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Uncoiling the Gut of Eleutherodactylus coqui: Characterization of the Anatomical Development and Proliferation Pattern

Carrie Elizabeth Langer

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Uncoiling the Gut of *Eleutherodactylus coqui*: Characterization of the Anatomical Development and Proliferation Pattern

A Thesis
Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements
For the Degree of Masters of Science

By

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Thesis Title: Uncoiling the Gut of *Eleutherodactylus coqui*: Characterization of Anatomical Development and Proliferation Pattern

Degree: Masters of Science

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ABSTRACT

Among the frogs there are two forms of development: biphasic and direct. *Xenopus laevis*, a biphasic developer, develops from an embryo into a tadpole, and then metamorphoses into an adult frog. *Eleutherodactylus coqui* is a direct developer, meaning that it lacks a tadpole stage. The embryo develops directly into the adult form. Comparing development between these two frogs provides insight on the evolution of these groups. I present an anatomical description of the gut development of *E. coqui*, and compare my findings to the development of *X. laevis*. *E. coqui* does not have a highly coiled gut like that of *X. laevis* tadpole, and the composition and gut size differs from *X. laevis* as well. In addition, I examined the proliferation pattern in the *E. coqui* gut by immunocytochemistry. The gut of *X. laevis* has a directional proliferation pattern during metamorphosis. The gut of *E. coqui* does not have this proliferation pattern. Instead, the anterior and posterior tubes have high levels of proliferation throughout development, and the other regions of the gut exhibit lower and variable levels of proliferation. I also examined the proliferation pattern of *X. laevis* tadpole intestines during metamorphosis, induced in pre-feeding tadpoles (NF 46/47) with exogenous thyroid hormone. Thyroid hormone induces precocious remodeling as shown by shortening of the intestine and proliferation in selective regions.
ACKNOWLEDGEMENTS

It is said that it takes a village to raise a child, well the same can be said for creating a thesis. Here is my village:

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INTRODUCTION

Direct Development ..................................................1
Biphasic Development ..............................................3

Eleutherodactylus coqui .............................................4
Origin and Development ...........................................4
Hormonal Control ....................................................6

Gut Formation .......................................................7
General Amniote ....................................................7

Xenopus laevis ..........................................................8
Endoderm Specialization and Regionalization ...................8
Metamorphic Remodeling of the Gut ..............................9
Small Intestine ......................................................10
Stomach .............................................................11

Hormonal Regulation of the Remodeling Gut ..................11

Thesis Objectives ...................................................14

MATERIALS AND METHODS .......................................15

Animals and Embryos ..............................................15

Xenopus laevis ......................................................15
Eleutherodactylus coqui .............................................16

Tissues: Treatments, Dissections, and Fixations ...............16

Xenopus laevis ......................................................16
Thyroid Hormone Treatment and BrdU Injections ...............16
Fixations ............................................................17
Dissections ..........................................................18
Tadpole Tail Removal ..............................................18
Colchicine Treatment ..............................................19

Eleutherodactylus coqui .............................................19
Anatomical Characterization of Gut Development ...............19
Fixation ..............................................................20

Sectioning and Embedding .......................................21

Immunocytochemistry and DAPI staining .......................22
Anti-BrdU ............................................................22
Anti-phospho histone H3 ...........................................22
DAPI Staining .......................................................23
### Mounting and Visualization

- *Xenopus laevis* ................................................................. 24
- *Eleutherodactylus coqui* .................................................. 24

### Statistical Analysis

- ................................................................. 25

### RESULTS

- Assaying cell division in frog embryos .................................. 28
- Thyroid Hormone Induced Remodeling of *X. laevis* Small Intestine .... 29
- Anatomical Description of *E. coqui* Gut development .................. 31
- Proliferation Pattern of the developing Gut of *E. coqui* ............... 34

### DISCUSSION

- Thyroid Hormone Induction of Intestinal Remodeling .................. 48
- Comparisons Between Anatomical Gut Development of *E. coqui* and *X. laevis* ................................................................. 50
  - Coiling and Size .................................................................. 50
    - *X. laevis* .................................................................... 51
    - *E. coqui* ..................................................................... 51
  - Composition ........................................................................ 52
- Proliferation Pattern Comparisons Between *E. coqui* and *X. laevis* .. 54
- Future Directions ................................................................. 57

### REFERENCES

- .................................................................................. 59

### APPENDIX

- .................................................................................. 62
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1:</td>
<td>Diagram of <em>E. coqui</em> Gut Dissection</td>
<td>26</td>
</tr>
<tr>
<td>Figure 2:</td>
<td><em>X. laevis</em> Intestinal Measurements</td>
<td>27</td>
</tr>
<tr>
<td>Figure 3:</td>
<td><em>X. laevis</em> Tail Regeneration Experiment</td>
<td>37</td>
</tr>
<tr>
<td>Figure 4:</td>
<td>Four Day T₃ Treated <em>X. laevis</em> Intestine</td>
<td>38</td>
</tr>
<tr>
<td>Figure 5:</td>
<td>Effects of T₃ on Intestinal Proliferation</td>
<td>40</td>
</tr>
<tr>
<td>Figure 6:</td>
<td>Diagram of <em>E. coqui</em> Gut Development</td>
<td>41</td>
</tr>
<tr>
<td>Figure 7:</td>
<td>Proliferation Pattern of a TS 12 <em>E. coqui</em> Gut</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8:</td>
<td>Proliferation Pattern of a TS 15 <em>E. coqui</em> Gut</td>
<td>43</td>
</tr>
<tr>
<td>Figure 9:</td>
<td>Proliferation Pattern of a TS 15₃ <em>E. coqui</em> Gut</td>
<td>44</td>
</tr>
<tr>
<td>Figure 10:</td>
<td>Proliferation Scale for <em>E. coqui</em> Gut Development</td>
<td>45</td>
</tr>
</tbody>
</table>
LIST OF TABLES

**Table 1**: Statistical Analysis of *X. laevis* Intestinal length .................. 39
**Table 2**: Average Levels of Proliferation of *E. coqui* Gut ..................... 46
**Table 3**: Comparisons of Gut Development between
*X. laevis* and *E. coqui* ................................................................. 47
INTRODUCTION

Among the frogs, the anuran amphibians, there are two main modes of development: biphasic and direct. Biphasic development consists of two transformations. The fertilized egg forms a motile feeding larva, and the larva undergoes a remodeling process, known as metamorphosis, to become an adult (Jennings and Hanken, 1998). Direct developers do not have a larva stage. The egg provides the necessary nutrition to sustain life, and upon hatching, an adult form emerges (Callery et al., 2001). Direct development is believed to be a derived state (Fang and Elinson, 1996). Comparing different aspects of development, such as RNA localization, limb formation, and gut development, between the two developmental modes provides insight into evolution within anurans.

I. **Direct Development:**

Direct development is widespread among anuran amphibians. The tadpole stage has been completely eliminated from the life cycle, and the embryo completes development via oogenic energy sources (Thibaudeau and Altig, 1999). In direct developing anurans, such as *Eleutherodactylus coqui*, the only obvious larval features are two pairs of small gills present for less than one third of development and a tail. The tail is believed to be involved in respiration and completely degenerates after hatching (TS 15) (Thibaudeau and Altig, 1999; Callery et al., 2001). The tail bud forms at Townsend and Stewart (TS) (1985) 4 and elongates from TS 6-10. Also during this time tail fins develop. The fins regress at TS 12, and are reabsorbed two days after hatching. It is unknown how direct development evolved (Callery et al., 2001). Terrestrial adaptation of certain frogs has been linked to a decrease in water. This environmental pressure has changed the way
frogs lay their eggs. Some frogs lay their eggs in small collections of water, such as in basins. Other frogs cover their eggs with foam to protect them from dehydration and predation (Lamotte and Lescure, 1977). Callery et al. (2001) postulated that when environmental pressures on larvae increase, shorter larval periods evolved concurrent with developmental modifications. The larval periods eventually disappeared in some frog species.

A noticeable developmental modification, believed to arise from terrestrial adaptation, is an increase in egg size. For example, *Eleutherodactylus coqui* possesses a 3.5 mm diameter egg, which is twenty times larger than that of *Xenopus laevis*, a biphasic developer (Callery et al., 2001). On average, direct developers have larger eggs than biphasic developers; however, there are some direct developers that have small eggs, such as *Sooglosus gardineri* (1.8 mm) (Callery et al., 2001). Because the fertilized eggs are so large and full of yolk, cleavages that cut through the vegetal hemisphere are slow. As a result, there are many complete cells at the animal pole before the cleavages extend vegetally (Ninomiya et al., 2001)

Accompanying an increased egg size is an increase in vegetal cytoplasm (Callery et al., 2001). Endoderm forms from the yolk cells at the vegetal pole of the blastula in *X. laevis* (Clements and Woodland, 2003). Arendt and Nübler-Jung (1999) hypothesized that an increase in vegetal cytoplasm led in evolution to an expansion in nutritive endoderm in contrast with the definitive endoderm. Nutritive endoderm only serves as a nutrient source for the embryo. Definitive endoderm will ultimately form the gut, liver, pancreas, thyroid gland, and thymus. Elinson and Beckham (2002) speculated that the expansion of nutritive endoderm was significant in the evolution from anamniote
development, characteristic of amphibians, to amniote development, characteristic of reptiles, birds, and mammals.

Direct developers also lack tadpole specific structures, particularly those involved in feeding. Lateral line organs for motion detection as well as cement glands needed for surface adhesion are absent in direct developers (Callery et al., 2001). The jaw muscles and teeth are not as specialized in the tadpole stage of biphasic developers. Furthermore, Smith et al. (2000) speculated that the gut of the direct developer lacks the extensive coiling in response to yolk as the main source of nutrition as compared to algae and debris consumed by biphasic herbivores.

Direct development is only present in nine out of the twenty-one anuran families (Duellman and Trueb, 1986). *Leiopelma* and *Eleutherodactylus* are two examples of genuses containing direct developers (Stephenson, 1951). *Eleutherodactylus* is perhaps the most well studied genus consisting of 500 members, all of whom are direct developers (Callery et al., 2001).

II. **Biphasic development:**

Biphasic development is a successful life history occurring throughout the animal kingdom (Callery et al., 2001). The first wave of development transforms the egg into a motile feeding larva. The larva represents a specialized phase that is adapted to different habitats and resources from the adult (Smith et al., 2000). The larva undergoes a metamorphosis transforming it into the adult form.

In amphibians, thyroid hormone (T₃ or TH) triggers the onset of metamorphosis with more morphological changes occurring in order to create the adult form. These
morphological changes include cell death and absorption of the tail and gills, growth and cell differentiation of the limbs, and remodeling of the tadpole intestine, skin, and brain (Schreiber et al., 2001). The completion of metamorphosis produces the adult.

*X. laevis* is the current model for anuran biphasic development because the developmental stages can be followed under a microscope and can be easily manipulated. Numerous researchers throughout the world have extensively studied the histology, gene expression, and molecular pathways of the African Clawed frog *X. laevis*

### III. *Eleutherodactylus coqui:*

#### A. Origin and Development:

*E. coqui* is a terrestrial Puerto Rican tree frog belonging to the family Leptodactylidae (Callery et al., 2001). Males produce a musical repetitive call during courtship to attract the female to the nest. Amplexus occurs between the two frogs and continues to the following day. The female leaves the nest after laying the eggs, and the father attends to the clutch of approximately 40 eggs (Michael, 1995). After three weeks, a froglet emerges from the jelly capsule (Callery et al., 2001). Hatching frogs measure 4-6 mm snout vent length (SVL). The father remains with the froglets for several days until they start to disperse (Michael, 1995).

Townsend and Stewart (TS) (1985) characterized the gross anatomical development of *E. coqui* from fertilization to hatching. Developmental stages were characterized by the embryo’s limbs, eyes, tail, gills, endolymphatic calcium deposits (ECD), yolk reserve, pigmentation, and eye progression. Upon reaching TS 4, neurulation is complete, and the limb buds become evident. Movement is first seen at TS 7 by a thrashing of the tail.
Blood vessels are first apparent in a TS 8 embryo and remain visible throughout development. The yolk sac is enclosed by a thin membrane, and over the course of development, TS 9-12, gradually becomes covered by the pigmented body wall (Fang and Elinson, 1996). A modified structure, the egg tooth, forms at TS 12 and is used at hatching to break through the jelly capsule (Thibaudeau and Altig, 1999). The pigment completely covers the yolk sac at TS 13. The tail becomes useless, and the limbs are the sole source of movement at TS 14. At TS 14, the eyes are fully developed, and a banding pattern is present on the animal’s legs. At hatching (TS 15), the froglet still has 1/2 or less of its tail, and the yolk reserves are very prominent (Townsend and Stewart, 1985). No staging exists after TS 15.

Currently, there are two hypotheses on the evolution of direct development of *E. coqui* (Elinson, 1990). The “compression” hypothesis postulates that the larval state has been significantly reduced. Larval features are not obvious in early stages of development but with detailed morphological and molecular analyses, larval characteristics may be revealed. The “excision” hypothesis states that all larval characteristics have been deleted. Further examination would prove fruitless for there are no tadpole specific patterns of gene expression or morphological characteristics (Elinson, 1990). Presently, several researchers are testing the two hypotheses by examining various molecular pathways and gene expression patterns in *E. coqui*.

**B. Hormonal Control:**

Hormonal controls are involved in the metamorphosis of amphibians from the larval to the adult form. Studies have mainly focused on the role of thyroid hormone in
development. Thyroid hormone is a member of the steroid family and is capable of diffusing through the cell membrane. TH is made and released from the thyroid gland and is considered to be the ‘master-switch’ for metamorphosis. Since *E. coqui* does not metamorphose, the role of TH is not well understood. Studies have been performed on the ontogeny of the thyroid gland and the targets of TH in hopes to reveal its role in *E. coqui* development (Callery and Elinson, 2000; Jennings and Hankem, 1998).

The thyroid gland is unlike other endocrine glands because it stores the hormone it produces extracellularly in the follicles (Jennings and Hanken, 1998). The thyroid gland of *E. coqui* first appears at TS 10, two-thirds of the way through embryogenesis (Jennings and Hanken, 1998). In other non-direct developing frogs, the thyroid gland does not form until well after hatching during the larval period (Jennings and Hanken, 1998). The histology of the *E. coqui* thyroid gland changes from poorly organized follicles with no colloid present in TS 10 to well developed follicles with colloids at TS 12 (Jennings and Hanken, 1998).

Once TH is released from the gland, it binds to thyroid hormone receptors (TRs) located in various cells of the body. TRs modulate gene expression in response to ligand stimulation (Callery and Elinson, 2000). The receptors are members of a nuclear hormone receptor superfamily of transcription factors. RNA for the two TR forms, $\alpha$ and $\beta$, are present at different times in development of *E. coqui*. TR $\alpha$ RNA is present early in development and levels remain constant. TR $\beta$ mRNA expression is low in early embryogenesis and increases when the thyroid gland matures. TR $\beta$ RNA remains high during the last one third of embryogenesis (Callery *et al*., 2001). This pattern of expression has also seen in biphasic anurans. The receptors are present in oocytes and
TH is present in eggs suggesting the possibility of early regulation by TH (Callery et al., 2001).

The onset of thyroid development in direct developers differs from the timing of development in biphasic anurans. There are two hypotheses as to the evolution of hormonal control. Lynn and Peadon (1955) proposed that there is early embryonic activation of the same hormonal axes that control metamorphosing frogs. The second hypothesis is that the tissues that were dependent on metamorphic hormones may have been freed from hormonal control (Hughes, 1966; Callery et al., 2001). Methimazole inhibits TH synthesis and is known to prevent metamorphosis in *X. laevis*. When Callery and Elinson (2000) treated *E. coqui* TS 8-10 embryos with methimazole, the embryos arrested in development at TS 12. Absence of TH affected skin, limbs, jaw musculature and cartilage, tail, and axial musculature. Furthermore, rescue experiments were conducted by adding TH to methimazole treated embryos. The embryos developed normally proving that the remodeling events of *E. coqui* are TH dependent and supporting the first hypothesis for hormonal control (Callery and Elinson, 2000).

IV. **Gut Formation:**

A. **General Amniote:**

The amniote gut is a complex, organized structure, functioning in digestion. There are several critical processes required to form a functional gut including induction and patterning of the endoderm, recruitment of the mesoderm and tube formation, proliferation and specification of sections along the gut tract, and renewal of the gut epithelium (McBride et al., 2003). After endoderm specification of the epithelium, a
simple gut forms by two invaginations appearing on either end of the embryo. The anterior intestinal portal (AIP) and the caudal intestinal portal (CIP) move inward into the amniote embryo transporting the specified endoderm with them. As a result, a primitive tube forms at either end of the gut. In the middle of the embryo, fusion occurs, and the midgut rolls up taking the endoderm and the surrounding mesoderm with it to form a continuous tube. The final product is a gut tube divided into three main regions: foregut, midgut, and hindgut. The foregut will produce the esophagus, stomach, liver, pancreas, and duodenum. The midgut gives rise to the small intestine, including the ileum and jejunum. The caudal colon is produced by the hindgut (McBride et al., 2003; Wallace and Pack, 2003).

B. *Xenopus laevis:*

1. **Endoderm Specialization and Regionalization:**

The formation of the three germ layers, ectoderm, endoderm, and mesoderm, is characteristic of the development of triploblasts. In amphibians, the layers are formed along the animal vegetal axis (Yasuo and Lemaire, 1999). The endoderm, arising from the vegetal pole becomes invaginated and will form the digestive and respiratory tract. The organs of the digestive system are the pharynx, oesophagus, pancreas, liver, gall bladder, stomach, and intestines (Chalmers and Slack, 2000).

The development of the gut is a three-step process, beginning with embryonic cells committing to become endoderm. Endoderm formation is complete by the end of neuralation (Chalmers et al., 2000; Horb and Slack, 2001). The second stage, regional specification, is the commitment of each tissue section to their fate. The endoderm cells
are informed of their positioning in relation to the anterior-posterior, dorsal-ventral, and right-left axes (Horb and Slack, 2001). Beside endoderm, organs of the gut are composed of an outer layer of mesoderm-derived mesenchyme. The mesoderm will develop into the connective tissue and smooth muscle of the gut (Chalmers et al., 2000). Horb and Slack (2001) recently determined that while the formation of the endoderm is autonomous, its regional specialization is not. Endoderm specification occurs only when there is simultaneous development of mesoderm. Specification of the cells along the anterior-posterior axis is established in *X. laevis* between Nieuwkoop and Faber (NF)(1967) stage30-35 (Horb and Slack, 2001).

Following regional specification, differentiation of the endoderm cells occurs. By this stage, the cell’s fate is irreversible. Specific functional proteins and mRNAs are formed for the particular cell type or organ. Cell differentiation begins at NF 30 and continues until metamorphosis is completed (Horb and Slack, 2001).

At day three, Nieuwkoop and Faber (NF) (1985) NF 40/41, of development, the gut of *X. laevis* is a simple, thick tube with undifferentiated cells. The intestine is a large mass completely lacking coils. Within 24 hours, the intestine elongates and the liver and gall bladder can be recognized. Between day 5 (NF 45/46) and day 7 (NF 46/47), intestinal coiling greatly intensifies. Maturity of the other organs is also occurring during this time (Chalmers and Slack, 1998).

2. **Metamorphic Remodeling of Gut:**

Amphibian metamorphosis begins around NF 60 and is completed at NF 66 (Ishizuya-Oka and Ueda, 1996). During this time the gut, mainly the small intestine and stomach,
undergo a remodeling process converting an herbivorous tadpole into a carnivorous adult. A transition from larval to adult epithelium is accomplished by apoptosis of the larval epithelium and proliferation of the adult epithelium (Ishizuya-Oka and Ueda, 1996). Apoptosis is identified by the condensation of nuclear chromatin preceding the fragmentation of the nucleus. Proliferation is detected by DNA synthesis during S phase of mitosis (Ishizuya-Oka et al, 1997a). The two organs in the gut that exhibit these cellular processes are the stomach and small intestine.

i. **Small Intestine:**

Prior to metamorphic climax at NF 59, the larval epithelium consists of few larval apoptotic cells and a moderate number of proliferating adult cells (Ishizuya-Oka and Ueda, 1996). Between NF 60-62, apoptotic cells increase in number, and there is an increase in the number of adult epithelial cells. In addition at NF 62, the basal lamina, consisting of the extracellular matrix (ECM) is noticeably thicker as a result of its folding (Ishizuya-Oka and Ueda, 1996; Ishizuya-Oka et al., 1997a).

The intestine is composed only of simple columnar adult epithelium in both the proximal and distal regions at NF 63. Only a few apoptotic cells remain in the distal region (Ishizuya-Oka et al., 1997a). The basal lamina, now lining the adult epithelium, dramatically decreases in thickness. It is interesting to note the correlation between the folding of the basal lamina and the expression of both apoptosis and proliferation. Ishizuya-Oka and Ueda (1996) proposed that the later cellular events are regulated by basal lamina modifications. The overall result of apoptosis of larval epithelium and
proliferation of adult epithelium in the small intestine is a dramatic reduction in length coinciding with the emergence of a carnivorous adult (Chalmers and Slack, 1998).

ii. **Stomach:**

Similar to the small intestine, the larval stomach experiences cellular changes at the beginning of metamorphic climax due to apoptosis and proliferation. Apoptotic cells are scattered throughout the stomach. Adult primordia are located in clusters lining the basal lamina and exhibit an increase in proliferation. The basal lining of the stomach thickens. By NF 62, apoptotic cells decline in number coinciding with a decrease in the larval/ adult epithelium ratio (Ishizuya-Oka *et al*., 1998). Glands emerge in the connective tissue of the adult epithelium and are fully formed by NF 66. At this point, the adult *X. laevis* stomach is composed of two distinct histological regions. Anteriorly, the stomach possesses a thick, folded epithelium and a large number of gastric glands. Posteriorly, the stomach is made of a thin layer of columnar epithelium and a decrease in glands. In addition, the adult now has a pyloric sphincter composed of thick muscle. The sphincter serves as marker for the transition between the stomach and the small intestine (Smith *et al*., 2000).

3. **Hormonal Regulation of Remodeling of Metamorphic Gut:**

The remodeling of the gut in *X. laevis* is initiated by thyroid hormone (TH / T₃). The active form of TH is 3, 5,3’-triiodothyronine (T₃) (Schreiber *et al*., 2001). The characteristics of both TH as well as α and β receptors are the same as mentioned previously for *E. coqui*. About six weeks after fertilization, the thyroid gland becomes
functional and metamorphosis begins. The receptors bind to chromatin and repress certain genes (Su et al., 1997). When TH is released, it binds to the receptor converting it from a repressor to an activator (Su et al., 1997). Thyroid hormone receptor-alpha (TRα) RNA can be detected shortly after hatching and expression reaches maximize levels well before metamorphic climax. Expression of thyroid receptor-beta (TRβ) correlates with metamorphosis. It is expressed at low levels at the beginning of metamorphosis, and reaches a peak in late prometamorphosis (Yaoita and Brown, 1990). Amphibian metamorphosis ceases in the absence of TH. Interestingly, no two tissues of the larva display the same response to the TH (Su et al., 1997). Upon completion of metamorphosis, the intestine has new functional and structural constituents due to apoptosis and proliferation in response to TH (Tata, 1999).

Genes responsible for remodeling the intestine encode transcription factors, ECM proteins, adhesion molecules, lectins, morphogenetic proteins, growth factors, immunosuppressant-binding proteins, enzymes, members of the ubiquitin proteasome pathway, RNA binding proteins, and proteins related to signal transduction (Su et al., 1997; Amano et al., 1998). Genes are classified as either early or late thyroid response genes. An early response gene involved in the remodeling process is Sonic hedgehog (Shh). Shh is expressed throughout the gut endoderm at developmental stages corresponding to the formation of the gut tube (Ishizuya-Oka et al. 2001a; Zhang et al., 2001). An example of a late response gene is the bone morphogenetic protein (BMP-4). Intestinal fatty acid binding protein (IFABP) is considered to be both an early and late TH response gene (Ishizuya-Oka et al., 1997a; Ishizuya-Oka et al., 2001b; Su et al., 1997).
BMP-4 is a member of the transforming growth factor β superfamily. It was the first TH-responsive growth factor identified in X. laevis intestine (Ishizuya-Oka et al. 2001b). During metamorphosis, X. laevis exhibits an up-regulation of BMP-4 RNA in the intestinal connective tissue in the presence of TH coinciding with the proliferation of the adult epithelium. BMP-4 RNA levels begin to decline during the final stage of metamorphosis when the adult epithelium is completing replacement. Furthermore, BMP-4 is one of the targets of Sonic hedgehog (Shh) signaling. (Ishizuya-Oka et al., 2001a; Smith et al., 2000).

The intestinal fatty acid binding protein (IFABP) shares high sequence similarity to the mammalian IFABP. IFABP is used as a marker for cellular differentiation due to its pattern of expression. Its mRNA and protein are up-regulated in the larval intestinal cells during pre-metamorphosis, down-regulated with apoptosis of the larval cells at the onset of metamorphosis, and re-expressed with the differentiation of the adult intestinal cells at the completion of metamorphosis (Beck and Slack, 1999; Ishizuya-Oka et al., 1997a)

The basal lamina, a specialized ECM, separates the intestinal epithelium from the underlying mesenchyme. It serves in tissue support and maintaining the integrity of an organ. As previously mentioned, the basal lamina undergoes remodeling during metamorphosis. Macrophages migrate across the basal lamina to the degenerating larval epithelium at the same time as lamina remodeling. The macrophages eventually ingest the apoptotic bodies, and upon completion, they undergo apoptosis as well. The question is raised as to how adult cells escape apoptosis. Su et al. (1997) proposed that the ECM not only inhibits T3 induced larval apoptosis, but that the new ECM formed by the adult cells has this function as well (Su et al., 1997).
V. Thesis Objectives:

Currently, there is no information available on the development of the gut of *E. coqui*. In this thesis, I intend to examine gut formation of *E. coqui*, and compare these findings to *X. laevis*. My comparisons will be based on the anatomical development as well as the proliferation pattern of cells in the gut. I will first establish a proliferation assay in order to view mitotic cells. I will induce metamorphosis by introducing exogenous TH in pre-feeding *X. laevis* tadpoles, and compare the proliferation pattern to control tadpoles. I will characterize the anatomy of *E. coqui* gut development. I will determine gut proliferation pattern during *E. coqui* development. Characterizations of *E. coqui* gut anatomy and proliferation pattern will be compared to *X. laevis*. 

MATERIALS AND METHODS

I. Animals and Embryos:

A. *Xenopus laevis*

Embryos of the African Clawed frog, *X. laevis*, were obtained by *in vitro* fertilization. Females were injected with 50 IU pregnant mare serum (PMS) and kept at 18 °C. Between 1-3 days after PMS injection, the females were injected with 600 IU of Human Chorionic Gonadotropin (HCG) and maintained at 15°C overnight. The following day, a male was sacrificed and his testes were removed. The testes were maintained in 200% Steinberg’s Solution at 4°C until needed. Steinberg's Solution is prepared from two different stock 20X solutions; Stock A, 1.16 M NaCl, 13mM KCl, 17mM MgSO₄•7H₂O with one liter of deionized (DI) water and Stock B, 9.98mM Tris dissolved in deionized water and adjusted to a pH of 7.4 with 1N HCl. For 200% Steinberg’s solution 100 ml of stock A and 100 ml of stock B were made to a liter with DI water. All Steinberg’s Solutions, 20%, 80%, 100%, and 200%, were made from these stocks.

A small piece of the testis was chopped up in a petri dish containing 80% Steinberg's Solution in order to release the sperm. Injected females were squeezed releasing eggs into the petri dish containing the sperm; the sperm and eggs were gently swirled in the petri dish. After three minutes, 20% Steinberg's Solution was added, flooding the eggs. About two hours later, the 20% Steinberg's Solution was removed and 2.5% cysteine pH 8, was added to dejelly the eggs. The eggs were swirled in the cysteine for about 5 minutes or until the eggs touched each other. The cysteine was discarded, and the eggs were washed 5-6 times with 20% Steinberg's, ensuring the complete removal of cysteine.
The embryos were reared in 20% Steinberg's Solution and staged according to the table created by Nieuwkoop and Faber (NF) (1956). For *X. laevis* tail removal and natural metamorphosis, tadpoles at NF 48-51 and 59-62 respectively, were obtained from Nasco Scientific.

B. *Eleutherodactylus coqui:*

*E. coqui* embryos were obtained from natural matings in a laboratory colony and staged according to Townsend and Stewart (TS) (1985). After fertilization, the eggs were removed from the guarding male and placed in a petri dish containing filter paper and 2-5 ml of 20% Steinberg's Solution. The embryos were examined daily for the appropriate stage, and 20% Steinberg's Solution was added as needed to maintain a moist environment. Upon reaching the desired stage, the embryos were flooded with 20% Steinberg's Solution, and their jelly and fertilization membrane were removed with forceps.

II. **Tissues: Treatments, Dissections, and Fixations**

A. *Xenopus laevis*

1. **Thyroid Hormone (T₃) Treatment and BrdU injections:**

Tadpoles were obtained by *in vitro* fertilization as previously described (Section I). Upon reaching NF 46/47, approximately 7 days after fertilization, tadpoles were placed in a 10 nM T₃ solution. Control tadpoles were kept in 20% Steinberg's Solution without T₃. Tadpoles were left in T₃ for 2, 3, or 4 days. On day three of treatment, gross anatomical differences between the two groups were noticeable. The control and experimental
solutions were changed every other day, and anesthetized in a petri dish containing a mixture of ice and 20% Steinberg's solution. After 10 minutes, both T₃ and non- T₃ treated tadpoles were removed from the dish and placed in a new petri dish with an agar base. The tadpoles were microinjected in their abdomen with 1.25µl of 20mM BrdU following the protocol of Marsh-Armstrong et al. (1999). Tadpoles were placed in 20% Steinberg's Solution and left overnight at room temperature to allow BrdU incorporation. Metamorphosing tadpoles, NF 59-62, were injected with BrdU in the abdomen in the same manner.

2. **Fixations:**

BrdU injected specimens were fixed in 2% TCA for two hours. The TCA was removed, and deionized (DI) water was added. The vials were gently rocked for 12 minutes. The water was poured off, and the specimens were dehydrated through 70% and 100% methanol series at room temperature with gentle rocking for 12 minutes each. The 100% methanol was drained off; Dent’s fixative (20%DMSO, 80% methanol) was added, and the specimens were stored at -20°C.

Tadpoles processed for anti-phospho histone H3 immunocytochemistry (ICC) were fixed in a different manner. After T₃ and/or colchicine treatment, the tadpoles were fixed in 4% paraformaldehyde for 30 minutes. The fixative was removed, and the tadpoles were washed in 70% methanol for 10 minutes followed by a 100% methanol wash and stored at -20°C until needed. The tadpoles were then bleached in 1:2 30% H₂O₂: methanol overnight at room temperature.
3. **Dissections:**

After fixation, the eyes and intestines of BrdU injected NF 59-62 tadpoles were removed. The skin surrounding the intestinal region was removed with forceps exposing the intestines. The intestine was pulled out of the body cavity, paying careful attention to anterior vs. posterior. Both eyes of each tadpole were removed with forceps as well. In order to examine tissues for BrdU incorporation, they were embedded and sectioned as described later (Section III). Tadpole intestines from earlier stages NF 46/47 were dissected out after processing for immunocytochemistry (ICC) with anti-phospho-H3-histone as described later. The skin surrounding the intestinal region on the ventral side was cut open exposing the intestine. The posterior intestine was disassociated from the tadpole with forceps. Next, the anterior region was clipped off completely detaching the intestine.

4. **Xenopus laevis Tadpole Tail Removal:**

Control, T₃ and/or colchicine treated *X. laevis* tadpoles from NF 48-51 were anesthetized by placing them in 200% Steinberg's Solution with ice. After 10 minutes, the tip of the tail was cut off. The tadpoles were then placed in 20% Steinberg's and allowed to regenerate the missing tail for three or four days. Upon the designated day of regeneration, the tadpoles were fixed as previously described. Tadpoles with intact tails served as controls and were fixed along side the experimental specimens.
5. Colchicine Treatment:

Colchicine traps cells in mitosis. It was used to increase the number of proliferating cells in order to verify the ability of the primary antibody, anti-phospho histone H3, to detect mitotic cells. A working solution was made by diluting 10 µl of the 1 µg/ml colchicine stock solution with 150 ml of 20% Steinberg's Solution. Tadpoles intended for ICC were divided into eight petri dishes: (1) No T₃ and no Colchicine (2) T₃ and no Colchicine (3) No T₃, no Colchicine and no tail (4) T₃, no Colchicine and no tail (5) no T₃ and Colchicine (6) T₃ and Colchicine (7) T₃, Colchicine and no tail (8) no T₃, Colchicine and no tail. "No tail" means that the tip of the tail of the tadpole was cut off and allowed to regenerate for three days. Colchicine containing dishes were treated for 10 hours. After treatment the tadpoles were fixed as described above.

B. Eleutherodactylus coqui:

1. Anatomical Characterization of Gut Development in E. coqui:

To isolate the gut of E. coqui, embryos were anesthetized in a mixture of 200% Steinberg's Solution and ice. The skin and abdominal muscles were cut open without disturbing the natural position of the gut. The orientation, yolk concentration, size, tube formation, blood supply, and general appearance were recorded. The gut was then dissected out of the frog as follows. The posterior tube of the gut was freed from the body. A dot of Carmine stain was placed on the posterior half to distinguish it from the anterior (Fig. 1 Step 1). The anterior tube was cut completely releasing the gut tube from the embryo. The guts were placed on a microscope slide and covered with a thin layer of 200% Steinberg's Solution. An incision was made in the anterior region below the
anterior tube. Utilizing two forceps, the gut was cut along the perimeter (Fig. 1 Step 2). Upon reaching the area below the posterior tube, the gut was cut in a horizontal line connecting to the initial incision. The ventral layer was removed and discarded (Fig. 1 Steps 3-4). The remaining section of the gut was swirled in a petri dish containing 200% Steinberg's Solution releasing loose yolk cells from the gut.

Staging of *E. coqui* by Townsend and Stewart (1985) ends at stage 15; therefore staging after TS 15 was based on the amount of yolk present in the gut as well as a green substance appearing later in gut development. Stages are denoted by subscripts with 15₂ representing the most amount of yolk and 15₆ indicating the absence of yolk. TS 15₂ - TS 15₆ froglets were dissected in a slightly different manner from earlier stages. Posterior and anterior regions were easily distinguishable due to their anatomical structure; therefore, Carmine stain was not needed for this purpose. All other steps in the dissection procedure were kept the same (Fig. 1). Diagrams of gut formation were made using Adobe Illustrator. Digital images were captured with the LEICA MZ6 dissecting microscope and Q-capture digital camera. Images were processed and refined in Adobe Photoshop.

2. **Fixation:**

Guts processed for immunocytochemistry with anti-phospho histone H3 were transferred to vials containing 4% paraformaldehyde. After thirty minutes, the fixative was removed. The guts were washed in 70% methanol for 10 minutes and stored at -20°C in 100% methanol. All steps of fixation were carried out while gently rocking.
III. **Embedding and Sectioning:**

Dent's fixative was removed from each sample intended for ICC with BrdU, and DI water was added for 10 minutes at room temperature. Next, the specimens went through an ethanol series: two washes for 20 minutes each with 70% ethanol and with 95% ethanol, followed by three washes for 20 minutes each with 100% ethanol. The ethanol was drained off, and the samples were incubated with three changes of xylene for ten minutes each. Once the xylene was completely removed, melted paraplast (Fischer Scientific) was added to the specimens, and they were transferred to a 65°C oven for 10 minutes. The specimens were taken out of the oven and the paraplast was removed, keeping it liquid over a flame. This process was repeated two more times. Paper boats were constructed from index cards to embed tadpoles, eyes, and intestines. After the third wash in paraplast, the samples were placed in the paper boats and orientated. Paraplast was poured on top of the specimens and the boats were placed in a container of cold water to solidify it. Pieces of paraplast containing a tadpole, an eye, or an intestine were cut in the shape of a trapezoid and mounted on wooden blocks. They were sectioned at 10 µm on a Spencer 820 Microtome and mounted on slides coated with aminoalkysilane (Sigma). The slides were left on a slide warmer at 60°C overnight to dry. It is important to note that each slide did not come from the same ribbon of sectioning. The ribbon was divided between four slides per one specimen.
IV. **Immunocytochemistry and DAPI Staining:**

A. **Anti-BrdU:**

Slides were prepared from T₃ and non- T₃ treated *X. laevis* whole tadpoles and dissected eyes and intestines of metamorphosing frogs. The slides were placed in 100% xylene for 20 minutes to remove the paraplast. Xylene was removed, and fresh xylene was added for an additional 20 minutes. Next the slides were run through a methanol series: 100% for 5 minutes, 95% for 2 minutes, 70% for 2 minutes, 30% for 2 minutes, and phosphate buffered saline (PBS) for 2 minutes. The PBS was removed, and 0.1% Triton X-100 in PBS was added to the slides for 10 minutes. The solution was changed three times. After the four washes with Triton-X, the slides were rinsed in PBS and placed in 10mg/ml BSA in PBS to block non-specific sites. The slides were incubated overnight at 4°C with the mouse monoclonal anti-BrdU primary antibody diluted 1/1000 with 10mg/ml BSA in PBS (Sigma). The primary antibody was washed off the following day with three one hour washes of PBT (2mg/ml BSA, 0.1% Triton-X 100 in PBS). A final wash of 10mg/ml BSA in PBS was done at room temperature for 30 minutes. The secondary antibody, FITC labeled goat anti-mouse antibody (Jackson Immunoresearch Laboratories Inc), was diluted 1/1000 with 10mg/ml BSA in PBS, and incubated with specimens either overnight at 4°C or at room temperature for 1 hour. The secondary antibody was washed off with three 30 minute washes of PBT. Two sets of controls were performed in conjunction with this experiment. No primary antibody was added to one eye, intestine, and tadpole control for non-specific binding of the secondary antibody. Another set of specimens did not receive the secondary antibody to control for autofluorescence of the specimen.
B. Anti-phospho histone H3:

Dissected *E. coqui* guts and *X. laevis* intestines were drained of 100% methanol and placed in PBT for 16 hours at RT. The PBT was removed, and a blocking solution of 10 mg/ml BSA, 0.1% Triton X-100 in PBS was added for 3 hours. The polyclonal antibody, anti-phospho Histone H3 (Upstate Pharmaceutical), was diluted 1/300 with 10mg/ml BSA, 0.1% Triton X-100 in PBS and incubated with the specimens overnight at 4°C. After the overnight incubation, the primary antibody was left on for an additional hour at room temperature before washing with PBT for three 2 hour washes. The PBT was removed, and, Alexa-Fluor 488 labeled goat - anti-rabbit secondary antibody (Molecular Probes) was added to the specimens for an overnight incubation at 4°C (1/400 in 10 mg/ml BSA, 0.1% Triton X-100 in PBS). The secondary antibody was removed by three two hour washes with PBT. As controls, either the secondary or primary antibody was not added to the guts.

C. DAPI Staining:

DAPI staining was carried out on various specimens to show the location of nuclei. A 0.6 mg/ml stock solution of DAPI (Sigma) was obtained from Dr. John Doctor. A working solution was made by adding 2.5 µl of the stock to 25 ml of PBS. The PBT was drained from the specimens and replaced by 4% formaldehyde in PBS for 10 minutes. The specimens were washed three times in PBS for 10 minutes each. DAPI was added to the vials for 20 minutes. The stain was removed with three washes of PBS for 10
minutes each. The specimens were maintained in 0.02% sodium azide in PBS until viewing.

VI. **Mounting and Visualization:**

A. **Xenopus laevis:**

Sectioned specimens, labeled with BrdU, were mounted in propylgallate (1g propylgallate, 20 ml glycerol, 1.25 ml 0.2M phosphate buffer, pH 8, brought to 25 ml with DI water). The specimens were covered with a cover slip and excess solution was removed with filter paper. The edges were sealed to the slide with clear nail polish. Experimental and control tadpole intestines were placed on slides in the proper orientation, anterior to posterior, and covered in propylgallate mounting media. Tadpoles from the tail removal experiments were mounted in depression slides in propylgallate, and a cover slip was placed on top. All specimens were examined with the Nikon Eclipse CF160 Microscope equipped with a 100W / Mercury Light source for fluorescence. Digital images were processed with the QED Imaging System and Adobe Photoshop.

B. **Eleutherodactylus coqui:**

In order not to harm the guts, Vaseline was placed on the slide in the shape of a square using a glass pipette. The gut was placed in the center of the square, and a diagram was drawn indicating orientation of the gut. Propylgallate mounting medium was added to the Vaseline well, and a cover slip was placed on top of the Vaseline square. Specimens were examined with Nikon Eclipse CF160 Microscope equipped with a 100W Mercury light source for fluorescence. Photographs were processed with the QED Imaging
System and Adobe Photoshop. Location in the gut of each picture was denoted by the creation of a numerical mapping system beginning in the anterior (1) and rotating counterclockwise to the middle (6) section of the gut. The anterior and posterior tubes were labeled A, and P, respectively.

VII. **Statistical Analysis:**

To quantitate the proliferation rate in *X. laevis* intestines, they were flattened between the slide and the cover slip when mounted. The flattening allowed the intestinal area to be calculated with the surface area of a rectangle (SA = length X width) rather than the surface of a cylinder. The first and last 0.95 mm were omitted on every specimen. The midpoint of the intestine was calculated by measuring the length of the intestine and dividing it by two. The anterior and posterior region counted for each intestine were 0.95 mm in length (Fig. 2). Cells in the middle region were counted 0.95 mm above and 0.95 mm below the mid point (Fig. 2). The average width of each position was calculated and used in determining the surface area. Once the surface area was determined, the number of proliferating cells per mm² was calculated by dividing the number of cells stained by anti-phospho histone H3 antibody in that region by the surface area. To determine its statistical significance, one-tailed T-tests were performed by using a computer-based program found at [http://www.graphpad.com](http://www.graphpad.com). Results were in graphed Cricket Graph.
Figure 1: Diagram of *E. coqui* Gut Dissection. After the abdominal skin and muscle were cut open, the posterior (P) tube was freed from the body. A dot of Carmine stain (red) was placed on the upper posterior half. The anterior (A) half was released from the body and the guts were placed on a microscope slide. After the initial incision was made below the anterior tube (Step 1), the gut was cut along its perimeter until reaching the Carmine stain (Step 2). A horizontal cut was made across the gut connecting the initial incision (Step 3). The top layer was removed exposing the interior of the gut (Step 4).
Figure 2: *X. laevis* intestinal measurements used in calculating the number of mitotic cells stained with the anti-phospho histone H3 antibody.
RESULTS

I. Assaying cell divisions in frog embryos:

BrdU (5-bromo-2-deoxyuridine) is a thymidine analogue that is incorporated into the cell’s DNA during S-phase of the cell cycle. Ishizuya-Oka et al. (1997a) used BrdU to measure cellular proliferation in the intestine of natural metamorphosing X. laevis. In an attempt to repeat their assay, a 20mM BrdU solution was injected into the abdominal region of anesthetized X. laevis tadpoles. The following day the tadpoles were fixed, and processed for ICC. Incorporation of BrdU was visualized by fluorescent microscopy. Successful BrdU labeling was seen in some of the sectioned specimens. The assay proved to be unreliable, however, perhaps due to the injection technique. Due to the failure of the BrdU assay, an antibody was examined for its ability to detect mitotic cells.

Anti-phospho histone H3 antibody is a polyclonal antibody, which is specific for histone H3 phosphorylated on serine 10 near the N-terminus. Phosphorylated histone H3 is detected in late G2 phase and continues to be present in prophase, metaphase, and at low levels in anaphase, and telophase of the cell cycle (Hendzel et al., 1997). Anti-phospho histone H3 can be used on fixed specimens, which provides an advantage over BrdU. BrdU first had to be incorporated into living tadpoles followed by fixation and detection by ICC.

Anti-phospho histone H3 ability to detect mitotic cells was tested in a X. laevis tail tip regeneration experiments. X. laevis tails regenerate when cut off (Ishino et al., 2003). Regeneration includes an increase of cell proliferation (Ishino et al., 2003). Tail tips from NF 48-51 tadpoles were cut off and allowed to regenerate for three or four days. After the allotted regeneration time, the tadpoles were processed for ICC. Specimens were also DAPI stained in
order to determine the location of the cells. The cells stained by anti-phospho histone H3 were
detected against a field of DAPI stained cells, and each antibody-stained cell was also DAPI
stained (Fig. 3).

In an attempt to increase the number of mitotic cells, colchicine was used before fixation.
Colchicine traps cells in mitosis. Control and experimental tadpoles did not show a considerable
difference in the number of cells stained (data not shown). Due to this result colchicine was not
used in further experimentation.

II. **Thyroid hormone induced remodeling of *X. laevis* small intestine:**

Thyroid hormone is responsible for the initiation of remodeling of the gut of *X. laevis* during
metamorphosis. One of the steps in the remodeling process is the proliferation of the adult
epithelium (Ishizuya-Oka and Ueda, 1996). Exogenous thyroid hormone is able to induce
metamorphosis in pre-feeding *X. laevis* tadpoles. Induced metamorphosis produces a tadpole
with an elongated jaw due to excessive growth of Meckel’s cartilage, early limb bud formation,
gill absorption, and increased brain width (Schreiber *et al.*, 2001). From this knowledge, the
question arises: Will thyroid hormone induce intestinal remodeling of pre-feeding tadpoles as
shown by an increase in proliferation of the intestinal epithelium?

Tadpoles were treated with 10 nM T₃, fixed, processed for ICC with anti-phospho histone H3,
and dissected. Initially, 235 tadpoles were incubated with T₃, and 100 were used as controls.
After two days of T₃ treatment, nine T₃ treated tadpoles, and two control tadpoles died. The
incidence of death increased for both experimental and control tadpoles on the third day of
treatment with fourteen and three deaths respectively. The most deaths occurred on day four of
incubation with T₃, when sixty-six T₃ treated tadpoles died. Only three control tadpoles died on
day four. Out of the initial 235 tadpoles incubated with T₃ only 136 survived whereas 92 out of 100 control tadpoles survived. The lethality caused by 10nM T₃ has to be considered in interpreting the results, particular at the day four treatment.

In naturally metamorphosing X. laevis, the emerging adult intestine is only 10% of the length of the tadpole gut (Chalmers and Slack, 1998). Induced metamorphosis by T₃ also produced shorter intestines with the length decreasing with increased time of treatment. On average, day two treated intestines were 13.0 ± 2.3 mm in length, day three treated intestines were 7.5 ± 2.5 mm in length, and day four intestines were 5.6 ± 2.0 mm in length (Table 1). Although they are not statistically significant, these decreases in length are highly suggestive.

Two days of T₃ incubation produced an intestine that contained the highest number of proliferating cells in the anterior region, the least in the middle, and a moderate number in the posterior intestinal region (Fig. 5). The anterior intestinal region of the experimental tadpoles contained on average, three and a half times more proliferating cells than the control tadpoles (Fig 5). The middle intestinal region contained a similar amount of proliferation in the control and T₃ treated tadpoles (Fig. 5). The posterior region contained five and a half times more proliferating cells in the experimental tadpoles compared to the control.

After three days of treatment with T₃, the same pattern of proliferation was seen. The anterior region had the highest level of proliferation, the middle region the least, and the posterior region contained a moderate amount of proliferating cells (Fig. 5). The anterior and posterior of the experimental tadpoles contained four times more proliferating cells than the controls. The middle contained twice as many proliferating cells as the controls (Fig. 5).

Four days of treatment produced a different pattern of proliferation. The posterior region of the intestine had the highest levels of proliferation with six times more mitotic cells than the
controls. The anterior region contained a moderate amount of proliferating cells, and the middle
region still had a low level of proliferating cells (Fig. 5). Both the anterior and middle regions
contained similar amounts of proliferating cells in comparison to the control specimens (Fig. 5).

While there were differences in proliferation between T₃ treated and control intestines, only
the anterior and posterior intestinal regions treated with T₃ for two days were significantly
different from control levels. Days three and four of treatment did not produce any statistically
significant differences (Fig. 5, Table 1 and Appendix Tables 3, 4, 5, and 6).

III. Anatomical description of *E. coqui* gut development:

Development of the gut in *E. coqui* has not been described. The gut including the liver,
stomach, small and large intestine, and gall bladder arises from a yolk sac contained in the
embryo’s abdomen. Until TS 9, the gut remains a yolk filled sac with no distinct morphology.
At TS 9, the gut contains a large amount of yolk with orange blood vessels on its surface. The
structures protruding from either end of the gut are thin tubes that are the same off white color as
the yolk. The anterior tube is seen on the ventral half of the gut and continues to the dorsal side
before extending upward toward the head of the embryo (Fig. 6). The distal portion of the
posterior tubes begins on the dorsal side of the gut and extends downward. The anterior and
posterior tubes are criss-crossed when you examine the dorsal side (Fig. 6). There are no twists
or turns (Fig. 6). The posterior and anterior ends of the gut, not to be confused with the thin
tubes, are only seen on the dorsal side of the embryo (Fig. 6). The posterior end is located on
the right side of the embryo and the anterior end is located on the left side of the embryo.

TS 10 gut has a similar morphology to TS 9. The amount and color of yolk as well as the
tube length remain the same. The posterior and anterior ends of the gut on the dorsal side
increase in length and extend posterior. The ends of the gut are folded facing the dorsal side of the embryo (Fig. 6). The anterior end of the gut increases in length and is seen on the ventral side of the embryo at TS 11. The anterior end of the gut twists approximately 90° clockwise. The posterior tube remains on the dorsal side and twists approximately 90° counterclockwise. Both of the tubes remain thin and the same color as the yolk (Fig. 6).

The anterior and posterior tubes remain the same in morphology and color in a TS 12 gut. The twisting of the gut ends intensifies, which increases the length of the gut. The anterior and posterior ends twist an additional 90° clockwise and counterclockwise, respectively (Fig. 6). Blood vessels are red in color and span the whole gut. An incision in a TS 12 gut results in the release of yolk. The yolk cells at this stage seem to be loosely associated with each other and the epithelium of the gut.

By TS 13, the anterior and posterior tubes of the gut are thinner and contain less yolk than the previous stages. The blood vessels are red in color. The looping of the gut progresses. The anterior end twists to the left and loops another 90° clockwise. The posterior end twists to the right and also loops another 90° counterclockwise (Fig. 6). The anterior and posterior ends make one complete revolution. This is the maximum amount of looping seen throughout development. The anterior end produces one loop that faces the ventral side of the embryo. The posterior tube also makes one loop but it faces the dorsal side of the embryo (Fig. 6).

A TS 14 gut resembles a TS 13 gut in the amount of yolk present, appearance of blood vessels, length, and tube morphology. The main difference between the stages is the appearance of intestinal folds in TS 14. The ends of the gut are more convoluted and appear to be untwisting making the gut a more linear structure. There is a decrease in the amount of yolk present compared to the previous stages (Fig. 6).
As the gut develops to TS 15, a green substance appears in its center. The substance is located internally and is only seen when the gut is punctured. The origin of the substance is unknown at this time; however, the gall bladder is the same color as this substance and may be its source. The anterior and posterior tubes increase in diameter and there is a decrease in yolk concentration. The gut continues to uncoil and increase in the degree of folding and creasing. The orientation of the gut at this stage resembles TS 10 (Fig. 6).

The staging of *E. coqui* by Townsend and Stewart (1985) ends at TS 15; therefore, I created another staging table based solely on gut development and the concentration of yolk and green substance. TS 15₂ has a decrease in the amount of yolk. The amount of the green substance increases and the substance darkens in color. The blood vessels are faint in color, and the anterior and posterior tubes are more translucent indicating a decrease in the amount of yolk. The diameter of the tubes continues to increase. The posterior tube contains a small amount of the green substance. The morphology of the gut resembles TS 9 except for the size of the gut; TS 9 is shorter and rounder than TS 15₂ (Fig. 6).

The gut of a TS 15₃ frog shows a further decrease in the amount of yolk. Yolk is absent from the central top region of the gut and the green substance is visible there. The gut is beginning to resemble a more tubular structure characteristic of the adult morphology. The anterior and posterior tubes increase in diameter and there is a distinct border between the tube region and the yolky center of the gut. Blood vessels are not noticeable at this stage (Fig. 6).

By the time the gut reaches 15₄, 1/₃ to 1/₂ of the yolk has been replaced by the green substance. The remaining yolk is diffuse in nature. The anterior and posterior tubes continue to widen, becoming almost as wide as the central gut region. The posterior tube contains an increased amount of the green substance (Fig. 6). In a TS 15₅, less than 1/₃ of the yolk present is replaced.
by the green substance. The yolk is present only close to the intestinal epithelium and is not located deeper in the gut. The posterior tube continues to widen and increase in the amount of green substance (Fig. 6).

A 15₅ gut is completely devoid of yolk, and resembles the adult morphology. The amount of the green substance decreases, and the blood vessels are not visible. The anterior and posterior tubes are similar in size to the rest of the gut. The anterior tube remains clear while the posterior still contains the green substance, although the intensity of the green color dramatically decreases (Fig. 6).

IV. Proliferation pattern of the developing gut of E. coqui:

The proliferation pattern of the E. coqui gut was determined by the use of immunocytochemistry with anti-phospho histone H3. Once the guts were processed for ICC, they were mounted on slides, and a map was created of the gut (Fig. 7, 8, and 9). The mapping system included all of the regions of the gut. TS 9-15₂ stages were all measured via the same mapping system. A more simplified mapping system was used for TS 15₃ –15₅ due to the morphology of the gut during these stages. Pictures were taken at each map position and scored using a proliferation scale developed from examining numerous E. coqui guts (Fig. 10).

Stage TS 9 contained moderate to high amounts of proliferation in all of the areas of the gut (Table 2). The highest levels of proliferation were seen in the tube regions (A₁ and P₁). The lowest levels of proliferation were seen in the central region (Position 6) and the posterior end (position 5). Compared to TS 9, TS 10 contained lower levels of proliferation in all the regions except positions 5 and 6 (Table 2). Again, the anterior and posterior tubes had the highest levels
of proliferation in TS 10. The anterior regions of the gut (positions 1 and 2) had the lowest levels of proliferation, while the rest of the gut contained a moderate proliferation level (Table 2). At TS 11, the tubes remained high in proliferation. The anterior regions of the gut (positions 1 and 2) contained low levels of proliferation. The posterior side region of the gut (position 4) and the middle region (position 6) had moderate levels of proliferation. The pattern of proliferation was unclear in positions 3 and 5 because the four samples demonstrated different amounts of proliferation in those regions (Table 2).

TS 12 had a similar proliferation pattern to TS 11. All regions from the anterior end to the posterior end (positions 1-5) were proliferating at a low to moderate level. Once again the tubes were proliferating at high levels (Fig. 7 and Table 2). The transition to TS 13 halted the proliferation process. At TS 13, there were low levels of proliferation in all regions of the gut except the tubes, which contained a moderate number of proliferating cells. Due to the dramatic decrease in proliferation, many samples were viewed for this stage in order to verify that the absence of proliferation was indeed a characteristic of this stage rather than some other factor. Map position three contained the fewest number of proliferating cells and the anterior tube contained the most cells (Table 2). As the gut continued to develop the number of proliferating cells increased. Almost all of the regions of a TS 14 gut doubled the level of proliferation compared to TS 13. The posterior end of the gut experienced the least proliferation and the anterior tube continued to contain the most mitotic cells (Table 2).

As the gut is uncoiled at TS 15, proliferation decreased once again. All regions of the gut contained low levels of proliferation except the tubes. The anterior tube remained high in the number of proliferating cells (Fig. 8 and Table 2). Stage TS 15.2 demonstrated low levels of proliferation in positions one and three. Low to moderate levels of proliferation was seen in the
posterior end of the gut. The tubes consistently had high levels of proliferation as seen in the other stages of development (Fig. 9). The anterior tube, however, contained the highest number of proliferating cells.

The transition into TS 153 produced a gut with a different morphology than seen previously. The morphology lacked some of the map positions of the gut examined in stages TS 9-152. From this stage on, I made comparisons only between map positions A1, 1, 3, 5, and P1. TS 153 increased in proliferation in the anterior end of the gut as compared to TS 152. The remaining regions of the gut contained low to moderate amounts of proliferation but they were slightly lower than the previous stage. As the gut progressed into its adult form, proliferation was at low levels from TS 154-156. All three stages of gut development showed extremely low levels of proliferation (Table 2).
Figure 3: *X. laevis* Tail Regeneration Experiment. Cells outlined in white are in focus and in mitosis. Arrows indicate cells in insets (A’ and B’). A, A’, and C were stained for mitotic cells by anti-phospho histone H3. B, B’, and D were DAPI stained in order to show the location of the nuclei. The tip of the tail is shown in pictures A, A’, B and B’. The right side of the tail is shown in C and D.
Figure 4: Four day T\textsubscript{3} treated \textit{X. laevis} intestine stained for anti- phospho histone H3 (green). Posterior portion was increased in magnification in order to visualize the stained proliferating cells.
### Day 2 Day3 Day 4

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<th>+T&lt;sub&gt;3&lt;/sub&gt;</th>
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*p* values

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**Table 1:** Statistical analysis of intestinal length comparing the length of three TH treated intestines to three control intestines on three different days of treatment. *p* values were determined by using a two-tailed paired t-test. T<sub>3</sub> treated intestines were also compared between each day of treatment. A *p* value less than 0.05 is considered significant.
Figure 5: The effects of T3 on intestinal proliferation. Three intestines were counted for control and experimental on each day of treatment. The averages of those samples are represented in the graph. Light speckled bars indicate T3 treated intestines and solid bars indicate control intestines. Paired one tailed T-tests were performed between T3 treated and control for each region “a” and “b” indicates data that is statistically significant with p < 0.05. Comparisons between different regions of T3 treated intestines within a day were not significantly different as determined by paired two-tailed T-test (see Appendix Tables 3, 4, and 5 for data and Tables 6 and 7 for p values).
Figure 6: Diagram of *E. coqui* Gut Development. (A) = Anterior, (P) = Posterior. Both the ventral and dorsal views are shown for TS stages 9-15. Only the ventral side is diagramed for TS 15₂ – 15₆ because the dorsal side is the same as the ventral only flipped.
Figure 7: Proliferation pattern of a TS 12 E. coqui gut. Mitotic cells are stained via ICC with anti-phospho histone H3 (green). Location of cells were found by utilizing DAPI stain for nuclei (blue). The morphology of the gut is drawn above the microscope pictures, and map locations on the drawing correspond to numbers above each picture pair. $A_t =$ anterior tube $P_t =$ Posterior tube
Figure 8: Proliferation pattern of a TS 15 E. coqui gut. Mitotic cells are stained via ICC with anti-phospho histone H3 (green). Location of cells were found by utilizing DAPI stain for nuclei (blue). The morphology of the gut is drawn above the microscope pictures, and map locations on the drawing correspond to numbers above each picture pair. A_t = anterior tube P_t = Posterior tube on map.
Figure 9: Proliferation pattern of a TS 15, E. coqui gut. Mitotic cells were stained via ICC with anti-phospho histone H3 (green). Location of cells were found by utilizing DAPI stain for nuclei (blue). The morphology of the gut is drawn above the microscope pictures, and map locations on the drawing correspond to numbers above each picture pair. \( A_t = \) anterior tube \( P_t = \) posterior tube
Figure 10: Proliferation scale for *E. coqui* gut development. The scale was used to determine the level of proliferation for each position of the *E. coqui* gut. Pictures show mitotic cells detected by ICC with anti-phospho histone H3.
Table 2: Average level of proliferation of *E. coqui* gut from stages TS 9- TS 15. Averages using the proliferation scale (Fig. 10) were taken from the number of specimens viewed for each stage (n). (A<sub>t</sub>) = anterior tube (P<sub>t</sub>) = posterior tube. Red numbers indicate high levels of proliferation. Black numbers indicate moderate levels of proliferation. Blue numbers indicate low levels of proliferation. Numbers in () indicate high variability in proliferation among samples, so these numbers are less reliable.

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<td>Large amount of yolk present throughout development depleted after hatching Green substance possible biliverdin</td>
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*Table 3: Comparisons of gut development between X. laevis and E. coqui. X. laevis development was characterized by Chalmers and Slack (1998).*
DISCUSSION

I. **Thyroid Hormone Induction of Intestinal Remodeling:**

Thyroid hormone is responsible for initiating metamorphosis in *X. laevis*. Tadpoles, NF 46/47, incubated with exogenous T₃ experience precocious metamorphosis (Schreiber *et al.*, 2001). A key event in natural metamorphosis is the remodeling of the tadpole intestine into the adult form. Precocious metamorphosis was induced in NF 46/47 tadpoles by T₃, and the length and proliferation pattern of T₃ treated intestines were compared to control intestines to determine whether intestinal remodeling occurred. As T₃ treatment of NF 46/47 tadpoles progressed, the intestinal length decreased (Table 1 and Appendix Tables 4, 5, and 6). A shortening of the intestine is known to occur during metamorphosis (Ishizuya-Oka *et al.*, 1997a). Although no significant differences were found in my experiments because of the large standard deviation, I suspect further examination of more intestines would lead to significant results (Table 1 and Appendix Table 8).

The proliferation levels were compared between T₃ treated and control intestines on the same day of treatment. Only the anterior and posterior intestinal regions of a day two treated intestine had significantly highest number of proliferation (Fig. 5 and Appendix Table 7). The anterior region contained the most amount of proliferating cells compared to the controls and the other intestinal regions (Fig. 5 and Appendix Table 4). The middle region of a two day treated intestine contains similar numbers of mitotic cells as the controls. Day three of treatment produced similar results to day two. The anterior region continued to contain the most proliferating cells, with moderate amounts in the
posterior, and low amounts in the middle region (Fig. 5 and Appendix Table 5). No regions of a three day T3 treated intestine were significantly different from controls. A different proliferation pattern was observed for four days of treatment. The posterior contained the highest levels of proliferation, the anterior contained moderate levels, and the middle had the lowest levels of proliferation (Fig. 5 and Appendix Table 6). This may indicate that the T3 only induces remodeling in the anterior and posterior portions of the intestine, and that the middle region either requires a higher dosage of T3 or is unresponsive to T3. The different regions of T3 treated intestines were compared to each other, but no statistical significances were observed (Appendix Table 8).

Ishizuya-Oka et al. (1997a) determined that during natural metamorphosis, the intestine of *X. laevis* remolds along the anterior-posterior axis. Proliferation of the adult epithelium (AE) increases in the anterior region of the intestine in comparison to the posterior during the early periods of metamorphosis (NF 59/60). Simultaneously, the larval epithelium (LE) in the posterior intestine experiences an increase in apoptosis. As metamorphosis continues (NF 62/63), AE proliferation increases in the posterior and decreases in the anterior region of the intestine. The number of apoptotic cells in the LE begins to increase in the anterior and decrease in the posterior intestine (Ishizuya-Oka et al., 1997a).

My results on precociously induced metamorphosis bear some resemblance to the findings of Ishizuya-Oka et al. (1997a). Inducing precocious remodeling of *X. laevis* intestine results in an increase of proliferation in the anterior region of the intestine, and as T3 treatment progresses, proliferation levels decrease in the anterior and increase in
the posterior. Furthermore, a shortening of the intestine was observed in response to T₃
treatment, which is characteristic of natural metamorphosing X. laevis tadpoles.

A dose response test would have proven useful in determining the concentration of T₃
that induces precocious metamorphosis but does not interfere with survival rate. The
number of incidences of death increased the longer the tadpole is incubated with T3, It is
important to note that different parts of the tadpole respond differently to T₃. For
example, in the early events of tadpole development, such as limb growth and DNA
replication in the brain, the T₃ and TRβ levels are low. On the other hand, during the
climax of metamorphosis when the gill and tail are absorbed and the intestine remodells,
both T₃ and TRβ levels dramatically increase (Schreiber et al., 2001). Schreiber et al.
(2001) used the same concentration of T₃ (10nM) that I did but in their case the tadpoles
survived for at least 7 days. I am uncertain why my tadpoles had a high incidence of
death.

II. **Comparisons Between Anatomical Gut Development of E. coqui and X. laevis:**

The biphasic developer X. laevis utilizes two different feeding habits to accommodate
its two distinct developmental phases. The tadpole of X. laevis is herbivorous and feeds
on debris and algae. During metamorphosis, the emerging adult is carnivorous and feeds
on insects (Smith et al., 2000). E. coqui, as mentioned previously, does not have a
tadpole stage, and its nutrient source throughout development is yolk. Due to the
different intestinal functions and developmental modes of the two anurans, I
hypothesized that their gut development would differ with respect to coiling, size, and
composition.
A. Coiling and Size:

1. *X. laevis*:

Initially at NF 40/41, the gut of *X. laevis* is a thick tube lacking coils (Chalmers and Slack, 1998). On day four of development (NF 44/45), the intestine greatly elongates and forms a looped structure. Chalmers and Slack (1998) define a coil as two loops. The following day (NF 45/46), the intestine forms a double-coiled structure. The first coil loops twice counterclockwise. At the end of the first coil, the intestine starts another coil clockwise forming a smaller internal coil (Chalmers and Slack, 1998). At the end of the second coil, the intestine turns and runs posteriorly along the dorsal wall of the body cavity. A NF 46/47 tadpole contains the maximum number of coils, and tadpole intestinal development is complete (Chalmers and Slack, 1998). The tadpole gut of *X. laevis* is extremely thin and long. The extensive coiling obscures the length of the gut. During metamorphosis the intestine uncoils and apoptosis of the larval epithelium produces a significantly shorter intestine (Ishizuya-Oka *et al.*, 1997a). The adult intestine is a thin tube that is only 10% the length of the tadpole intestine (Smith *et al.*, 2000).

2. *E. coqui*:

Up until TS 11, there are no twists or loops in the gut of *E. coqui*. The gut is a yolk filled sac with no distinct morphology. At TS 11, the anterior end of the gut twists approximately 90° clockwise, and the posterior end twists approximately 90° counterclockwise (Fig. 6). The gut twists again in the same fashion in a TS 12 gut, and also in a TS 13 gut. It is at this point that the gut reaches its maximum amount of coiling (Fig. 6). In comparison to the *X. laevis* gut, the gut of *E. coqui* contains two loops, one
starting at the anterior end facing the ventral side, and the second starting at the posterior end facing the dorsal side (Table 3). According to Chalmers and Slack’s (1998) definition of a coil, the gut of *E. coqui* makes only one complete coil of two loops. The gut of *X. laevis* makes two complete coils of four loops total. The gut of *E. coqui* contains half as much coiling as *X. laevis* (Table 3).

The transition into TS 14 produces a gut similar to TS 13 but containing folds in the anterior and posterior ends of the gut (Fig. 6). The folds of the gut might be compared to the missing loops in *E. coqui* compared to *X. laevis*. I believe that they are not loops but rather an unfolding of the gut to create the adult form. The ventral and dorsal loops created by the anterior and posterior ends are absent in a TS 14/15 gut. The gut becomes more linear in the later stages of development. Throughout development, the gut of *E. coqui* is much wider than that of *X. laevis* due to the presence of yolk. The anterior and posterior tubes of *E. coqui* are comparable in width to the gut of *X. laevis*. The adult gut is a thin tube similar in appearance to the adult form of *X. laevis* (Table 3).

B. Composition:

The different modes of development, biphasic and direct, modified the contents of the gut during development. *X. laevis* contains a small amount of yolk cells early on in development, which are depleted before the formation of the tadpole gut (Table 3) (Smith *et al.*, 2000). The tadpole gut is yolk-free and functions similarly to the adult form (Ishizuya-Oka *et al.*, 1997b). *E. coqui*, on the other hand, contains a large amount of yolk, and a yet to be identified green substance. The yolk is the embryo’s main nutritional source, and the yolk reserve is emptied upon reaching the adult form of the
gut. Throughout the developmental stages, the yolk is broken down and processed for nutrients (Table 3). The depletion of yolk is first evident by a disassociation of yolk from the epithelium and other yolk cells in a TS 12 gut. Packard et al. (1996) analyzed dry yolk mass in *E. coqui* embryos and concluded that yolk mass declines steadily throughout development. The greatest decrease in yolk mass is in the last one third of embryogenesis, TS 12-15.

Beginning at TS 15, a green substance is found in the center of the yolky gut (Fig. 6), which I propose is biliverdin. Biliverdin is a bile pigment derived from hemoglobin degradation. Erythrocytes contain a large amount of the protein hemoglobin, the universal oxygen carrying protein in vertebrates. Oxygen loading and unloading is carried out through changes in the structure of the protein (Vander et al., 1998). During vertebrate development, oxygen requirements change and new hemoglobins with different properties are produced. Hemoglobin switching in mammals occurs at the transition from an embryo to a fetus and again from a fetus to an adult (Collins and Weisman, 1984; Grosveld et al., 1993).

In metamorphosing amphibians, hemoglobin switching coincides with metamorphic change and is governed by the thyroid hormone (Johansen and Lenfant, 1972). An aquatic lifestyle requires globin with higher oxygen affinity because of the lower oxygen levels in water (Johansen and Lenfant, 1972). When the tadpole develops into a terrestrial frog, its requirements change. It needs a lower oxygen affinity globin in order to ensure a more efficient unloading of the oxygen in the tissue level. The frog, *Rana catesbelana*, has been show to have this physiological pattern of hemoglobin switching at metamorphosis (Johansen and Lenfant, 1972).
When there is a switch in hemoglobin type, the old hemoglobin is destroyed. The destruction of hemoglobin occurs in the liver, and results in the formation of bile pigments. The bile pigments are then secreted by hepatocytes to the gall bladder and released into the small intestine via the bile duct (Vander et al., 2001). Biliverdin is a green bile pigment formed from the destruction of the heme group of hemoglobin (Vander et al., 1998). Tan (1996) stated there is at least one globin transition during *E. coqui* development from a larval-like globin to an adult like one. Two different results were found by Tan (1996). In the first, the switch to adult hemoglobin was not observed. Froglet hemoglobin was retained four to five weeks after hatching. The second set of results indicated that the beginning of the switch in globin protein is between 20-26 days after hatching, corresponding to the absorption of the yolk sac. I propose that prior to TS 15, there is a hemoglobin switch resulting in the destruction of primitive hemoglobin. It is broken down to biliverdin and released into the intestine of *E. coqui*. In support of this proposal, the gall bladder of *E. coqui* is a similar green color to that found in the gut.

**III. Proliferation Pattern Comparisons Between *E. coqui* and *X. laevis*:**

The adult gut of *X. laevis* is formed by a distinctive pattern of cellular proliferation and apoptosis along the anterior-posterior axis. Ishizuya-Oka et al. (1997b) outlined the proliferation pattern that occurred during metamorphosis. At NF 60, the adult epithelium of the anterior region of the intestine begins to proliferate, and proliferation continues at NF 61. The proliferation rate in the anterior intestine at this point is much higher than in the posterior. As metamorphosis continues, posterior adult epithelium, begins to
proliferate rapidly, and surpasses the anterior at NF 63. By NF 64, the larval epithelium is completely replaced by the adult epithelium, and intestinal remodeling is complete (Ishizuya-Oka et al., 1997a).

The gut proliferation pattern of *E. coqui* is different than the proliferation pattern of *X. laevis*. The development of *E. coqui* gut does not experience a directional proliferation pattern, but rather a regional proliferation pattern. Before the formation of the thyroid gland, at TS 9 (Jennings and Hanken, 1998), proliferation levels are high in all of the gut regions. The thyroid gland forms at TS 10 (Jennings and Hanken, 1998), and the proliferation levels in regions 1-6 continue to show no discernable pattern (Table 2). The anterior and posterior tubes, however, continue to have high levels of proliferation (Table 2).

A hypothesis can be formulated from these results. It is possible that regions 1-6 (Table 2 and Figs. 7, 8 and 9) are not under thyroid hormone control at this time, and therefore T_3 does not affect their levels of proliferation. The only regions affected by T_3, in this case, are the anterior and posterior tubes. T_3 maintains high levels of proliferation in the tubes throughout development. In order to test this hypothesis, *in situ* hybridization using a probe for TRβ, could be performed. If high levels of TRβ RNA are only found in the tube regions of TS 10-12 guts, my hypothesis would be supported. If TRβ RNA is found at high levels throughout the gut from TS 10-12, my hypothesis would be rejected. A second experiment, inhibition of T_3 production by methimazole, would aide in determine the regions of *E. coqui* gut that are or are not controlled by T_3. Methimazole inhibits the synthesis of T_3 (Callery and Elinson, 2000). If a similar proliferation pattern occurs in regions 1-6 of methimazole treated guts, it is possible that
these regions are not under control of T₃. If the proliferation pattern of the tubes is affected by T₃ inhibition, it is reasonable to conclude the tube regions are governed by T₃. Furthermore, if exogenous T₃ is introduced early in *E. coqui* gut development, and development remains unaffected, it is possible that T₃ is not playing a significant role in gut development. If the gut experiences induced morphological and molecular changes, it could be concluded that TH is involved in *E. coqui* gut development.

TS 13 proved to be the most interesting stage due to its dramatic decrease in proliferation levels throughout all regions of the gut. It is during this time that the gut reached its maximum amount of coiling; yet it had the least amount of proliferation (Fig. 6 and Table 2). The question could be raised: Could the embryo reduce proliferation to allow cell movement? If so, is the decrease in proliferation due to T₃? It is possible that regions 1-6 are not under T₃ control until TS 13. At this time, T₃ may act as a down-regulator of proliferation to allow for coiling of the gut. To examine this further, another round of *in situ* hybridization could be performed to determine if there is an increase or decrease of TRβ RNA in regions 1-6 as compared to previous stages. A change in the level of TRβ RNA levels could indicate whether or not T₃ is affecting the proliferation pattern.

The transition to TS 14 doubles the amount of proliferation in regions 1-6 of the gut compared to TS 13 (Table 2). The anterior and posterior tubes continue to have high levels of proliferation. It is unclear what is causing the dramatic increase of proliferation from TS 13 to TS 14. A change in TRβ RNA levels could be responsible for than increase in proliferation between these two stages, or there may be a change in sensitivity
to T3 levels. The remaining developmental stages TS 15-156 show a decrease in proliferation in all gut regions, including the tubes (Table 2).

T3 has varying effects on different organs during *X. laevis* development (Smith *et al.*, 2000). T3 could also have varying effects on different regions of the same organ. During *E. coqui* gut development, T3 may be stimulating proliferation in the anterior and posterior tubes of the gut while suppressing proliferation in the rest of the gut. There are two possible ways that proliferation could be regulated by T3. The first is by selective regional regulation. T3 binding to TRβ in regions 1-6 would signal proliferation to decrease. Alternatively, the binding of T3 to TRα in either the anterior or posterior tubes may trigger proliferation to occur. It is also possible that the amount of TRβ present in each of these areas is affecting the proliferation levels. The anterior and posterior tubes may contain more TRβ, which would allow more T3 to bind leading to an increase in proliferation. The other regions of the gut may have low levels of TRβ, therefore, not as much T3 is able to bind and stimulate proliferation. The inhibition of T3, by methimazole, would determine if T3 has varying effects on the gut. Proliferation in the anterior and posterior tubes would decrease, while the proliferation in regions 1-6 would increase if regional regulation is occurring.

V. **Future Direction:**

Gut development of *E. coqui* is relatively unexplored. In order to fully understand how the gut develops other experiments must be conducted. In this thesis, the proliferation pattern of the gut of *E. coqui* has been determined; however, it would also be beneficial to determine the apoptotic pattern of the gut and to compare these findings
to *X. laevis*. Does it follow the anterior posterior axis? Is the apoptotic pattern similar to the proliferation pattern of *E. coqui*? Examining the expression of molecular gut markers, such as IFABP and endodermin, could provide insight to cell differentiation and organ development.

Furthermore, it is unknown what regions of the gut are composed of nutritive or definitive endoderm (Arendt and Nübler-Jung, 1999). Creating fate maps with substances, such as FDA or DiI, could identify the fate of the endodermal cells and categorize them as either nutritive or definitive endoderm. Lastly, determining if the green substance present at TS 15 is indeed biliverdin by biochemical assays would not only provide further insight on gut development of *E. coqui*, but also in hemoglobin switching.
REFERENCES


### Table 4: Two day TH treated intestines measurements and cell counts.

Three TH treated and three control intestines total length and width were measured for each region, anterior, posterior, and middle. The number of mitotic cells was counted for each region and divided by the SA in order to determine the total number of mitotic cells per SA.

<table>
<thead>
<tr>
<th>Two Day Intestine</th>
<th>Total length (mm)</th>
<th>Position</th>
<th>Width (mm)</th>
<th>Length (mm)</th>
<th>Surface Area ((\text{mm}^2)^2)</th>
<th># Mitotic Cells</th>
<th>Cells per SA ((\text{mm}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>#1</strong></td>
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<td></td>
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<td>0.494</td>
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</tr>
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<td>0.95</td>
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</tr>
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<td></td>
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</tr>
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<td>Width (mm)</td>
<td>Length (mm)</td>
<td>Surface Area (mm$^2$)$^2$</td>
<td># Mitotic Cells</td>
<td>Cells SA (mm$^2$)</td>
</tr>
<tr>
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<td>------------------</td>
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<td>------------</td>
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<td>1.064</td>
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<td>50</td>
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<tr>
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<td>0.95</td>
<td>1.083</td>
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<td>34.2</td>
</tr>
</tbody>
</table>

**Table 5:** Three day TH treated intestines measurements and cell counts. Three TH treated and three control intestines total length and width were measured for each region, anterior, posterior, and middle. The number of mitotic cells was counted for each region and divided by the SA in order to determine the total number of mitotic cells per SA.
<table>
<thead>
<tr>
<th>Thyroid Hormone Treated</th>
<th>Day Four Intestine</th>
<th>Total length (mm)</th>
<th>Position</th>
<th>Width (mm)</th>
<th>Length (mm)</th>
<th>Surface Area (mm²)²</th>
<th># Mitotic Cells</th>
<th>Cells SA (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>#1</strong></td>
<td>4.14</td>
<td>Anterior</td>
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<td>0.95</td>
<td>0.513</td>
<td>27</td>
<td>50.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Middle</td>
<td>0.30</td>
<td>1.9</td>
<td>1.14</td>
<td>19</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>Posterior</td>
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<td>0.464</td>
<td>61</td>
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<td>4.72</td>
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<td>0.95</td>
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<td></td>
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<td>0.570</td>
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<td>20.7</td>
<td></td>
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<td></td>
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<td>0.95</td>
<td>2.128</td>
<td>66</td>
<td>124.1</td>
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<tr>
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<td>Anterior</td>
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<td>0.95</td>
<td>0.532</td>
<td>6</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Middle</td>
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<td>1.9</td>
<td>0.513</td>
<td>45</td>
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<tr>
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<td></td>
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<td>2.052</td>
<td>22</td>
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<td></td>
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<td></td>
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<td>1.501</td>
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<td>4.7</td>
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</tr>
<tr>
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<td>Anterior</td>
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<td>0.95</td>
<td>0.76</td>
<td>36</td>
<td>47.4</td>
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</tr>
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<td></td>
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<td>1.634</td>
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<td>8.6</td>
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</tr>
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<td></td>
<td></td>
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<td>0.95</td>
<td>0.475</td>
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<td>0</td>
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</tr>
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<td>Anterior</td>
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<td>17</td>
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<tr>
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<td>1.045</td>
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<td></td>
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</table>

**Table 6**: Four day TH treated intestines measurements and cell counts. Three TH treated and three control intestines total length and width were measured for each region, anterior, posterior, and middle. The number of mitotic cells was counted for each region and divided by the SA in order to determine the total number of mitotic cells per SA.
<table>
<thead>
<tr>
<th>Region</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.33</td>
</tr>
<tr>
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</tr>
<tr>
<td>Posterior</td>
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<td>0.06</td>
</tr>
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</table>

*Table 7:* p values for the anterior, middle, and posterior regions of *X. laevis* intestine. A p value less than 0.05 (*) is statistically significant. P values were calculated with [http://www.graphpad.com](http://www.graphpad.com).
<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th></th>
<th>Day 3</th>
<th></th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior/Posterior</td>
<td>Middle/Posterior</td>
<td>Anterior/Middle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>116.0</td>
<td>61.0</td>
<td>30.6</td>
<td>51.0</td>
<td>116.0</td>
</tr>
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<td>52.1</td>
<td>10.3</td>
<td>10.2</td>
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<td>52.1</td>
</tr>
<tr>
<td>Standard Error mean (SEM)</td>
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<td>5.9</td>
<td>13.2</td>
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<tr>
<td>p value</td>
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<td>0.3028</td>
<td>0.1739</td>
<td></td>
<td></td>
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</tbody>
</table>

| Mean  | 130.7 | 97.9 | 60.3 | 97.9 | 60.3 | 130.7 |
| Standard deviation (SD) | 75.5 | 75.4 | 25.9 | 75.4 | 75.5 | 25.9 |
| Standard Error mean (SEM) | 43.6 | 43.5 | 14.9 | 43.5 | 43.6 | 14.9 |
| p value | 0.2131 | 0.3165 | 0.1477 |       |       |

| Mean  | 44.5 | 77.9 | 19.8 | 77.9 | 44.5 | 19.8 |
| Standard deviation (SD) | 30.2 | 56.2 | 2.7 | 56.2 | 30.2 | 2.7 |
| Standard Error mean (SEM) | 17.5 | 32.4 | 1.6 | 32.4 | 17.5 | 1.6 |
| p value | 0.1573 | 0.2216 | 0.3051 |       |       |

*Table 8: Two-tailed paired T-test results comparing the different regions of TH treated X. laevis intestines. A p value less the 0.05 is statistically significant. Three samples were taken for each day of treatment. p values were determined by using [http://www.graphpad.com](http://www.graphpad.com).*