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Embryonic and post-embryonic HoxA13 expression in the four-toed salamander, Hemidactylium scutatum.

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Embryonic and post-embryonic *HoxA13* expression in the four-toed salamander, *Hemidactylium scutatum*.

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Abstract

The process of somitogenesis occurs during embryological development and results in the definition of persisting axial segments. The four toed salamander, *Hemidactylium scutatum,* exhibits post-embryonic segmentation while most other vertebrates stop segmentation at the end of embryological development. *HoxA13,* a transcription factor expressed along the developing anteroposterior axis, plays a role in specifying caudal segmental identity, cell ingression into the pre-somitic mesoderm (PSM), and PSM truncation. *HoxA13's* responsibilities in influencing embryological development make it an interesting candidate for involvement in post-embryonic segment addition mechanisms. This study explores the role of *HoxA13* in tail segmentation in *H. scutatum* embryonic, larval, and adult tail tip tissues using RT-PCR techniques. The results of this study indicate that *HoxA13* is expressed throughout all life stages. Most importantly, these results suggest that *HoxA13* determines segment identity during post-embryonic tail segmentation as it is expressed in the tips of *H. scutatum* larval and adult tails.

Introduction

Embryonic segments assemble during vertebrate neural development in a process known as somitogenesis. Formation of the pre-somitic mesoderm (PSM) begins at the end of gastrulation when cells ingress from the mesoderm of the gastrula through the blastopore lip. After these cells ingress, they stay at the location they exited as the axis grows posteriorly causing earlier ingressing cells to be located anteriorly and later ingressing cells to be located more posteriorly along the axis. Timing of cell addition to the PSM determines their location on the anterioposterior (AP) axis, which ultimately directs differentiation of somites into the proper axial structures along the developing spinal cord. During later stages of somitogenesis, cells ingress through the tail bud to form posterior PSM, which will give rise to tail segments (Gomez and Pourquie, 2009). Somitogenesis continues in the posterior PSM resulting in the formation of a discrete number of somites unique to each vertebrate species (Gomez *et al.*, 2008). All somites go on to develop three distinct regions, the sclerotome, myotome, and dermatome, which differentiate into derivatives such as skin, bone and skeletal muscle, resulting in the vertebral axis and associated tissues (Ordahl, 1993).

Hox genes direct somite specification by controlling the identity of sclerotomal, dermatomal and myotomal cells within each somite. Each of the 13 *Hox* genes have up to four paralogues (A-D) expressed sequentially along the AP axis (Liang et al., 2011; Pick and Heffer, 2012; Dubrulle et al., 2001; Pollock et al., 1995). More posterior *Hox* genes delay ingression of cells into the tailbud, inhibiting the formation of somites in the most posterior regions AP axis terminating segmenation (Denans *et al.*, 2015). *Hox* genes transcriptionally activate a signaling cascade of genes that signal the specification of segment identity (Mallo *et al.*, 2010). Determination of the most posterior somites involves the *Hox13* paralogues, A-D. Each paralogue has similar

functions; however, expression domain and timing of expression may differ between paralogues. The expression of the *HoxA13* paralogue occurs before the other paralogues and coincides with the termination of PSM elongation (Denans *et al*., 2015; de Santa Barbara and Roberts, 2002). Removal of *Hoxb13* expression in mice result in tail vertebrae that are thicker, longer, and more numerous suggesting that endogenous *Hoxb13* halts axial elongation (Young *et al*, 2009; Economides *et al*, 2003). Gain of function studies in mice show that *Hox13* paralogues act to truncate developing mice tails when expressed early in development. Posterior segments exhibit abnormal morphologies with sacral vertebrae that are thinner and partially fused in addition to complete loss of caudal vertebrae (Young *et al*., 2009). *Hoxc13* and *Hoxd13* loss of function mutations in mice also resulted in the addition of caudal vertebrae, even in *Hox13* heterozygote loss of function mutants (Godwin and Cappechi, 1998). Overall, these results suggest that the *Hox13* paralogues control segment morphology and inhibit posterior segmentation.

Unlike most vertebrates, segmentation in Plethodontid salamanders does not terminate during embryogenesis and continues throughout all life stages (Vaglia *et al*., 1997; Vaglia *et al*., 2010). Previous work suggests that *H. scutatum* embryonic and adult tail tips express the putative tail organizer, *Wnt8* and the myotomal marker gene, *Myf5* (Rossbach, unpublished). Since both of these genes play key roles in embryonic segmentation, these data suggest that similar molecular pathways may drive segmentation throughout all life stages. This study examines *HoxA13* expression in *H. scutatum* tail tip tissues to determine if *HoxA13* expression occurs during embryonic tail development and if so, continues post embryonically in larval and adult stages. *HoxA13* expression occurs in the tail tips of most vertebrates at the end of embryogenesis when segmentation typically terminates. If *HoxA13* plays a role in segment termination in *H. scutatum*, we would not expect to see expression in larval or adult tail tips thus allowing the continued

segmentation of the tail. However, if *HoxA13* is acting to control specification of segment morphology and ingression of cells to the PSM or a PSM-like tissue, expression of *HoxA13* will occur in embryonic, larval, and adult segmenting tails.

Methods

Embryonic and Larval Tissue Collection

Embryos were collected from the George Washington National Forest and reared in Provosoli Medium to stages 22, 24, and 28 in a lab in the Bioscience Building on James Madison University campus (Hurney *et al.,* 2015). Samples were frozen in liquid nitrogen and stored at 80°C. Prior to storage, stage 22 and 24 embryos were manually dissected using sterile scalpels into body and tail tissues. Stage 28 specimens were dissected into head, body, tail base, middle tail, and tail tip. Small, 4cm portions of the tips of adult tails, were sectioned into three parts – tip (1), middle (2), and base (3). Dissected embryonic tissue samples were stored in separate tubes at -80°C.

Total RNA extraction and purification

TRIZOL reagent was added to tissues and homogenized with a power homogenizer. Tissue debris was discarded after centrifugation. Chloroform was used to separate TRIZOL and remaining solid tissue and fat from the aqueous phase. RNA from the aqueous phase was precipitated with ispropanol in the presences of glycogen (2.5 µg/µL). Pellets were washed with 75% ethanol and resuspended in DEPC-treated water. Precipitation with LiCl was performed to eliminate DNA. Samples were stored at -80°C. RNA purity and concentration were analyzed using a BioTek Synergy H1 Hybrid Reader and the Gen5 software.

Primer Design

Primers were designed using a protein and nucleotide consensus sequence, generated using Geneius alignment software, of amphibian HoxA13, HoxB13, HoxC13, and HoxD13 protein sequences. The protein consensus sequence was used to design degenerate primers (Figure 1; Table 1). Primers designed using the protein consensus sequence were then compared to the nucleotide consensus sequence to eliminate unnecessary degeneracy. Primers for RT-PCR were designed using the cloned *HoxA13* sequence (Figure 1; Table 2). All primers were at least 20 nucleotides long and ended in guanine or cytosine with a melting temperature around 60°C.

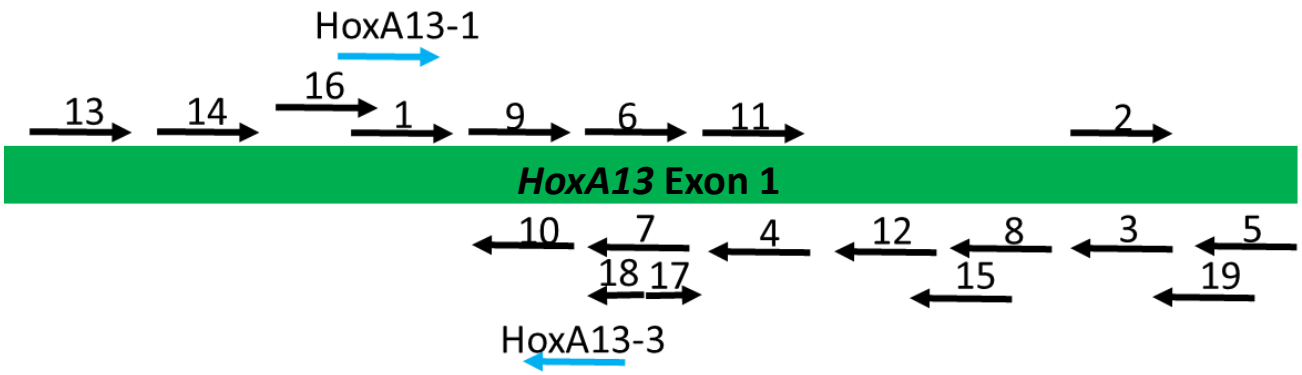

Figure 1. Representation of primer locations. The green box represents exon 1 of the vertebrate *HoxA13* gene. Black arrows represent primers designed based on consensus sequences. Blue arrows represent primers designed from the cloned *H.* scutatum *HoxA13* sequence.

Table 1. *Hox13* cloning primer combinations**.**

Primer Name	**Sequence**	**Degeneracy**
Hox13-1	5’-GCN GAY AAR TAY ATG-3’	32
Hox13-2	5’-CAR GTN ACN ATH TGG-3’	96
Hox13-3	5’-CCA DAT NGT NAC YTG-3’	96
Hox13-4	5’-YTG NCC RTT CCA NCC-3’	64
Hox13-5	5’-YTT YTT YTC YTT NAC-3’	64
Hox13-6	5’-ATG GAR GGN TAY CAR CCN TGG-3’	128
Hox13-7	5’-CCA NGG YTG RTA NCC YTC C-3’	128
Hox13-8	5’- YTT RTT NTG NGC RTA YTC- 3’	256
Hox13-9	5’- GNG CAN ARG ART TYG C-3’	128
Hox13-10	5’- GCR AAY TCY TTN GCN C-3’	352
Hox13-11	5’- GGN TGG AAY GGN CA-3’	32
Hox13-12	5’- NAC YTT NGT RTA NGG-3’	256
Hox13-13	5’- ATG TYY TNT AYG AYA A-3’	16
Hox13-14	5’-GAA GCC GGC AAG CAG TGC-3’	0
Hox13-15	5’-CTC CAG YTC CTT CAG CTG-3’	2
Hox13-16	5’-TTC GCC GAC AAG TAC ATG GAC ACG-3’	0

Table 1. Continued.

Hox13-17	5’-CCC ATG GAG GCC TAC CAG CCC TGG GCC-3’	0
Hox13-18	5’-GGC CCA GGG CTC GTA GGC CTC CAT GGG-3’	0
Hox13-19	5’-GAT GAC CTT TTT CTC YTT GAC-3’	2

Table 2. Primer combinations specific to cloned *H. scutatum HoxA13* sequence.

Primer Name	**Sequence**	**Degeneracy**
HoxA13-1	5’- TGT CCA TGT ACT TGT CGG CG-3’	0
HoxA13-3	5’- CGC TCG GGT GGT GAA CTC-3’	0

First-Strand cDNA Synthesis

Reverse transcriptase was used to synthesize first strand cDNA by priming 1 µg of total RNA at 55°C for 1 hour. Reaction termination occurred at 85°C for 5 minutes. Samples were stored at -20°C for up to 2 weeks.

Polymerase Chain Reaction

All PCR reactions were held at 95°C for 5 minutes followed by 35 cycles of denaturation, annealing, and elongation. PCR parameters varied based on primer annealing temperatures, DNA source, and length of predicted products. RT-PCR reaction conditions were 94°C for 0:15, 55°C for 0:30, and 72°C for 1:30 with a final elongation at 72°C for 30:00. PCR reactions to confirm

the presence of inserts in vectors from *E. coli* stocks were identical except for an annealing time of 0:30 seconds. *Myf5* primers were used as a positive PCR control and were ran separately using the same parameters as above, with the exception of the annealing temperature, which was 60°C. PCR products were stored at 4°C.

Gel Electrophoresis

All PCR products and plasmids were analyzed on 1.2% agarose gels buffered with 1X TAE buffer and run at 130 volts. Gels were stained with Ethidium Bromide (10mg/1mL) and visualized under using a ChemiDoc gel imager.

Ligation, Transformation, and Plasmid Isolation

PCR products excised from 1.2% agarose gels were purified using the Roche High Pure PCR product purification kit. Purified products were ligated into pGEM-T plasmids at room temperature for 2 to 5 days. Following ligation, competent DHα *E coli* cells were transformed with the plasmids plus inserts using heat shock for 90 seconds at 42°C. Cells were revived and allowed to grow in LB broth at 37°C for 90 minutes. Cells were plated on LB/Amp/XGAL/IPTG plates and allowed to grow at 37°C for 48 hours. Colonies that had not developed a blue color were chosen for insert PCR screening. Stocks of the chosen colonies were maintained in LB/Amp broth. The M13 primer combination, which flanks the insert annealing sites on the pGEM-T vector, was used to detect presence of an insert. Plasmids were isolated using standard QIAprep Miniprep Spin Kit.

Sequence Analysis

Isolated plasmids were sent to Eurofins MWG Operon for sequencing via Sanger method. BLAST analysis was used to determine similarity of cloned sequences to known *Hox13* sequences.

DNase treatment

Previously extracted RNA precipitated with LiCl was treated with 1uL RNAse-free, DNase I (1U/µL) for 30 min at 37°C. Reactions were terminated by incubation at 75°C for 10 min.

Results

Cloning of H. scutatum HoxA13

Cloning of *HoxA13* was required to generate primers specific to *H. scutatum HoxA13* for use in RT-PCR. Amino acid analysis of Hox13 paralogues from amphibian vertebrate species revealed consensus areas common among Hox13 paralogues. These were used to generate sets of degenerate primers to amplify a Hox13 paralogue (Figure 1; Table 1). Using a primer pair with no degeneracy, HoxA13-16 and HoxA13-18, a 300 bp band was isolated, cloned into pGEM plasmid vector, and sequenced (Figure 2). The top two results following a BLAST analysis of the sequence of 212 base pairs revealed 90% similarity with portions of the *HoxA13* gene from two Plethodontid salamander species, *Eurycea bislineata* and *Eurycea cirrigera* (NCBI/BLAST, Figure 2). The translated version, 64 amino acids in length, of the clone sequence shares 89% amino acid similarity with both *Eurycea* species' HoxA13 proteins (Figure 3). The third result from the BLAST search showed the nucleotide sequence of the cloned insert also shares 88%

with *HoxA13*, exon 1 from *Desmognathus fuscus* (Figure 2). The amino acid sequence of the cloned gene also shared 89% similarity with *D. fuscus* HoxA13 (Figure 3). There is a substantial gap between the first AA and the area where the majority of the alignment begins when aligned with all three salamander species mentioned (Figure 3).

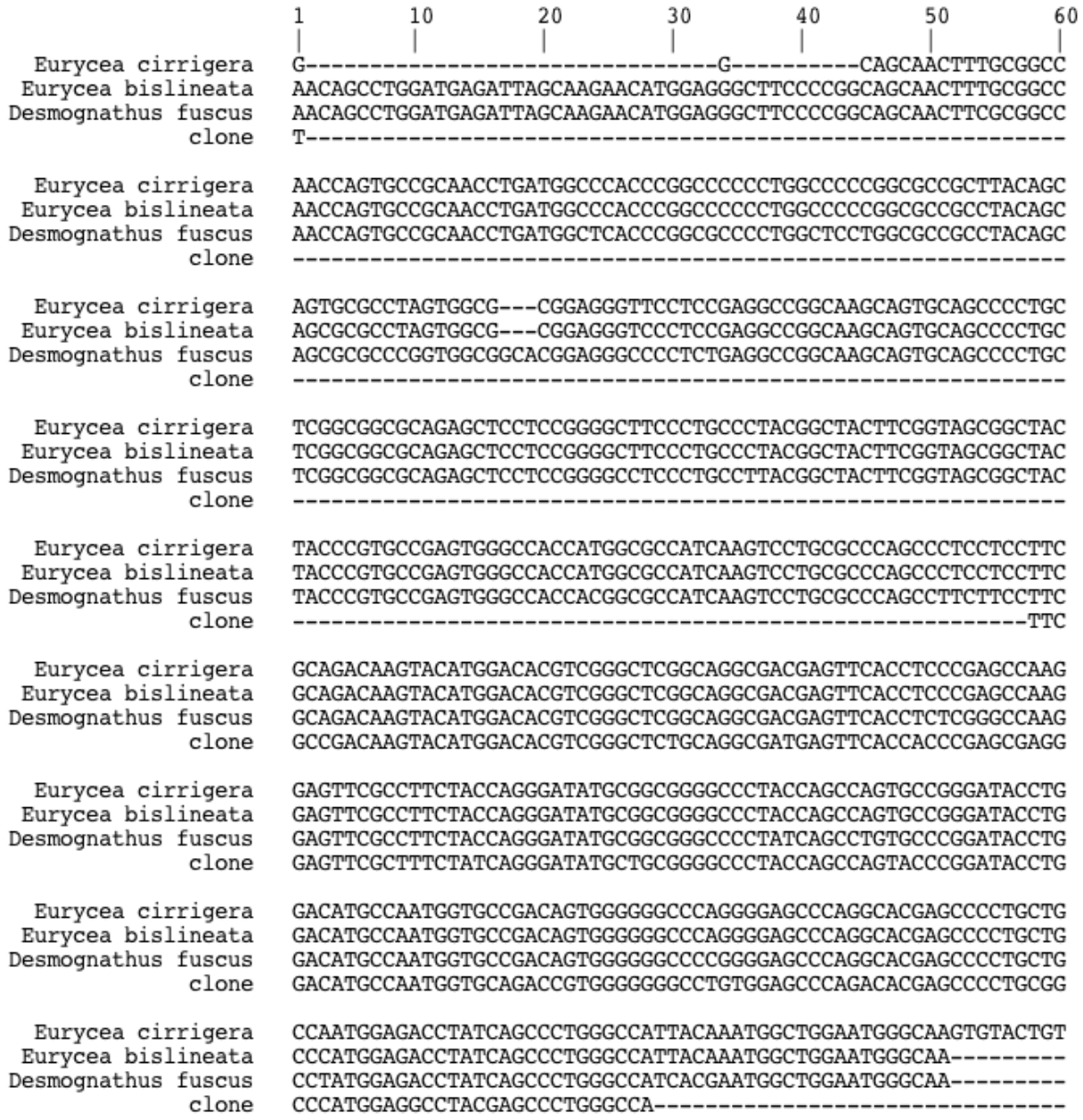

Figure 2. *HoxA13* gene alignment generated using Geneious. The clone nucleotide sequence, 212 base pairs in length, aligned with exon 1 of *HoxA13* from *Eurycea cirrigrea* (90% similarity), *Eurycea bislineata* (90% similarity), and *Desmognathus fuscus* (88% similarity). The lines indicate gaps in the alignment.

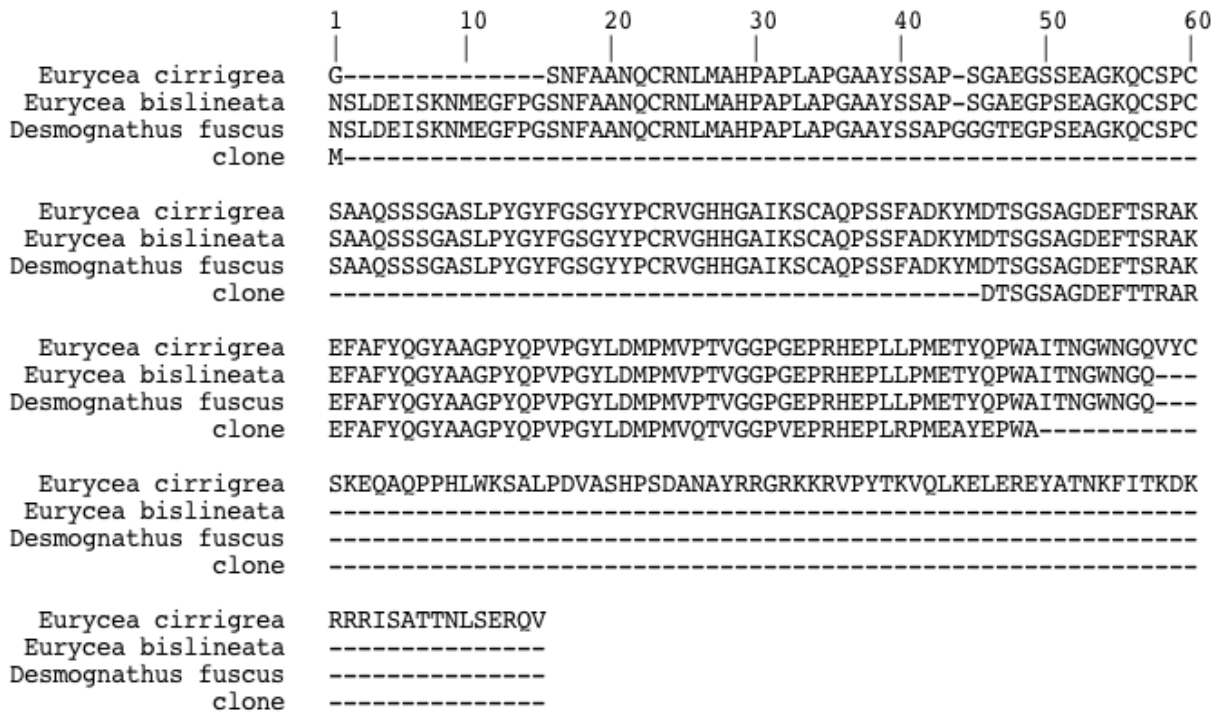

Figure 3. HoxA13 amino acid alignment generated using Geneious. The cloned sequence, 64 amino acids in length, aligned with similar sections of HoxA13 from *Eurycea cirrigrea* (89% similarity), *Eurycea bislineata* (89% similarity), and *Desmognathus fuscus* (89% similarity). The lines indicate gaps in the alignment.

Expression of *HoxA13* during embryonic, larval and adult stages in *H. scutatum*

RT-PCR techniques revealed *HoxA13* expression in *H. scutatum* embryonic, larval, and adult tissues. Primers designed for RT-PCR based on the cloned sequence amplified a 170 base pair sequence from embryonic, larval, and adult tissues. Embryonic stage 22 tail tip and body tissues showed expression of *HoxA13* (Figure 4). Stage 24 tail tip tissues did not exhibit *HoxA13* expression. These results are confirmed by the positive *Myf5* control using St. 24 tail tip tissues (Figure 5). The *Myf5* control for Stage 24 body tissues was negative, indicating denatured St. 24 body cDNA as a cause for a negative experimental result (Figure 5). Larval stage 28 tail tip, tail base, and body tissues all exhibited expression of *HoxA13* (Figure 6). Adult tail tip tissues showed expression of *HoxA13* (Figure 7).

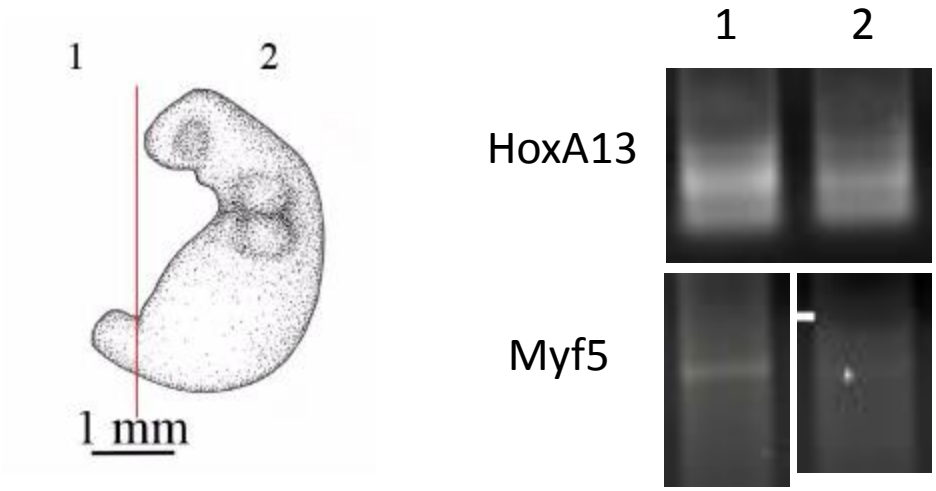

Figure 4. Gene expression in Stage 22 *H. scutatum* embryos. The red line through the embryo tissue diagram indicates dissection line. The top gel row is *HoxA13* RT-PCR55 product and the bottom gel row if *Myf5* RT-PCR60 product. *HoxA13* is expressed in tail tip (Section 1) and body tissues (Section 2). *Myf5* is expressed in the tail tip of *H. scutatum* (Section 1).

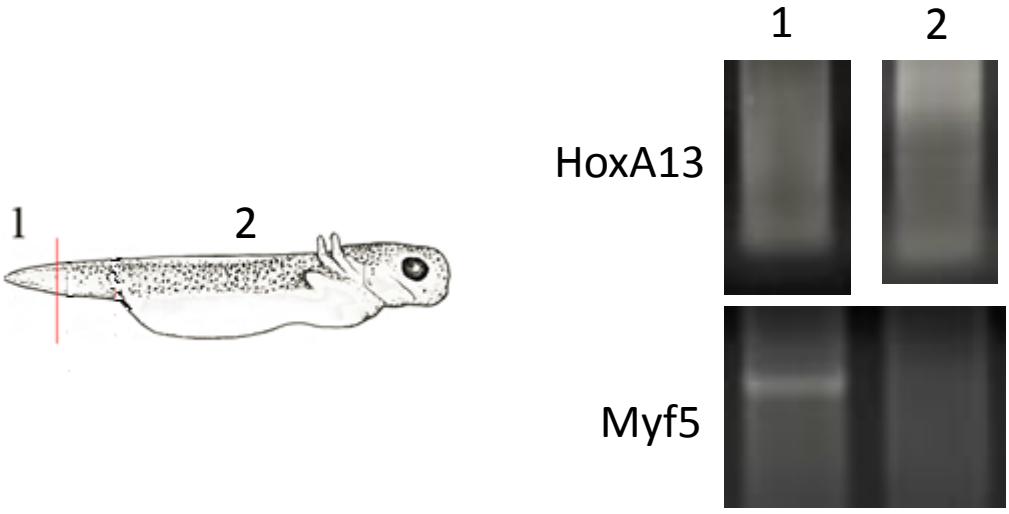

Figure 5. Gene expression in Stage 24 *H. scutatum* embryos. The red lines indicate dissection lines. The top gel row is *HoxA13* RT-PCR55 product and the bottom gel row is *Myf5* RT-PCR60 product. *HoxA13* does not appear in either tail tip (Section 1) or body tissues (Section 2). *Myf5* is expressed in St. 24 tail tissues (Section 1).

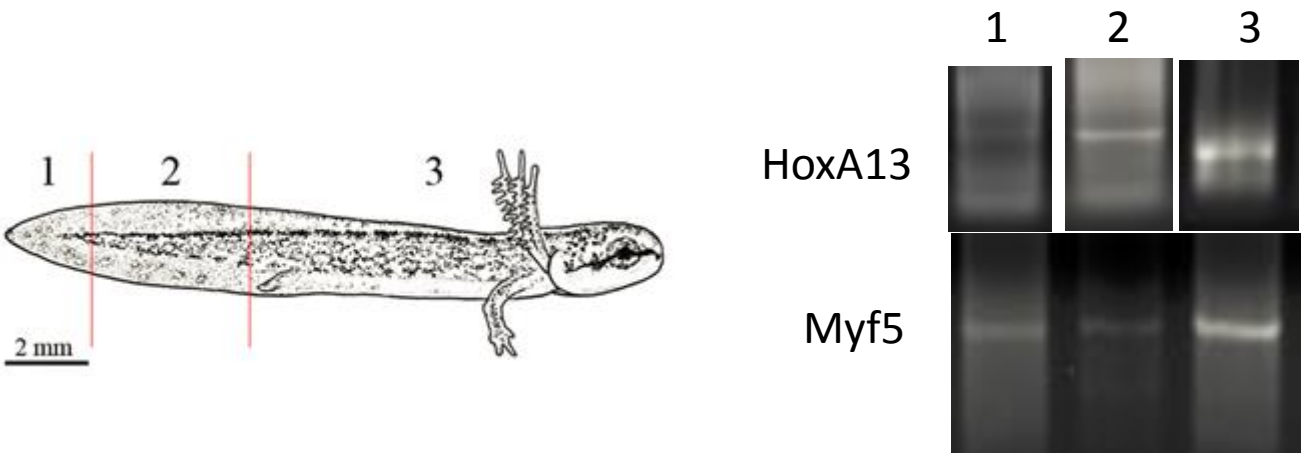

Figure 6. Gene expression in Stage 28 *H. scutatum* larva. The red lines indicate dissection lines. The top gel row is *HoxA13* RT-PCR55 product and the bottom gel row is *Myf5* RT-PCR60 product. *HoxA13* is expressed in all St. 28 tissues (Sections 1-3). *Myf5* is expressed in all St. 28 tissues.

Figure 7. Gene expression in adult *H. scutatum* tail tip tissue. The box indicates the dissected section of the tail. The top gel row is *HoxA13* RT-PCR55 product and the bottom gel row is *Myf*5 RT-PCR60 product. *HoxA13* is expressed in adult tail tips (*HoxA13* section).

Discussion

Cloning of HoxA13

A 212 base pair section of exon one of *HoxA13* was cloned in this study from *H. scutatum* embryonic St. 21 tail tip tissue. St. 21 tail tip tissue was the best candidate location for expression of *Hox13* paralogues based on inferences from typical trends in vertebrate development. Although portions of the *Hox* gene sequences are relatively conserved, the more 5' regions of the gene are typically the most variable and include in a domain on the first exon specific to each *Hox* paralogue (Mann *et al.*, 2009). The cloned gene shared sequence homology with exon 1 of *HoxA13* in many salamander genera including *Eurycea, Desmognathus,* and *Ambystoma*, all of which are Plethodontid salamanders that exhibit segmental addition in all life stages (Babcock *et al.*, 2001; Vaglia *et al.*, 2010). This suggests the presence of a Pletheodontid specific domain in HoxA13 that could be part of the explanation for post-embryonic segmentation in Plethodontid salamanders.

Expression of HoxA13 *in* H. scutatum *Tails*

Expression of *HoxA13* in embryonic, larval, and adult tail tips occurs both embryonically and post-embryonically in *H. scutatum*. Experimental results suggest that *HoxA13* is involved in mechanisms allowing post-embryonic segment addition in Plethodontid salamanders. Body tissue expression in embryonic stage 22 and larval tissue tested was likely due to *HoxA13's* involvement in urogenital and limb development (Imagawa *et al*., 2014). Expression patterns in tail tip tissues revealed in this study were consistent with the expected role of *HoxA13* in specifying caudal most segment morphology and its role during cell ingression to the PSM. Expression patterns found in tail tip tissues were inconsistent with what was expected based on

HoxA13's role in terminating embryonic segmentation. Termination of embryological segmentation implies a halt in the mechanism used for continual segmentation. Therefore, it is interesting that *HoxA13* expression is present in *H. scutatum* embryonic tail tip tissues because *H. scutatum* and other Plethodontid salamanders segment throughout their life. Further study on the activities of *HoxA13* in other Plethodontid salamanders is essential in understanding if *HoxA13* deviates from its traditional role in terminating PSM growth during typical vertebrate embryological development.

Early Embryonic (Stage 22)

HoxA13 expression occurred in embryonic stage 22 tail tip tissue. Early embryonic tail tip tissue was the best candidate for *HoxA13* expression because vertebrate embryological segmentation is taking place as cell ingression is occurring and the morphologies of caudal segments are in the process of being specified. *HoxA13* expression in St. 22 tissues indicates occurrence of typical vertebrate segmentation mechanisms. *HoxA13* expression in tail tip tissues at St. 22 may mark *HoxA13* influencing timing of cell ingression into the PSM and specification of segment morphology.

Late Embryonic (Stage 24)

Expression of *HoxA13* is not present in later embryonic stage 24 tail tip tissue examined. RT-PCR data marks an absence of *HoxA13* mRNA, however, the data cannot determine levels of active HoxA13 protein. One explanation for presence of *HoxA13* in these late embryonic tissues is that HoxA13 in the tail tip reaches a concentration lower than that required for termination of

PSM growth (Woltering, 2012). *HoxA13* expression may be inhibited or stifled to allow for persistence of the PSM in embryonic stage tissues. Examination of *Hoxb13* expression in Axolotl salamanders, a Plethodontid salamander that participates in post-embryonic segmentation, revealed termination of *Hoxb13* tail tip expression just before hatching (Carlson, et al, 2001; Vaglia *et al.*, 2010). Findings from this study may indicate existence of an alteration in *HoxA13* expression pattern relative to typical vertebrate patterns as it relates to *HoxA13's* role in caudal segment identity specification and cell ingression to add to the population of cells creating new segments.

Larval (Stage 28)

Tail tip expression of *HoxA13* in larval, stage 28, tissue following a halt in late embryo tail tissues may be evidence for a post-embryonic segmentation event. Following late embryonic termination of *HoxA13* transcription, a mechanism to turn on *HoxA13* sometime after deactivation may occur. In this way, PSM truncation would be inhibited because of controls on the concentration of *HoxA13*, however, HoxA13 proteins would be replenished to continue segmentation. PSM replacement during hatching may allow for post-embryonic *HoxA13* expression in tail tips. The persistence or absence of somites and PSM tissues has not been studied in Plethodontid salamanders, however, it is expected that tail tip tissue is embryonic in nature. Cells in the tail tip may share a molecular fingerprint with PSM tissue and therefore act in a similar way to allow for post-embryonic segmentation. Expression of *HoxA13* in the base of the larval tail was also observed, which may be due to the dissection location allowing *HoxA13* from the tail tip to extend the tail base tissues. Alternatively some of the *HoxA13* expressed in the urogenital tract may be included in tail base tissues.

Adult

HoxA13 expression was found in adult tail tip and base tissues indicating that *HoxA13* is active post-embryonically and may be active in the mechanism of post-embryonic segmentation. PSM tissues or PSM like tissue comprised of a stem cell population may exist in adult tail tips to allow for a continued segmentation program.

Conclusion

HoxA13 expression is evident in embryonic (St. 22), larval (St, 28), and adult tail tissues and is likely acting to specify segment morphology and to direct cells to developing segments. *H. scutatum* follows the typical program of vertebrate embryonic segmentation during early neurulation but seems to deviate in late embryonic development. Inhibition of axis length truncation could occur through transcriptional regulation of *HoxA13.* Alternative *Hox* gene actions have been characterized and there may be another mechanism for persistence of the PSM or a PSM-like tissue and post-embryonic *HoxA13* expression. Vertebrates that form a large number of segments during somitogenesis, such as snakes and zebrafish, use a reduced number of active *Hox13* paralogues during somitogenesis in an effort to maintain the PSM (Gomez et al., 2008). This allows for a slow accumulation of Hox13 paralogue concentration in the tail tip and therefore a long lived PSM and a larger area for somites to form.

An exception to the posterior dominance principle could allow for *HoxA13* to specify caudal most segments without resulting in PSM, or PSM-like tissue, truncation. When *Hox* gene expression domains overlap, the posterior most *Hox* gene typically dominates over all other *Hox* genes expressed within that tissue acting on that segment (Denans *et al.,* 2015). Exceptions to

this principle posterior dominance exist in invertebrates and are an area of continuing research (Durston, 2012). An exception to posterior dominance in these salamanders overriding Hox13 paralogues ability to initiate truncation of the PSM may be occurring. *Hox12* paralogues could be dominant over *Hox13* paralogues in the truncation mechanism to stop *Hox13* from truncating the PSM, or PSM-like tissue.

Hox13 may also have evolved expression patterns exhibited by other *Hox* genes. A possible explanation for suppressed rib formation in snakes is *Hoxa10* and *Hoxc10* have lost activity in influencing snake rib formation (Di-Poi et al, 2010). Relaxed or altered use of *Hox13* paralogues could allow for prevention of axial truncation in *H. scutatum* and other Plethodontid salamanders.

*HoxA13'*s role in segmental addition is beyond the scope of this study, however, the results point to the fact that *HoxA13* is expressed in *H. scutatum* tissues during the stages the gene is known to be active in embryonic segmentation in common vertebrate development. A better understanding of the mechanisms of axial elongation in Plethodontid salamanders would involve comprehensive study of proteins expressed in embryonic, larval, and adult tail tip and body tissues. This would be achieved via generation of a proteome and a following analysis for proteins, such as HoxA13, that are likely to be involved in embryonic and post-embryonic segmentation.

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