

1 An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish

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Comparative analyses of Hox gene expression and regulation in teleost fish support the long-entrenched notion that the distal region of tetrapod limbs, containing the wrist, ankle and digits, is an evolutionary novelty^{1–4}. Data from fossils support the notion that the unique features of tetrapod limbs were assembled over evolutionary time in the paired fins of fish⁵. The challenge in linking developmental and palaeontological approaches has been that developmental data for fins and limbs compare only highly derived teleosts and tetrapods; what is lacking are data from extant taxa that retain greater portions of the fin skeletal morphology considered primitive to all bony fish^{6,7}. Here, we report on the expression and function of genes implicated in the origin of the autopod in a basal actinopterygian, *Polyodon spathula*. *Polyodon* exhibits a late-phase, inverted collinear expression of 5' HoxD genes, a pattern of expression long considered a developmental hallmark of the autopod and shown in tetrapods to be controlled by a 'digit enhancer' region. These data show that aspects of the development of the autopod are primitive to tetrapods and that the origin of digits entailed the redeployment of ancient patterns of gene activity.

The acquisition of hands and feet (autopodia) was a seminal event in tetrapod evolution, facilitating terrestrial invasion and habitation. Digits—elongate, segmented rods with individuated morphologies—have no clear antecedents in extant fish fins, and so the autopod has generally been regarded as a tetrapod innovation. During early stages of teleost and tetrapod appendage development, 5' members of the homeobox A (HoxA) and HoxD gene clusters are expressed in a conserved, spatiotemporally collinear fashion; more 5' genes are progressively activated, and expressed in domains progressively restricted along the proximodistal and anteroposterior axes, respectively. However, at later stages when autopodial elements are being determined and patterned, a distinct tetrapod-specific 'late phase' of 5' HoxD expression is activated with inverted spatial collinearity along the anteroposterior axis. Concomitantly, *Hoxa11* and *Hoxa13* resolve into mutually exclusive domains, with *Hoxa13* expressed throughout the autopod. Late-phase Hox expression is not observed in teleosts, and it has been proposed that these regulatory changes in Hox expression may underlie the origin of the autopod^{1,2}. Indeed, a DNA regulatory element recently identified in mouse controls late-phase autopodial Hox expression, and has been dubbed the 'digit enhancer'; this element has not been found in teleosts³. An alternative hypothesis is that teleosts may have lost or modified portions of an ancestral Hox program that is retained in tetrapods. Morphological data lends support to this notion: teleosts appear to have lost skeletal structures in the fin that are homologous to tetrapod limb bones^{6,7}.

To address these issues, we have analysed genes of the *Sonic hedgehog* (*Shh*) pathway and HoxA and HoxD cluster genes in a basal actinopterygian, the paddlefish *Polyodon spathula*. *Polyodon* possesses

pectoral fin endoskeletal elements considered homologous to both teleosts radials and tetrapod limb bones^{6,7} (see also Supplementary Information). Furthermore, analysis of fin development in *Polyodon* reveals a mosaic pattern of endoskeletal condensation and chondrogenesis, exhibiting aspects of both teleost and tetrapod appendage development (ref. 6 and Supplementary Information). For example, the posterior portion of the fin, the metapterygium, contains branched radials comparable to sarcopterygians such as *Eusthenopteron* and *Tiktaalik*⁵. In addition, the boundary between proximal and distal radials is comparable to the proximal portion of the autopod. In both taxa, a boundary between rod-like endochondral bones and more distal nodular bones transverses the width of the appendage. All of these features are general to many vertebrates, so it may well be that the development of these regions is reflected in common genetic patterns among the tetrapod autopod and different regions of fish fins.

As is expected from analysis of *Shh* expression in teleosts¹ and chondrichthyans⁸, major components of the *Shh* pathway in limbs are conserved in *Polyodon*. *Shh* expression is restricted to a posterior domain comparable to the zone of polarizing activity (ZPA) of model taxa (Fig. 1a). Exposure to retinoic acid (RA) before fin formation (stages 37–39) results in a broadened anterior expression of *Shh* (Fig. 1e, see also ref. 8) comparable to RA-affected zebrafish⁹, but differing from the focused 'ectopic ZPA' described in tetrapods¹⁰. As was predicted, exposure to a cycloamine analogue (SANT-1) inhibited or downregulated *Shh* expression below detectable levels (Fig. 1f) and buds failed to develop a fin skeleton (data not shown). We also assessed expression of the *Shh*-associated transmembrane protein *Ptc1*. Wild-type expression of *Ptc1* is similar to that of *Shh* (Fig. 1b). *Ptc1* expression in RA-affected fins is broadened anteriorly, as is *Shh* (Fig. 1g). In SANT-1 fins, *Ptc1* expression is similarly inhibited or downregulated (Fig. 1h). To support further the interpretation that the appendage *Shh* pathway in *Polyodon* is conserved, we looked at the expression of the transcription factor *dHAND*, thought to be an upstream activator of *Shh*¹¹, and *Gli3*, which is considered a direct intracellular mediator of *Shh*¹². *dHAND* is expressed posteriorly in the mouse limb before *Shh*, but in a broader domain than that of *Shh*/ZPA¹¹, and at later stages is expressed in the interdigital tissue¹³. Mutations in the *Hand2* locus in zebrafish lack anteroposterior patterning in the fin¹⁴. In *Polyodon*, *dHAND* is expressed early in the posterior mesoderm, but then expands anteriorly across the distal bud (Fig. 1c, stages 42 and 43). As in tetrapods¹³, late-stage expression of *dHAND* in *Polyodon* is restricted to the distal-most cells of the bud, in this case to the cells that will form the distal radials (Fig. 1c, stage 46). *Gli3* is expressed throughout the early mouse limb before 5' Hox-mediated expression of *Shh* restricts *Gli3* to the anterior bud¹⁵. In *Polyodon*, *Gli3* is initially expressed throughout the fin bud, and later becomes restricted to distal cells in a similar manner to *dHAND* (Fig. 1d).

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Early *HoxA* expression is remarkably similar in tetrapods and zebrafish. In both, *Hoxa11* and *Hoxa13* exhibit a proximodistally nested collinear expression, with *Hoxa13* the most distally restricted^{1,2,16}. Later expression is markedly different: during the stages of digit specification, tetrapod *Hoxa11* becomes restricted to the zeugopod region and *Hoxa13* becomes restricted to the developing autopod^{1,2}. In zebrafish, *Hoxa11* and *Hoxa13* remain nested and overlapping with no observed proximodistal segregation^{1,16}.

We looked at *Hoxa11* and *Hoxa13* expression in *Polyodon* from onset to cessation of expression to ascertain which of these conditions might be primitive. Early *HoxA* expression is similar to that of tetrapods and zebrafish (Fig. 2a, b; stage 40). *Hoxa11* is expressed through the posterior two-thirds of the fin bud and is also expressed proximally in the dorsal and ventral myoblasts (white arrowhead in Fig. 2a; see also ref. 17). *Hoxa13* expression is restricted to the distal-most

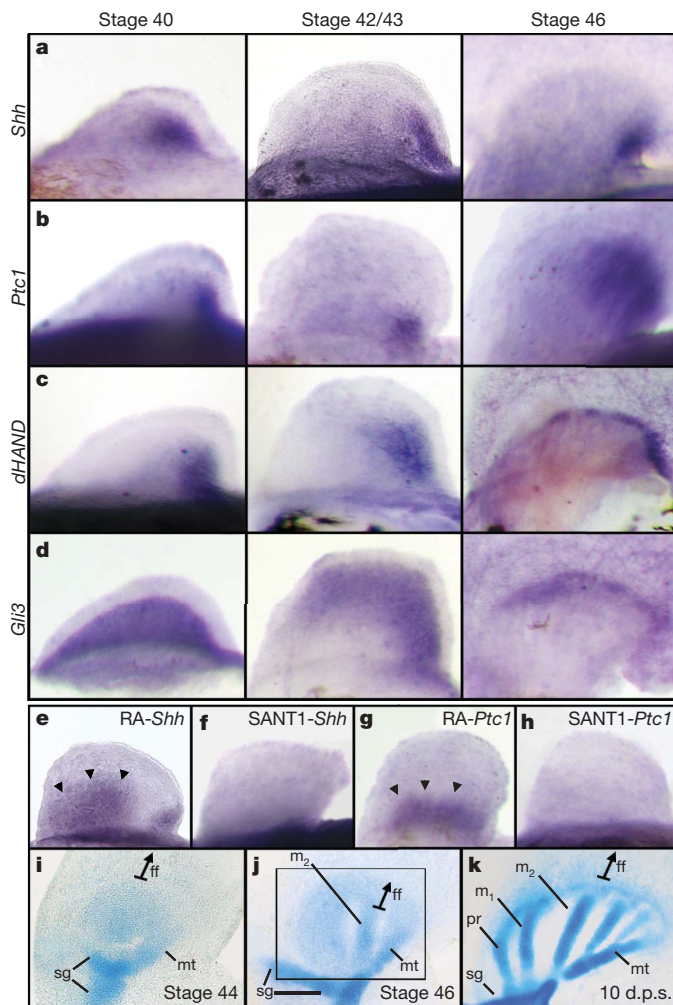


Figure 1 | Expression of *Shh* pathway genes in *Polyodon* pectoral fins by stage. Anterior to left; distal to top. **a–d**, *Shh* (**a**), *Ptc1* (**b**), *dHAND* (**c**) and *Gli3* (**d**). *Shh* and *Ptc1* remain in the posterior fin mesenchyme throughout development. *dHAND* expression is initially posterior (stage 40) but then becomes distally restricted (stage 46). *Gli3* is initially expressed throughout the fin mesenchyme (stage 40) before following a similar pattern of expression to *dHAND*. **e, f**, Exposure to RA (**e**) results in ectopic expression of *Shh* in the anterior two-thirds of the fin (arrowheads), while SANT-1 (**f**) downregulates normal *Shh* expression. **g, h**, Expression of *Ptc1* in animals exposed to RA (**g**) and SANT-1 (**h**) show similar ectopic expression (arrowheads) and downregulation respectively. **i–k**, Alcian Blue skeletal preparations of late larval stages (**i, j**) and a fry at 10 d.p.s. (**k**). See Supplementary Information for a more detailed series. Boxed region in **j** corresponds to stage 46 figures in **a–d**. ff, fin fold; m₁, mesopterygial radial 1; m₂, mesopterygial radial 2; mt, metapterygium; pr, propterygium; sg, shoulder girdle.

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fin bud and is never observed proximally. In subsequent stages, *Hoxa11* and *Hoxa13* become progressively more distally restricted (Fig. 2a, b; stages 44 and 46) and disappear after stage 46. Expression remains nested and overlapping throughout development, with no evidence of tetrapod-like proximodistal segregation.

Tetrapods and zebrafish exhibit a conserved early phase of *HoxD* expression, in which transcripts of the more 5' genes are expressed in a posteriorly restricted nested pattern^{1,18}, that may have evolved in the unpaired fins of early vertebrates¹⁹. In tetrapods, a late phase of *HoxD* expression extends anteriorly across the distal portion of the limb that will form the autopod^{4,18}. Zebrafish appear to lack this late phase of *HoxD* and also lack elaborate distal skeletal structures resembling digits. On the basis of these observations, it has been hypothesized that this late phase of *HoxD* expression is a tetrapod novelty correlated with the evolution of the autopod^{1,16}.

To test this hypothesis, we looked at expression of 5' *HoxD* genes in *Polyodon*. Early-phase expression of *Hoxd11–13* exhibits the same posteriorly nested pattern seen in other vertebrates, with *Hoxd13* being the most anteroposteriorly restricted (Fig. 3a–c; stage 40).

Remarkably, at later stages there is a distally restricted and inverted collinear phase of 5' *HoxD* expression in the fins of *Polyodon* (Fig. 3a–c; stage 46). At this stage, *Hoxd13* expression extends more anteriorly than either *Hoxd11* or *Hoxd12*. Later still (Fig. 3a–c; at ten days post-staging, 10 d.p.s.), 5' *HoxD* expression expands proximally into the region surrounding, but not including, the developing radials (Fig. 3g–i). *Hoxd11* and *Hoxd12* expression extends anteriorly to the inter-radial space between mesopterygial radials one and two (Fig. 3a–c; compare to Fig. 1k), while *Hoxd13* expression extends all the way to the anterior margin of the radial field, demarking the space between mesopterygial radial 1 and the propterygium and the space anterior to the propterygium (Fig. 3c, 10 d.p.s.). Exposure to RA results in ectopic anterior expansion of early-phase *Hoxd11* (Fig. 3d). Late-phase *Hoxd11* expression in RA-exposed embryos is expanded both anteriorly and proximally (Fig. 3e; compare with stage 46 in Fig. 3a), in agreement with the observed *Shh* responsiveness of late-phase *HoxD* in tetrapods¹⁸. Also consistent with results from tetrapods²⁰, SANT-1-mediated inhibition of *Shh* does not affect early-phase *Hoxd11* expression (Fig. 3f).

Polyodon shows a mosaic of primitive and derived features of skeletal pattern, appendage development, and gene activity. All bony fish surveyed to date share a conserved pattern of expression of *Shh* pathway, early-phase *HoxA* and *HoxD* gene expression, and appendage skeletons that can be generalized to an early developing proximal region and a later developing distal region. However, *Polyodon* and

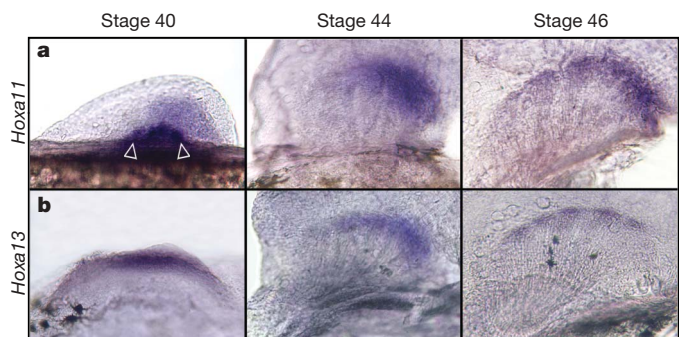


Figure 2 | Expression of *HoxA* genes in *Polyodon* pectoral fins. Anterior to left; distal to top. **a, b**, *Hoxa11* (**a**), and *Hoxa13* (**b**). At early stages (**a**, stage 40), *Hoxa11* is expressed throughout the posterior mesenchyme and strongly expressed in the nascent dorsal and ventral muscle buds (open arrowheads). *Hoxa11* then becomes increasingly restricted to the distal fin bud (**a**, stage 44) and disappears completely shortly after the onset of feeding (**a**, stage 46). *Hoxa13* is restricted to the distal-most fin bud cells from onset of expression (**b**, stage 40) until expression disappears (**b**, stage 46). *Hoxa11* and *Hoxa13* remain nested and overlapping throughout development. Compare stages 44–46 to skeletal development in Fig. 1i and j respectively.

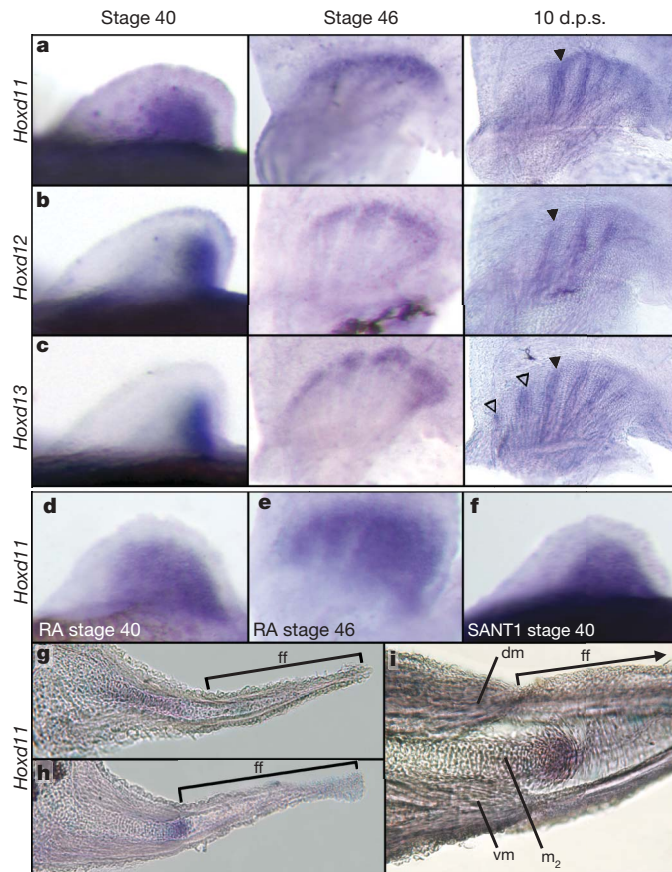


Figure 3 | Expression of HoxD genes in the pectoral fin of *Polyodon*. Anterior to the left; distal to top in **a–f**. Proximal to the left; dorsal to the top in **g–i**. **a–c**, Expression of *Hoxd11* (**a**), *Hoxd12* (**b**) and *Hoxd13* (**c**). Early HoxD expression (stage 40) exhibits the collinear pattern described for other vertebrates. Late-phase HoxD expression is initially restricted to the cells surrounding condensing radials (stage 46) and the nascent distal radials, but then extends proximally to the inter-radial cells (10 d.p.s.). The anterior limit of *Hoxd11* and *Hoxd12* expression is between m1 and m2 (closed arrowheads). In contrast, *Hoxd13* is expressed more anteriorly (open arrowheads). **d–f**, Expression of *Hoxd11* in animals exposed to RA (**d**, **e**) and SANT-1 (**f**). RA slightly upregulates early-phase *Hoxd11* expression (**d**, compare with stage 40 in **a**) and broadly upregulates late-phase *Hoxd11* expression (**e**, compare with stage 46 in **a**). SANT-1 has no effect on *Hoxd11* expression (**f**, compare with stage 40 in **a**). **g–i**, Transverse sections through 10 d.p.s. pectoral fins expressing *Hoxd11*. Expression is restricted to inter-radial (**g**, section at the anterior–posterior level of closed arrowhead in **a**) and in adjacent distal-most cells (**h**, section posterior to the level of the closed arrowhead in **a**). Magnification (**i**) reveals that *Hoxd11* expression is isolated to the mesenchymal cells distal to the proximal radials (red arrows). This region sits within the proximal fin fold and is the site of condensing distal radials (see Supplementary Information). dm, dorsal fin musculature; vm, ventral musculature.

tetrapods share a developmental hallmark that is not observed in teleost fins: an inverted collinear expression of HoxD genes in the distal region of the appendage. Novelty in the appendages of lobe-finned and ray-finned fish has arisen both by changes in regulation, as in tetrapods, and by loss of portions of an ancient and conserved pattern of Hox expression in teleosts.

METHODS SUMMARY

Embryos and staging. *Polyodon spathula* eggs were incubated at 18 °C (12 h:12 h light:dark cycle) in 25% Hank's Balanced Salt Solution (HBSS) until hatching, and grown thereafter at 22 °C. Embryos were staged as described⁶. Alcian Blue cartilage preparations and expression analyses were performed as previously described, respectively^{6,21}.

Polyodon embryos ($n = 100$) were treated with all-trans retinoic acid (1×10^{-6} ; Sigma R2625) in 25% HBSS + 0.5% dimethylsulphoxide (DMSO)

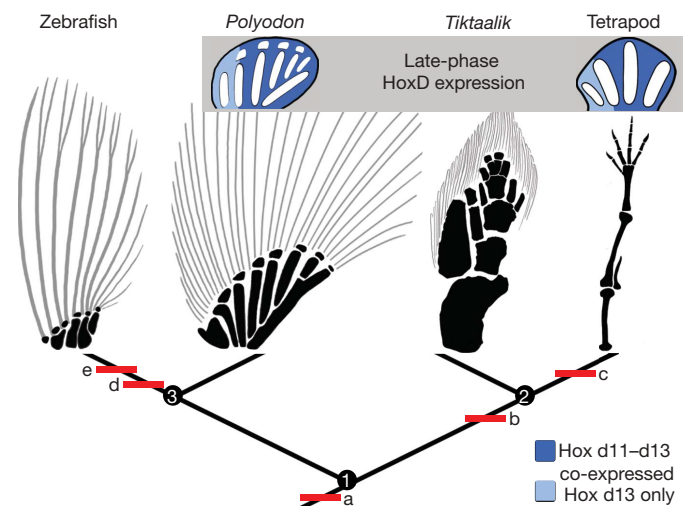


Figure 4 | Conservation of late-phase HoxD expression in bony fish (osteichthyes). Anterior to the left; distal to top. HoxD expression in *Polyodon* supports the notion that late-phase HoxD expression is primitive to tetrapods and to osteichthyes in general. Red bars denote: **a**, acquisition of late-phase HoxD expression; **b**, loss of non-metapterygial radials; **c**, loss of the dermal fin fold; **d**, loss of the metapterygium; **e**, loss of late-phase HoxD expression. Blue cartoons depict conserved late-phase HoxD seen in *Polyodon* and tetrapods (here represented by the hind limb of the chicken, *Gallus*).

for 30 min in the dark at stage 37 (pre-fin budding) or stage 39 (pectoral fin endoskeletal condensation). Embryos were returned to 25% HBSS after several washes to remove residual retinoic acid. Embryos exposed to the *Hedgehog* inhibitor SANT-1 (10 μ M in 25% HBSS + 0.5% DMSO; Calbiochem 559303) were maintained in this solution until harvesting. Control embryos ($n = 100$; 25% HBSS containing 0.5% DMSO) showed no differences from wild-type expression or skeletal pattern. Mortality rates were approximately 50% for both RA and SANT-1 exposure.

Isolation of *Polyodon* genes. *Polyodon* gene fragments were isolated by RT-PCR using the Expand High Fidelity PCR System (Roche) and manufacturer's instructions.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Sequences for *Polyodon Shh*, *Ptc1*, *dHAND*, *Gli3*, *Hoxa11*, *Hoxa13*, *Hoxd11*, *Hoxd12*, and *Hoxd13* are deposited in Genbank with accession numbers: *Shh* (1,006 bp, EF100659), *Ptc1* (1,253 bp, EF527815), *dHAND* (278 bp, EF527816), *Gli3* (1,045 bp, EF534083), *Hoxd11* (486 bp, EF527819), *Hoxd12* (420 bp, EF527820), and *Hoxd13* (863 bp, EF527821), *Hoxa11* (660 bp, EF527817), *Hoxa13* (833 bp, EF52718). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to N.H.S. (nshubin@uchicago.edu).

METHODS

Embryos and staging. Fertilized *Polyodon spathula* eggs were acquired at four days post-fertilization from Osage Catfisheries (Osage beach, Missouri). Larvae were reared (22 °C, 12 h:12 h light:dark cycle) in 25% HBSS and fed *Artemia* from the onset of feeding behaviour (stage 46) until harvesting. Embryos and larvae were staged as previously described^{6,22}. *Polyodon* were euthanized with a lethal dose of MS-222 (tricaine) and fixed for 24 h in 4% paraformaldehyde, dehydrated step-wise into 100% methanol, and stored until use at -20 °C.

Isolation of *Polyodon* genes. Amplified *Polyodon* gene fragments were cloned into PGEM-T Easy Vector (Promega). Primers were designed against conserved regions within the homeobox of *HoxA* and *HoxD* genes and against conserved 5' and 3' regions of Shh-pathway-related genes for mouse (*Mus*), chicken (*Gallus*), zebrafish (*Danio*), and horned shark (*Heterodontus*) available on GenBank.

Whole-mount *in situ* hybridization. These were performed as described²¹ with the following modifications: All washes took place in 1.5 ml Eppendorf tubes with liquid decanted each wash; an additional PBT (phosphate buffered saline + 0.2% Tween-20) wash day (eight washes) was added between anti-digoxigenin incubation and staining; and BM Purple (Roche Labs) was used to develop the signal instead of nitro blue tetrazolium + 5-bromo-4-chloro-3-indolyl phosphate. Specimens were cleared step-wise into 100% glycerol for imaging.

Skeletal preparations. Alcian Blue skeletal preparations were performed as previously described⁶.

Histology. Thick sections were prepared by hydrating whole-mount *in situ* stained specimens in phosphate-buffered saline and embedding in 17% gelatin (dissolved in 10% HBSS at 37 °C). Specimens were then fixed for 24 h in 4% paraformaldehyde, washed in phosphate-buffered saline, and mounted for sectioning. Sections were cut to 30 µm thickness on a Lancer 1000 vibratome, mounted on glass slides, coated with 75% glycerol, and photographed.

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