis, Wagner and Jockusch (2003) hypothesize that salamander limb regeneration may be contingent upon post-hatching formation of the limb during larval development and metamorphosis. They propose that limbs of metamorphosing salamanders possess a highly modular developmental autonomy, which allows limb formation well after "pleiotropic constraints" of the highly integrative phylotypic stage during embryogenesis. Such autonomy of limb development would thus constitute a prerequisite for the extensive regenerative abilities of adult salamander limbs. Direct-developing *Plethodon cinereus* is also capable of limb regeneration (Dinsmore & Hanken, 1984; Scadding, 1977; Sessions & Larson, 1987), though its forelimbs form during the phylotypic stage and the hind limbs form only slightly later (Kerney, 2011). It remains to be seen whether or how limb patterning of direct-developing salamanders is governed by the networked regulatory interactions that simultaneously occur within the embryo as opposed to the larvae of metamorphosing salamanders. Future studies of early limb development of *P. cinereus* may reveal the extent to which limb formation has changed during the evolution of direct development and its relation to those features that govern limb regeneration at later stages.

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