Examining tail development models in the four-toed salamander, *Hemidactylium scutatum*

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Examining Tail Development Models in the Four-toed Salamander, *Hemidactylium scutatum*

A Project Presented to
the Faculty of the Undergraduate
College of Math and Science
James Madison University

in Partial Fulfillment of the Requirements
for the Degree of Bachelor of Science

by Peter Daniel Rossbach

May, 2014

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>4</td>
</tr>
<tr>
<td>Abstract</td>
<td>5</td>
</tr>
<tr>
<td>Introduction</td>
<td>6</td>
</tr>
<tr>
<td>Methods</td>
<td>11</td>
</tr>
<tr>
<td>Results</td>
<td>18</td>
</tr>
<tr>
<td>Discussion</td>
<td>24</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>28</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myf5 primer design</td>
</tr>
<tr>
<td>2</td>
<td>Wnt8 primer design</td>
</tr>
<tr>
<td>3</td>
<td>2 % agarose gel of PCR fragments isolated from stage 24 body tissue</td>
</tr>
<tr>
<td>4</td>
<td>Gene expression in stage 21 <em>H. scutatum</em> embryos</td>
</tr>
<tr>
<td>5</td>
<td>Gene expression in stage 24 <em>H. scutatum</em> embryos</td>
</tr>
<tr>
<td>6</td>
<td>Gene expression in larval <em>H. scutatum</em></td>
</tr>
<tr>
<td>7</td>
<td>Gene expression in <em>H. scutatum</em> adult tail tips</td>
</tr>
<tr>
<td>8</td>
<td>Wnt8 gene alignment</td>
</tr>
<tr>
<td>9</td>
<td>Myf5 gene alignment</td>
</tr>
</tbody>
</table>

Tables

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tissue samples used for total RNA extraction</td>
</tr>
</tbody>
</table>
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Abstract

Tails are common among most vertebrates. The embryonic tails of most vertebrates grow into adulthood while other vertebrates absorb them. Interestingly, some species of salamanders have the ability to add axial segments to their tails post-embryonically. In the salamander, *Hemidactylium scutatum*, postembryonic growth of the tail is due to the development of new caudal vertebrae, as well as the growth of pre-existing segments. The mechanisms that drive the postembryonic segmentation in salamanders are relatively unexplored and may offer insights on the molecular nature of tail development in vertebrates. I found evidence that larval and adult tail growth could be utilizing the same mechanisms for tail segmentation that are used in embryonic tail development by comparing the expression of Wnt8 and My5 in the developing tails of *H. scutatum* embryos, larvae, and adults. Specifically, the expression of Wnt8 in the growing tails suggests that both larval and adult *H. scutatum* continue to segment through the use of a molecular oscillating clock and/or a tail organizer, which induces the tail to undergo somitogenesis and, with the aid of Myf5, develop skeletal muscle in the tail.
Introduction

Despite the fact that many adult vertebrates do not have tails, all vertebrates develop a tail as an embryo. The development and segmentation of these tails depends on the process of somitogenesis, which forms bilateral blocks of mesoderm on each side of the notochord that give rise to much of the skeletal muscle, the dermis, and the cartilage surrounding the spinal chord. While some vertebrates, like humans, absorb their embryonic tails as adults, other species, such as mice, retain and continue the development of their tail into adulthood through the elongation of pre-existing segments. Some species of salamanders, such as the four-toed salamander, *Hemidactylium scutatum*, are not only capable of extending pre-existing segments, but are able to form new segments at the caudal end (Babcock and Blais, 2001; Vaglia et al, 2012). The ability to continually add new segments into adulthood is a fascinating phenomenon and may function through similar mechanisms to those driving segmentation during embryonic development: a topic that has been an area of interest in research for close to a century (Holmdhal, 1925a; Pourquié, 2011)

Research regarding embryonic development of the vertebrate tail first began early in the 20th century when Holmdahl and Vogt proposed two opposing models of embryonic tail development. Holmdahl (1925) suggested that the vertebrate body and tail develop through separate mechanisms, where as Vogt (1926) suggested that the tail develops as a continuation of trunk and body development. In Holmdhal's model, the body and tail develop in two separate phases. During the first phase, primary body development, gastrulation drives the body and trunk to segment from the primary germ layers. In the second phase, secondary development, the tail forms directly from a group of cells, called
the tailbud, located at the caudal limit of the embryo. This tailbud does not incorporate cells originating from the primary germ layers, but rather is composed of a mass of homogenous mesenchymal cells that are directed to differentiate through morphogenetic signals from the apical ectodermal ridge (Handrigan, 2003). Research in mammals and birds support Holmdhal’s theory, as the tailbud is a mass of undifferentiated mesenchymal cells capable of forming somites, the neural tube, and the tail gut (Griffith, 1992; Schubert et al., 2001). This differs from what Vogt originally theorized, which is that the tail develops as a continuation of the trunk and body from the tissues formed during gastrulation.

Pasteel (1943) supported Vogt's idea when he found that a region of the notochord and spinal chord of the tail, called the chordoneural hinge, is the direct descendant of the dorsal blastoporal lip, where the involution and gastrulation of the embryo first occurs (reviewed in Handigran, 2003). Gont et al. further supported Vogt's claim when they followed the expression of two transcription factors, Xbra and Xnot2, in Xenopus from the blastoporal lip into the tailbud (Gont et al., 1993). Fate maps of cells located in the tailbud definitively show that cells contributing to the notochord, ventral spinal chord, and somites are direct descendants from the late blastopore (Gont et al., 1993). This supports that the tail does form from tissues found in the primary germ layers and develops as a continuation of trunk and body development, contrary to what Holmdahl had suggested. The conflict between the two ideas has continued to serve as an area of disagreement in vertebrate development, although more recently, it appears that the tail develops through a process that takes characteristics from both models.

More recent research defines a model that combines ideas from both Holmdhal and Gont, where aspects of tail development are a continuation of trunk development but also
under the influence of signals from the tailbud. A caudally located tail organizer, identified in zebrafish embryos, induces the growth of ectopic tails, which supports part of Holmdhal’s theory (Agathon et al., 2003). Additional studies show that a molecular oscillator drives the segmentation of the body, in all vertebrates, from the trunk towards the caudal limit of the embryo (Purquie, 2011). While this falsifies the claim that tail development is separate from the development of the trunk and body, it supports the hybrid model of tail development that incorporates the process of trunk segmentation under the influence of a posterior inducing tailbud.

Fibroblast Growth Factor (FGF), and the Notch gene families, as well as the canonical Wnt gene family, which regulates the accumulation of the transcription factor, b-catenin, in the nucleus, all play a role in the segmentation clock (Purquie, 2011). Cells residing in the posterior portion of the pre-somitic mesoderm (PSM) remain in an immature and undifferentiated state through the expression of high levels of canonical Wnt and FGF. Cells located in the anterior portion of the PSM, on the other hand, express low levels of Wnt and FGF. With each oscillation of the segmentation clock, cells residing in the PSM move towards the anterior end by a distance of approximately one somite. As cells move towards the anterior end, they reach a point, called the determination front, where FGF signaling is low enough that they become competent to respond to a periodic pulse of Notch issued by the segmentation clock (Dubrulle et al., 2001; Oginuma et al., 2010). The periodic signaling of the segmentation clock drives somitogenesis through the head, trunk, and tail of the developing embryo. The ability of the zebrafish tail organizer to induce the growth of secondary body tissues is dependent on three key genes: bone morphogenetic protein (BMP), Nodal, and, interestingly, the canonical Wnt gene, Wnt8. Ectopic addition of
these genes into early embryos forms secondary tails, while loss-of-function of any of these genes results in defects in tail development (Agathon et al. 2003). Of the three genes, Wnt8 expression is both necessary and sufficient for tail organizer function (Agathon et al., 2003). Thus, the expression of Wnt8 is a molecular marker for both the presence of a tail inducing activity as well as the activity of the somite clock.

Many of the cells that comprise the somite will go on to become skeletal muscle. Myogenic regulatory genes, such as Myf5 and MyoD, activate progenitor cells destined to become skeletal muscle become activated by the expression of myogenic determination genes, of which the canonical Wnts are a positive effector (Buckingham, 2001). In situ hybridization analysis of Myf5 expression in the salamander, Ambystoma mexicanum, reveals that developing somites express the Myf5 gene (Banfi et al., 2012). As such, Myf5 is another molecular marker of somitogenesis.

The possibility of a hybrid model of tail development being used in both embryonic and postembryonic segmentation is supported by the presence of an additional canonical Wnt gene, Wnt10a, in the adult tail of the salamander, Pleurodeles waltl (Caubit et al., 1997). Comparative analysis of the expression of Wnt8 and Myf5 in trunk and tail tissues from embryonic, larval, and adult H. scutatum specimens will further test the hybrid model of tail segmentation and address whether similar molecular processes support segmentation during all life stages. I predict to observe expression of Wnt8 and Myf5 in the posterior tips of developing tails if the tail inducer and/or the somite clock drive segmentation. The expression of Myf5 in the tailbud with no Wnt8 expression would falsify the possibility of the hybrid model functioning in the postembryonic tail, while expression of both transcripts would supports the presence of both the oscillator and the tail organizer.
Finally, the absence of Myf5 in the postembryonic tail tip would support the use of a different myogenic regulatory process during postembryonic muscle differentiation.
Methods

Egg and Adult Tail Tissue Collection:

*H. scutatum* embryos and adult tail tissue were collected from Big Branch Pond #1, located in the James River District of George Washington National Forest (VDGIF Scientific Collecting Permit 026507) and reared in the laboratory at 15°C in artificial pond water (JMU IACUC Protocol A16-12). Embryos were staged using the Normal Table of Embryonic Development in the Four-Toed Salamander, *Hemidactylium scutatum* (Hurney et al., Submitted 2014). Embryos were harvested at Stage 21, when the tailbud first begins to form, and Stage 24, once the tail is established and forming new somites, to test whether the embryos are utilizing Wnt8 and Myf5 in embryonic tail development. Embryos were removed from their embryonic envelope and the tail sections were separated from the trunk using a scalpel (Table 1). Embryo sections were placed into 1.5 ml microcentrifuge tubes, submerged in liquid nitrogen, and stored at -80°C until RNA isolation. Larvae were also harvested to test whether this early postembryonic stage is utilizing Wnt8 and Myf5 as well. Larval tail sections were separated from the body using a scalpel and frozen using the same methods as those used for the embryonic stages.

To collect adult tail tips, adult female *H. scutatum* were placed on top of an ice bath. Using a sharp razor blade, approximately one centimeter of the tail was cut from the caudal end and placed into separate 1.5 ml microcentrifuge tubes and stored on dry ice. Due to the ability of *H. scutatum* to regenerate their tail, this procedure did not cause any permanent harm to the salamanders. Tail segments were later stored at -80°C until RNA isolation, at which point they were cut into three sections (3 mm each) prior to RNA isolation. Only the caudal 3 mm of the tails were used in this thesis.
Total RNA Isolation

Sections of *H. scutatum* embryos (stage 21 and Stage 24), larvae and adult tail tissue (Table 1) were disrupted in TRIZOL reagent by homogenization with a power homogenizer and centrifuged to remove yolk, cellular debris, and other unneeded materials. Chloroform was added and the phases separated by centrifugation. After phase separation, the RNA in the upper aqueous phase was isolated, leaving the lower chloroform layer containing DNA and proteins. The RNA was precipitated in isopropyl alcohol at room temperature and stored at -80°C. The sample containing the tip of the larval tails was purified in lithium chloride solution to concentrate the RNA in this sample due to limited amount of tissue available. RNA was quantified using a BioTek Synergy H1 Hybrid Reader.

<table>
<thead>
<tr>
<th>Stage of development and section of tissue</th>
<th>Number of tissue samples</th>
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</thead>
<tbody>
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<td>Stage 21 Tail</td>
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<tr>
<td>Stage 21 Body</td>
<td>5</td>
</tr>
<tr>
<td>Stage 24 Tail Section 1 (Posterior Half)</td>
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</tr>
<tr>
<td>Stage 24 Tail Section 2 (Anterior Half)</td>
<td>3</td>
</tr>
<tr>
<td>Stage 24 Body</td>
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</tr>
<tr>
<td>Larval Tail Section 1 (Tail tip)*</td>
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</tr>
<tr>
<td>Larval Tail Section 2 (Anterior Tail)</td>
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<tr>
<td>Larval Body</td>
<td>1</td>
</tr>
<tr>
<td>Adult Tail Tip (Posterior 3mm)</td>
<td>3</td>
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</tbody>
</table>

Table 1. Tissue samples used for total RNA extraction. Embryos were staged according to “Normal Table of Embryonic Development in the Four-Toed Salamander, *Hemidactylium scutatum* (Hurney et al., Submitted 2014). The stage and section of each tissue sample is listed along with how many salamander samples were used for each. Samples were homogenized using TRizol and phases were separated using chloroform.
**Primer Design**

Primers were designed to be between 17 and 24 base pairs in length and to have a melting temperature above 60°C. Primers were designed from known sequences of *Hs-Myf5* and *Hs-Wnt8* to be used in PCR reactions (Lee, 2008; Burton, 2011). Primers were designed to span an intron to ensure that any fragments of mRNA that were transcribed would be distinguishable from any possible DNA contaminants remaining from the RNA isolation (Figure 1 and 2). Using these primers a fragment length of 316 base pairs was predicted for Wnt8, and a fragment length of 447 base pairs was predicted for Myf5 (Figure 3).
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>Myf5 1F</td>
<td>5’- CGAGGGGCTTGCCAGGTTCC - 3’</td>
</tr>
<tr>
<td>Myf5 E2R1</td>
<td>5’- ACTGTTGTTCTCCTGT TTCC - 3’</td>
</tr>
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![Myf5 Primer Diagram]

Figure 1. Myf5 primer design. Primers are based on the previously cloned sequence of *Hemidactylium scutatum* Myf5. The Myf5 gene is shown above. Boxes represent the exons and lines represent the introns.

<table>
<thead>
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<th>Primer Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Wnt8 3F</td>
<td>5’- AACTGCGGGCTGCAGACTCCAG - 3’</td>
</tr>
<tr>
<td>Wnt8 1R</td>
<td>5’- GCGCCTGGTCTGTCTTGAT - 3’</td>
</tr>
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</table>

![Wnt8 Primer Diagram]

Figure 2. Wnt8 primer design. Primers are based on the previously cloned sequence of *Hemidactylium scutatum* Wnt8. The Wnt8 gene is shown above. Boxes represent the exons and lines represent the introns.
Figure 3. 2 % agarose gel of PCR fragments isolated from stage 24 body tissue. The gel shows a cloned 316 base pair Wnt8 fragment in lane 1 produced by priming with the Wnt8 3F and Wnt8 1R primers, and a 447 base pair Myf5 fragment in lane 4 produced by priming with the Myf5 1F and Myf5 E2R1 primers. Lanes 2 and 5 are –RT controls of both the Wnt8 and Myf5 respectively. Lane 3 contains a 100 base pair ladder.
First-Strand cDNA Synthesis

First-strand cDNA was synthesized with reverse transcriptase by priming 0.5 μg of *H. scutatum* total RNA at 55°C for 1 hour for all stages except the larval tissues, which used 1 μg of *H. scutatum* total RNA. This discrepancy was due to limited tissue samples. Negative controls were performed without the addition of reverse transcriptase to ensure that only mRNAs were being detected rather than chromosomal DNA contaminants. Reactions were terminated at 85°C for 5 minutes and samples were stored at -80°C.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify portions of the *H. scutatum* Myf5 and Wnt8 genes using appropriate primer pairs (Figure 1 and 2) from first strand cDNA reactions described above. PCR reactions were initialized at 95°C for 5:00 minutes. 35 cycles of degeneration, annealing, and elongation were performed with the following parameters respectively: 94°C for 0:15, 60°C for 0:30, and 72°C for 1:30. A final elongation was performed at 72°C for 30:00. PCR reactions for bacterial colonies had identical cycling parameters but were annealed at 50°C. All samples were stored at 4°C following reaction.

Gel Electrophoresis

All PCR products were analyzed using 2% agarose gels buffered with TAE buffer. All gels contained 1 μl of ethidium bromide to allow visualization of PCR products. Gels were run at 250 volts and 300 milliamps for approximately 15 minutes.
Ligation, Transformation, and Plasmid Isolation

DNA fragments of interest were excised from the agarose gels using a sterile razor blade. DNA was purified from the gel slices using High Pure PCR Product Purification Kit (Roche Applied Science). DNA isolated from gel slices was ligated into the pGEM-T vector using T4 DNA ligase at room temperature for 24 hours. A portion of the ligation reaction was transformed into competent E. coli bacteria using heat shock at 42°C for 90 sec followed by the addition of 1 mL of LB broth and incubation at 37°C. A portion of these cells were plated on LB/ampicillin (100 µg/mL) plates and allowed to grow at 37°C overnight. Individual colonies were screened using PCR to detect insert length using SP6 and T7 primers that flank the multiple cloning sites in the pGEM-T vector. Cultures were grown from bacteria with inserts of interest in LB/ampicillin (100 µg/mL) and plasmids were isolated using standard QIAprep Miniprep Spin Kit.

Sequence Analysis

Sequencing of isolated plasmids containing inserts of interest was obtained by sending plasmids containing PCR products to Eurofins MWG Operon. BLAST analysis (http://www.ncbi.nlm.nih.gov) and DNASTar software alignment tools revealed sequence similarity of PCR products obtained using Myf5 and Wnt8 primer pairs to vertebrate genes and to the known H. scutatum Myf5 and Wnt8 sequences.
Results

RT-PCR

RT-PCR assays were performed to investigate the expression of Wnt8 and Myf5 in the body and tails of early tailbud stage embryos (Stage 21), late tailbud stage embryos (Stage 24), larvae (Stage 28) and adult tail tips. RT-PCR for Stage 21 embryos revealed that both Wnt8 and Myf5 are expressed in both budding tailbud (Section 1), and the body tissues (Section 2) (Figure 4). Expression pattern of Wnt8 and Myf5 in stage 24 tails (Sections 1 and 2) and body tissue (Section 3) was similar to the expression in samples from Stage 21 embryos. Specifically, both Wnt8 and Myf5 were expressed in the body, and both sections of the developing tail during these embryonic stages (Figure 5). Myf5 transcripts were detected in the body (Section 3) and all tail sections (Sections 1 and 2) of the larvae as well, but Wnt8 was expressed only in the tail (Sections 1 and 2) (Figure 6). Finally, the caudal tip (3 mm) of *H. scutatum* adult tails express both Wnt8 and Myf5. Adult body tissues were not collected for this round of experiments (Figure 7).
Figure 4. Gene expression in stage 21 *H. scutatum* embryos. Both Myf5 and Wnt8 are expressed in both the tailbud (Section 1) and the body (Section 2). Red lines signify where dissections occurred for RNA isolation.

Figure 5. Gene expression in stage 24 *H. scutatum* embryos. Myf5 and Wnt8 are both expressed in both the anterior tail (Section 1) and posterior tail (Section 2) as well as the body (Section 3). Red lines signify where dissections occurred for RNA isolation.
Figure 6. Gene expression in larval *H. scutatum*. Wnt8 is expressed in both the anterior tail (Section 1) and posterior tail (Section 2). Myf5 is expressed in the anterior tail (Section 1) and the posterior tail (Section 2), as well as the body (Section 3). Red lines signify where dissections occurred for RNA isolation.

Figure 7. Gene expression in *H. scutatum* adult tail tips. Wnt8 and Myf5 are expressed in the most caudal 3 mm of the tail of adult tails.
**DNA Sequencing**

RT-PCR was performed using primers designed from the known *H. scutatum* sequences of Myf5 and Wnt8. Following gel electrophoresis (Figure 1), the expressed bands were excised, purified, and sequenced to confirm the identity of the genes. Nucleotide alignment analysis confirmed that the 316 base pair fragment aligned with close to 100% similarity to cloned portions of *H. scutatum* Wnt8 gene (Burton, 2010; Figure 8) and the 447 base pair fragment aligned with close to 100% similarity to cloned portions of the *H. scutatum* Myf5 gene (Lee, 2008; Figure 9). The Wnt8 PCR fragment also shows 82% similarity to the *Cynops pyrrhogaster* Wnt8 gene and the Myf5 PCR fragment shows 92% similarity to the *Neomyia virdescens* Myf5 gene.
Figure 8. Wnt8 gene alignment. The 316 bp Wnt8 fragment cloned in PCR was aligned with the known *H. scutatum* Wnt8 gene. The two showed nearly 100% identical sequences.
Figure 9. Myf5 gene alignment. The 447 bp Myf5 fragment cloned in PCR was aligned with the known *H. scutatum* Myf5 gene. The two showed nearly 100% identical sequences.
Discussion

Evidence of the Hybrid Model of Tail Segmentation

My results support that a hybrid tail development model executes tail segmentation, growth and differentiation via the molecular cues provided by the somite clock and posterior inducer. The oscillating molecular somite clock functions in vertebrate embryos to periodically form somites and segment the trunk, body, and tail (Pourquié, 2011). The clock functions through the use of many genes with cyclical expression patterns. Among these the FGF, Notch, and the canonical Wnt families of genes play the key roles. For the cells in the posterior PSM to remain in an undifferentiated state preceding somite development, high levels of FGF and Wnt are expressed. High levels of Wnt expression is also a feature of the tail organizing function found in zebrafish. Thus, my results demonstrating that Wnt8 and Myf expression is present in all developing tails supports the hybrid model of tail development and lends insight into the molecular mechanisms driving vertebrate adult tail segmentation.

Given that the molecular oscillator drives embryonic segmentation in many vertebrates (Pourquié, 2011), it is likely this same mechanism drives embryonic segmentation in *H. scutatum*. Wnt8 expression observed in the tail tips of both stage 21 (Figure 4) and stage 24 embryos (Figure 5) is consistent with the molecular segmentation clock, under the control of canonical Wnts, working to form somites during embryonic trunk and tail segmentation in *H. scutatum*. The expression of Wnt8 in the embryonic tails of *H. scutatum* also supports that a posterior inducer assists with tail segmentation and development as observed in Zebrafish, where Wnt8 expression is both necessary and sufficient for tail organizer function (Agathon et al., 2003). If the tails of *H. scutatum* retain
the use of the tail organizer into the larval and adult stages of life, then the expression of Wnt8 within the tails of these later life stages would be expected. Again, my results are the first report of Wnt8 expression during vertebrate postembryonic tail segmentation (Figure 6 and 7), and together with the expression of Wnt10a in Pleurodeles waltl (Caubit et al., 1997), this offers new insights into the molecular aspects of vertebrate segmentation in adults and the possible conservation of molecular cues driving this developmental phenomenon.

Myf5 expression in the tips of H. scutatum embryonic, larval and adult tails (Figure 6 and 7) also supports the hybrid model of tail development. If a molecular oscillating clock controls postembryonic somitogenesis and segmentation, then Wnt8 expression would most likely drive the molecular clock to activate myogenesis by activating myogenic genes like Myf5. *In situ* hybridization analysis of Myf5 embryonic expression in the salamanders, Ambystoma mexicanum and H. scutatum, shows high levels of expression in trunk and tail somites (Banfi et al., 2012; Hurney and Konen, unpublished data). Thus, like somitogenesis within the embryo, somitogenesis in the larval and adult tails utilizes Myf5 to induce a myogenic fate within muscle precursor cells. Expression of Myf5 in the adult tail tips could also result from the posteriorizing influence of Wnt8 (Figure 7).

Although the expression of Wnt8 and Myf5 in the tail throughout all life stages in H. scutatum lends support to the hypothesis that postembryonic tails segment using the same mechanisms used during embryonic somitogenesis, an alternative explanation for the expression of Wnt8 and Myf5 also exists. It is possible that the Wnt8 expressed in larval and adult tail tissues is being expressed to repair any damaged muscle cells that may be present. Studies in adult mice show that while there is no detectable evidence of Wnt
signaling in uninjured muscle tissues, most myogenic progenitors in regenerating muscle signal through Wnt pathways (Barack et al., 2007). Similarly, Myf5 expression in adult tails could be due to the expression of Myf5 in stem cell populations present in adult vertebrates (Biressi et al., 2013). It is possible that muscle regeneration in *H. scutatum* utilizes Wnt pathways similar to those in mice, and if this is true, any injured cells within the tail could be actively expressing Wnt8 resulting in the activation of Myf5. While no visible damage was observed on the adult tail tips at the time of collection, it could be possible that some cells within the tissue were damaged and the muscle was undergoing regeneration, resulting in Wnt8 and Myf5 expression.

**Further Investigation**

Further research comparing the genes expressed during adult tail segment formation with genes involved in embryonic segmentation will extend our understanding of molecular mechanisms that keep the segmentation process working through adult life stages. Specifically, the examination of FGF, and Notch could be particularly insightful to the operation of a molecular operator, while the examination of Nodal and BMP could help to explore whether the tail organizer is working within the adult tails. The generation of a transcriptome containing sequences of all genes expressed in embryonic, larval, and adult tails of *H. scutatum* will facilitate this analysis by offering computing solutions to searching and comparing sequences that can give us information as to whether these, and many other possible genes, are being expressed within the post-anal tail.

Finally, it would be helpful to analyze the expression of tail segmentation genes in vertebrate tails that do not continue the segmentation process, such as mice or lizards. By comparing the expression of genes expressed in the tails of *H. scutatum* against that of a
non-segmenting vertebrate, we may identify genes only expressed by segmenting tails, thus adding support to the possibility a molecular oscillator and tail organizer function to drive postembryonic somitogenesis in vertebrates.
Literature Cited


