The ability of a species to adapt to changes in habitat is a prerequisite for survival. Adaptation to different environments is frequently accomplished by exploiting novel food sources. The feeding strategy of an organism is closely associated with features of its digestive organ morphology. Changes in feeding strategy are often accompanied by marked changes in digestive organ morphology and the evolution of novel features in digestive anatomy. Like the larvae of the African Clawed Frog, *Xenopus laevis*, most anuran tadpoles are omnivorous filter feeders and have a rudimentary stomach, as well as very long intestines with numerous coils. However, the larvae of the South American frog *Lepidobatrachus laevis* are obligate carnivores and have a digestive tract with a well-developed stomach and much shorter intestines with relatively little coiling. Embryos of this remarkable carnivorous South American frog provide a unique opportunity to study the evolution of novel features of digestive organs in anuran larvae. Here we investigate the molecular mechanisms that underlie the dramatic variation in digestive organ morphology in two anuran larvae with distinct feeding strategies. A chemical genetic approach was used to determine which signaling pathways were modified during the evolution of carnivore-like gut features.

Treating *Xenopus* embryos with compounds that inhibit retinoic acid signaling results in the development of foregut features similar to those of
*Lepidobatrachus* embryos, including an elongated gastroduodenal loop, as well as displacement of pancreatic tissue. Reciprocally, treating *Lepidobatrachus* embryos with ectopic retinoic acid induces the development of a foregut that more closely resembles that of *Xenopus* embryos, with a shortened gastroduodenal loop and more apparent pancreas tissue.

*Pitx2* is an evolutionarily conserved transcription factor involved in left-right specification and its expression domain is shifted posteriorly in *Lepidobatrachus* embryos, corresponding with the position of the more elongated gastroduodenal loop. Upon treatment with retinoic acid the *Pitx2* expression domain is shifted anteriorly whereas treating *Xenopus* embryos with a retinoic acid synthesis inhibitor results in the posterior expansion of the *Pitx2* expression domain. The shift in the *Pitx2* expression domain in the treated embryos correlates with the modification of foregut morphology in embryos exposed to small molecule modifiers of retinoic acid synthesis and signaling.

These results suggest that the evolution of the derived digestive anatomy in carnivorous *Lepidobatrachus* larvae may have been caused by alterations in retinoic acid signaling which, in turn, had an effect on the position of the *Pitx2* expression domain and the shape of foregut anatomy, including the gastroduodenal loop and pancreatic tissue.
A Small Molecule-Mediated Chemical Genetic Screen Reveals a Role for Retinoic Acid in Anuran Gut Evolution

by
Stephanie K. Bloom

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science

Comparative Biomedical Sciences

Raleigh, North Carolina
October 27, 2010

APPROVED BY:

_______________________________  ______________________________
Dr. Nanette Nascone-Yoder       Dr. Jonathan M. Horowitz
Committee Chair                   Minor Representative

_______________________________  ______________________________
Dr. James W. Mahaffey             Dr. Heather B. Patisaul
DEDICATION

For my parents, Valerie and Stephen Bloom.

Their never-ending love, encouragement, and support has been extraordinary and never ceases to amaze me.

In remembrance of Eugene H. Bloom, Robert E. Flick, and Virginia Epps.

Through their own examples, they instilled in me a sense of discipline and dedication towards my work.

Because of all of you I am always inspired to do my best.

I hope to make you proud.
BIOGRAPHY

I was born in the Commonwealth of Virginia to Stephen and Valerie Bloom and was raised in the small rural town of Emporia. Growing up I enjoyed playing the piano and tennis. My affection for science did not materialize until I was in high school, thanks to two fantastic science teachers, Mrs. Kern and Mrs. Wrenn. In the eleventh and twelfth grades I attended the Southside Regional Governor’s School for Global Economics and Technology, a half-day magnet school program with advanced classes and the opportunity to earn college credits while taking classes necessary to fulfill high school requirements. I graduated from Greensville County High School in 1999 and chose further my education at North Carolina State University. I graduated from NC State in 2003 and accepted a research technologist position in Research Triangle Park. After several years I decided to pursue an advanced degree and was accepted into the Comparative Biomedical Sciences Program at the NC State College of Veterinary Medicine in 2006. After joining the lab of Dr. Nanette Nascone-Yoder I began research into the molecular mechanisms involved in the embryonic development of the digestive system.
ACKNOWLEDGMENTS

I would like to start by acknowledging my advisor Dr. Nanette Nascone-Yoder for her support and guidance. Thank you for everything you have taught me both in and out of the lab.

In addition, I would like to thank the members of my advisory committee Dr. Jon Horowitz, Dr. Jim Mahaffey, and Dr. Heather Patisaul. Your input and participation has been greatly appreciated.

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And last, but certainly not least, I would like to thank the many family and friends who have provided encouragement and support, both professional and personal, especially, Vicki Flick, Louanne Flick, Brandon Mangum and Allison Morckel. I am truly grateful.

I couldn’t have done it without you!
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<th>Full Form</th>
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<tbody>
<tr>
<td>BBBA</td>
<td>Benzyl Benzoate:Benzyl Alcohol</td>
</tr>
<tr>
<td>BM</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>BMB</td>
<td>Boehringer Mannheim Blocking Reagent</td>
</tr>
<tr>
<td>DEAB</td>
<td>Diethylaminobenaldehyde</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>G</td>
<td>Gosner</td>
</tr>
<tr>
<td>GD</td>
<td>Gastroduodenal</td>
</tr>
<tr>
<td>ISH</td>
<td>In Situ Hybridization</td>
</tr>
<tr>
<td>LHRH</td>
<td>Luteinizing Hormone-Releasing Hormone</td>
</tr>
<tr>
<td>LPM</td>
<td>Lateral Plate Mesoderm</td>
</tr>
<tr>
<td>MAB</td>
<td>Maleic Acid Buffer</td>
</tr>
<tr>
<td>MEMFA</td>
<td>MOPS/EGTA/Magnesium Sulfate/Formaldehyde Buffer</td>
</tr>
<tr>
<td>MMR</td>
<td>Marc’s Modified Ringers Solution</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>N&amp;F</td>
<td>Nieuwkoop &amp; Faber</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature Tissue Freezing Medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PTw</td>
<td>PBS + Tween 20</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic Acid Response Element</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline Sodium Citrate</td>
</tr>
<tr>
<td>ST</td>
<td>Stomach</td>
</tr>
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</table>
INTRODUCTION

The vertebrate gut is one of the longest, largest, and most important organs in the body. The gastrointestinal tract is responsible for digesting food and absorbing the nutrients necessary to support life. During vertebrate embryogenesis, the primitive gut tube is formed by the folding of the embryonic disk into a simple tubular structure. As embryonic development proceeds the primitive gut is transformed from a short, straight tube into an elongated structure that must fold extensively to fit within the visceral body cavity (Moore and Hay, 1973). The primitive gut is divided into three parts, the foregut, midgut, and hindgut. The foregut will give rise to the esophagus, stomach, duodenum, liver, and pancreas. The derivatives of the midgut are the small intestines, except the duodenum, the cecum, appendix, ascending colon, and two-thirds of the transverse colon. The hindgut develops into the last one-third of the transverse colon, the descending colon, the sigmoid colon, and the rectum. During embryogenesis these three different regions are patterned into conserved functional domains in an anterior to posterior fashion (Moore and Hay, 1973; Bayha et al., 2009).

One of the first key morphogenetic events during this process is the formation of a concavity on the left side of the primitive foregut. As the foregut elongates, the curvature of the gastroduodenal (GD) loop is generated. Proper positioning of the GD loop correctly orients the future stomach, duodenum and accessory organs, such as the pancreas and liver. As embryogenesis proceeds, the C-shaped GD loop remains on the embryo’s right side, encompassing the pancreas. The midgut then
undergoes extensive lengthening. As the gut elongates and matures portions of the gut tube acquire asymmetric bends and undergo stereotypical looping and chiral rotations (Chalmers and Slack, 1998; Lipscomb et al., 2006). This series of looping and rotational events result in the proper positioning of the gut tube within the abdominal cavity. The final configuration of the digestive tract is critical for the proper mechanics of digestion (Chalmers and Slack, 1998; Muller et al., 2003).

The mechanics of digestive function can be affected when the failure or disruption of intestinal looping and rotation during embryogenesis leads to the abnormal positioning of digestive organs. Congenital intestinal malrotations occur very frequently – 1 out of every 500 live births in the United States – and can be life threatening (Liptak, 2006). Emergency surgery is often required to repair intestinal malformations; no other treatment is available (D’Agostino, 2007). Unfortunately, there is limited understanding of the morphogenetic mechanisms necessary for proper intestinal looping and rotation, so the cause of congenital intestinal defects is not known.

Development of the gastrointestinal tract is an intricate process involving numerous signaling pathways and modifications in multiple dimensions (anterior-posterior, dorsal-ventral, left-right, and radially). Determination of the distinct morphogenetic mechanisms that facilitate proper formation of the digestive tract will aid in the understanding of this complex developmental process and may provide insight into the molecular basis of congenital gut defects associated with intestinal malrotations.
Gut Morphology is Related to Diet

The organs of the digestive tract have been conserved throughout vertebrate evolution (Smith et al., 2000). However, the size and shape of digestive organs are known to vary among species (Smith et al., 2000; Caviedes-Vidal et al., 2007; Lavin et al., 2008; Lovegrove, 2010). There is a close association between the feeding strategy of an organism and features of its digestive organ morphology (Wells, 2007; Ledon-Rettig et al., 2008; Lavin et al., 2008). Distinct variations in diet and gut morphology can be seen in several anuran species (Wells, 2007), which develop highly coiled intestines analogous to mammalian intestines (KEMP, 1951).

The vast majority of anuran larvae are herbivorous or omnivorous with a diet consisting of plant matter and microorganisms. These tadpoles have a long, coiled gut specialized for feeding on detritus. However, there are carnivorous anuran tadpoles which feed on animal matter. Some carnivorous tadpoles feed on large prey and possess specialized morphology for a macrophagous diet. Other carnivorous tadpoles do not have specialized morphology and exhibit few adaptations for a macrophagous diet; these tadpoles mostly feed on algae and the eggs of other anuran species (Wells, 2007). Specialized larvae that feed on large particles of animal prey generally have a relatively short, uncoiled gut tube (Wells, 2007; Ledon-Rettig et al., 2008).

Anuran Gut Development

Anurans typically have a biphasic life history. Through the tadpole stages they are aquatic herbivores that feed by filtering very small pieces of algae, plant
matter and detritus from water passed over the gills (McDiarmid and Altig, 1999). They have a primitive stomach and very long, coiled intestines (Fry and Kaltenbach, 1999; McDiarmid and Altig, 1999). Anuran larvae are often described as lacking a “true” stomach as they do not secrete digestive enzymes until metamorphosis, although they do possess specialized tubular glands (McDiarmid and Altig, 1999). In most anurans, the lumen of the simple stomach does not expand, although the stomach wall becomes slightly thicker and more glandular as development proceeds to feeding stages (Smith et al., 2000). The portion of the gut tube posterior to the GD loop, the midgut, elongates extensively and several concentric intestinal coils are formed (Nieuwkoop and Faber, 1994; Chalmers and Slack, 1998; Muller et al., 2003). After metamorphosis and transition to the carnivorous adult form, frogs either continue to live in an aquatic environment or venture to terrestrial habitats (McDiarmid and Altig, 1999).

*Xenopus laevis*, the African Clawed Frog, is an established anuran model in which gut morphogenesis has been described (Nieuwkoop and Faber, 1994; Chalmers and Slack, 1998). Like most anurans *Xenopus laevis* tadpoles are primarily herbivorous and undergo the ancestral transitions seen in most other tadpoles. They have very long coiled intestines which are indistinguishable from other omnivorous tadpoles (McDiarmid and Altig, 1999), making *Xenopus* a good model for representative omnivorous anuran gut morphogenesis.
**Lepidobatrachus laevis**

Unlike *Xenopus*, tadpoles of the unique South American frog *Lepidobatrachus laevis* are predatory carnivores that are also known to be cannibalistic (Ruibal and Thomas, 1988). Once feeding begins the larvae only feed on live prey; ingestion is accomplished by suction and is very rapid. All prey is swallowed whole and the tadpoles are able to ingest prey that is equal to them in size. The ability to ingest such large prey is also known as megalophagy, and this phenomenon has not been seen in other taxa (Hanken, 1992). The distinctive diet of *Lepidobatrachus laevis* tadpoles is accompanied by the development of a digestive tract that is functionally and anatomically highly divergent from that found in the typical tadpole.

*Lepidobatrachus* tadpoles have a large, compartmentalized, J-shaped stomach that can undergo extreme extension to accept prey and a muscular pyloric sphincter. The stomach is adult-like in morphology and histology and has been shown to secrete digestive enzymes (Ruibal and Thomas, 1988; Carroll *et al.*, 1991). *Lepidobatrachus* larvae also have a very short intestinal tract which is only 1.5 times their snout-vent length whereas microphagous tadpoles have very long intestines that are many times longer (9x+ longer) than their snout-vent length (Ruibal and Thomas, 1988; Fry and Kaltenbach, 1999). The digestive morphology observed in *Lepidobatrachus laevis* tadpoles is highly specialized and similar to what is usually seen in adult vertebrates. Because of this, it is assumed that *Lepidobatrachus* evolved from an ancestor with a more typical anuran larva and that the unique characteristics seen in the *Lepidobatrachus* gut represent
specialization and derived characteristics (Ruibal and Thomas, 1988; Hanken, 1992).

**Small Molecule-Mediated Chemical Genetics**

The organs of the digestive tract in omnivorous *Xenopus* tadpoles and carnivorous *Lepidobatrachus* tadpoles are analogous structures because they perform the same function—food digestion. These organs are likely homologous structures because they are brought about by the same developmental processes that existed in a common vertebrate ancestor. When such pre-existing developmental mechanisms undergo modifications, the evolution of morphological novelties in derived species can result (Moczek, 2008). Slight alterations in evolutionarily conserved molecular and genetic developmental mechanisms are known to lead to diverse developmental outcomes (Moczek, 2008). The phenotypically diverse digestive anatomy in *Lepidobatrachus laevis* larvae may have arisen in this way. The goal of these experiments is to identify developmental mechanisms responsible for morphogenesis of the derived digestive anatomy in carnivorous *Lepidobatrachus* larvae.

Because *Lepidobatrachus* is a non-model species there are minimal molecular and genomic tools available to examine their embryonic development. To circumvent this limitation, a chemical genetic approach will be employed where living embryos are exposed to small molecules, or drug-like compounds, that modulate the activity of specific proteins or signaling molecules. In recent years, chemical genetic screens have been used to identify pathways of development by
identifying deviations from normal phenotypes in embryos exposed to small molecules that perturb specific signaling pathways. Anuran embryos are well-suited for these types of experiments since they develop ex vivo, and small molecules can be added to the aqueous growth media. One of the major advantages of this method is that it allows for the evaluation of protein function in real time rather than indirectly by manipulation of genes. It is also beneficial to have temporal control over modulation of gene function when studying developmental events because the timing of protein function is critical.

**Research Focus**

Changes in feeding strategy are often accompanied by novel digestive organ morphology. Most anuran tadpoles, including *Xenopus laevis*, are omnivorous filter feeders and have a rudimentary stomach and very long coiled intestines. However, tadpoles of the South American frog *Lepidobatrachus laevis* are obligate carnivores and have a digestive tract with a large, compartmentalized stomach, and short, uncoiled intestines. The goal of this study is to determine the developmental mechanisms underlying the evolution of the specialized digestive organ morphology seen in *Lepidobatrachus* larvae that allow for the exploitation of a novel food source. It is hypothesized that the evolution of the novel digestive anatomy in *Lepidobatrachus laevis* embryos is the result of an alteration in an existing signaling pathway and that a small molecule-mediated chemical genetic screen can be used to identify the modified signaling pathway(s).
**EXPERIMENTAL METHODS**

**Breedings and Developmental Staging**

*Xenopus laevis* embryos were obtained by *in vitro* fertilization. Ovulation was induced in the female frogs by injection of 700-800 units of human chorionic gonadotropin (hCG, Sigma-Aldrich, St. Louis, MO) into the dorsal lymph sac. The female frogs were then kept at room temperature (~23°C) overnight until egg laying was initiated. The following day, male frogs were sacrificed by injection of 1.0 mL 5% tricaine methanesulfonate (MS-222; Sigma-Aldrich) pH 6.5-7.5 into the abdominal cavity. Whole testes were dissected from the abdominal cavity and cultured in 1.0X Marc’s Modified Ringers (MMR, pH 7.4; 0.1 M NaCl, 2.0 mM KCl, 1 mM MgSO$_4$, 2 mM CaCl$_2$, 5 mM HEPES pH 7.8, 0.1 mM EDTA). Manual egg collection was performed by gently but firmly massaging the belly of the female frog. Eggs were collected into a clean, dry plastic petri dish and fertilized with a fragment of testis. After contact with the sperm, the eggs were covered with 0.1X MMR and reared at 16°C or 23°C. Embryos were sometimes reared at lower temperatures to prolong the time required to reach certain developmental stages. Developmental stages of *Xenopus* embryos were determined using the morphological criteria of Nieuwkoop and Faber (N&F, 1994).

*Lepidobatrachus laevis* embryos were obtained through natural matings of adults collected in Salta, Argentina and maintained in a breeding colony in James Hanken’s Laboratory at Harvard University, Cambridge, Massachusetts. Spawning was induced by injection of both male and female frogs with a luteinizing hormone-
releasing hormone (LHRH) analog (Sigma-Aldrich) at a dosage of 0.1 mg per kg body weight approximately 2 hours before the adult male and female of the selected breeding pair were introduced into a breeding chamber overnight. Embryos were reared at 22°C in 10% Holtfretter’s solution (Prepare 600 mM NaCl, 6 mM KCl, 9 mM CaCl₂ then mix 100 ml with 100 ml 25 mM NAHCO₃ and adjust volume to 1 liter). Developmental stages of embryos were determined using the morphological criteria of Gosner (1960).

**Small Molecule Chemical Treatments**

In collaboration with other members of the Nascone-Yoder laboratory *Xenopus laevis* embryos were exposed to known and unknown small molecules that activate or inhibit various signaling pathways. This method was used to elucidate the genetic and biochemical pathways underlying morphogenesis of the gastrointestinal tract. For this study, the methodology was employed to not merely identify signaling pathways that can perturb gut development, but to pinpoint the signaling pathways that can convert the trophic features of the omnivorous digestive anatomy of *Xenopus laevis* to resemble the morphological novelties that exist in the carnivorous digestive anatomy of the related species, *Lepidobatrachus laevis*.

Late stage *Xenopus laevis* embryos were exposed to multiple concentrations of small molecule chemical compounds at N&F stage 28, prior to initiation of gut looping morphogenesis. Treatment was continued until N&F stage 46 when intestinal looping has been completed. Twenty-one small molecule chemical
compounds were screened, including novel compounds generously gifted by Alex Deiters (North Carolina State University Chemistry Department, compounds denoted by *). Exposure to three of the compounds resulted in aberrant GD looping morphogenesis (Table 1). Of those three compounds all-trans-retinoic acid and diethylaminobenzaldehyde were chosen for further study because they could be used to manipulate the same signaling pathway.
<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Function</th>
<th>Elongated GD Loop</th>
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<tbody>
<tr>
<td>DEAB</td>
<td>RA Synthesis Inhibitor</td>
<td>30%</td>
</tr>
<tr>
<td>R041-5253</td>
<td>RA Receptor Inhibitor</td>
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</tr>
<tr>
<td>Retinoic Acid</td>
<td>Signaling Molecule</td>
<td>-</td>
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<tr>
<td>Rho Kinase Inhibitor</td>
<td>ROCK Inhibitor</td>
<td>-</td>
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<tr>
<td>Rho Kinase Inhibitor IV</td>
<td>ROCK Inhibitor</td>
<td>-</td>
</tr>
<tr>
<td>Cyclopamine</td>
<td>Hh Signaling Inhibitor</td>
<td>25%</td>
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<tr>
<td>Thyroid Hormone</td>
<td>Signaling Molecule</td>
<td>-</td>
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<tr>
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<td>SB-431542</td>
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</tr>
<tr>
<td>B1-61A+</td>
<td>unknown</td>
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</tr>
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<td>B1-61B+</td>
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<td>-</td>
</tr>
<tr>
<td>B1-160+</td>
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</tr>
<tr>
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</table>
All-trans-retinoic acid (RA, Sigma) and diethylaminobenzaldehyde (DEAB, Sigma) were prepared in ethanolic solvents. Stock solutions were diluted to working concentrations in either 0.1X MMR for *Xenopus laevis* treatments or 10% Holtfretter’s solution for *Lepidobatrachus laevis* treatments.

The retinoic acid synthesis inhibitor DEAB was used at concentrations of 4 x 10^{-4} M, 5 x 10^{-5} M, 6 x 10^{-4} M, and 7 x 10^{-4}M diluted from a 1.0M stock solution made in 75% ethanol. *Xenopus laevis* embryos were cultured in 10 ml of each DEAB solution for 24 hours at 16°C beginning at stage 18. Sibling controls were cultured in an appropriate dilution of ethanol alone to control for any teratogenic effects of the solvent. After 24 hours of treatment, embryos were removed from the treatment and washed at least 5 times in 0.1X MMR. Embryos were cultured in 0.1X MMR at 16°C or 23°C until N&F stage 32 or N&F stage 45. At N&F stage 32 some embryos were harvested and processed for in situ hybridization as described below. At N&F stage 45 embryos were fixed in MOPS/EGTA/Magnesium Sulfate/Formaldehyde Buffer (MEMFA, 0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO_{4}, 3.7% formaldehyde) and gut morphology was evaluated.

All-trans RA (Sigma) was used at concentrations of 5 x 10^{-8} M, 1 x 10^{-7} M, 5 x 10^{-7} M, and 1 x 10^{-6} M in 10% Holtfretter’s solution, diluted from a 0.01M stock solution prepared in 50% ethanol. *Lepidobatrachus laevis* embryos were cultured in 100 ml of each RA solution from either Gosner stage 16-19, 17-19, or 18-19. Sibling controls were cultured in an appropriate dilution of ethanol alone to control for any teratogenic effects of the solvent. At stage G19, embryos were removed from treatment and washed at least 5 times in 10% Holtfretter’s. Embryos were
cultured in 10% Holtfretter’s solution at 22°C until stage G19, G20, or G23. At stages G19 and G20, some embryos were harvested and processed for in situ hybridization as described below. At stage G23, embryos were either processed for in situ hybridization or fixed in MEMFA for analysis of gut morphology.

**Whole Mount In Situ Hybridization**

*Pitx2, Nkx2.5, Insulin,* and *Raldh2* expression were detected in both species using *Xenopus laevis* riboprobes. Antisense digoxigenin (DIG)-labeled RNA probes were synthesized from linearized plasmids containing cloned *Xenopus* cDNA sequences of the following genes and the SP6/T7 DIG RNA Labeling Kit (Roche): *Pitx2* cloned into pBluescript II KS was linearized with EcoRI and transcribed with T7 (Muller *et al.*, 2003), *XNkx-2.5* cloned into pGEM3z was linearized with *XbaI* and transcribed with T7 (Smith *et al.*, 2000), *XRaldh2* cloned into pBluescript II KS was linearized with *NotI* and transcribed with T7 (Lipscomb *et al.*, 2006).

The *Insulin* gene was amplified by Polymerase Chain Reaction (PCR) using reverse transcribed cDNA isolated from N&F stage 40 *Xenopus laevis* gut tubes. The *Xenopus laevis insulin* mRNA sequence (GenBank accession number NM_001085882) was used to determine primer sequences (forward primer: 5’- ATG GCT CTA TGG ATG CAG TGT CTG C – 3’; reverse primer: 5’ – CTA GTT GCA GTA ACT CTC CAG CTG GAA G – 3’). The fragment generated from PCR amplification was cloned into pCRII (Invitrogen). In order to prepare the antisense DIG-labeled RNA probe, the plasmid was linearized with *NotI* and transcribed with SP6.

Embryos were fixed in MEMFA for 2 hours at room temperature or overnight at 4°C, gradually dehydrated through a series of methanol washes and stored in
absolute methanol at -20°C until use. Embryos were gradually rehydrated from methanol into 1X PBS + 0.1% Tween-20 (PTw) in a series of washes, followed by several washes in PTw. Embryos were permeabilized in a 10 µg/ml Proteinase K solution and then washed in 0.1M triethanolamine prior to acetylation with 2.5 µl/ml acetic anhydride 2 times. Samples were then rinsed several times with PTw, refixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PTw for 20 minutes, and washed at least 2 more times in PTw. Prehybridization was carried out for at least 6 hours at 60°C in a minimum of 0.5 ml hybridization buffer (50% formamide, 5x SSC, 1 mg/ml Torula RNA, 100 µg/mL heparin, 1x Denhardt’s Solution (for a 100x solution: 20 g/l Bovine Serum Albumin, 20 g/l ficoll 400, 20 g/l polyvinylpyrrolidone), 0.1% Tween 20, 0.1% CHAPS, 0.01 M EDTA). After at least 6 hours, the prehybridization solution was removed and replaced with probe solution (1.0 µg/ml probe in hybridization buffer). Embryos were incubated in probe solution overnight at 60°C.

The probe solution was removed and replaced with 2X Saline-Sodium Citrate buffer (SSC pH 7.0; 0.3 M NaCl, 30 mM disodium citrate dihydrate). Embryos were washed 3 times for 20 minutes in 2X SSC at 60°C followed by 2-20 minute washes in 0.2X SSC at 60°C. Samples were washed several times in Maleic Acid Buffer (MAB pH 7.5; 100mM Maleic Acid, 150 mM NaCl) at room temperature and then incubated in 2% Boehringer Mannheim Blocking Reagent (BMB, Roche) in MAB for 1 hour or more at room temperature. The blocking reagent was removed and replaced with fresh 2% BMB solution and a 1/3000 dilution of alkaline
phosphatase conjugated-anti-digoxigenin Fab fragments (Roche). The embryos were incubated in antibody solution overnight at 4°C.

Embryos were washed at least 5 times for 1 hour each in MAB followed by several short washes in alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM MgCl$_2$, 100 mM NaCl, 0.1% Tween 20, 2 mM levamisole) to inhibit endogenous phosphatases. The last wash was replaced with BM Purple (Boehringer Mannheim Purple, Roche) which turns blue and precipitates in the presence of the antibody conjugated to alkaline phosphatase. The stain was developed quickly at room temperature or more slowly at 4°C. The chromogenic reaction was stopped with a short wash in MAB and embryos were fixed in MEMFA and stored at 4°C.

*Xenopus laevis* and *Lepidobatrachus laevis* embryos are inherently opaque and *Lepidobatrachus* embryos are highly pigmented, both of which interfere with visualization of antibody staining. Upon completion of the in situ hybridization procedure some embryos were bleached to remove pigment and cleared to render the sample transparent. After the chromogenic reaction was stopped with MAB, embryos were rinsed in buffered 70% ethanol at least 5 times. Embryos were incubated in bleaching solution (1.25 ml 20X SSC, 2.5 ml formamide, 2 ml 30% hydrogen peroxide, 45 ml distilled water) for 2+ hours at room temperature under direct light, followed by a 10+ minute wash in methanol. The embryos were then cleared in 2:1 benzyl benzoate: benzyl alcohol (BBBA) which has a refractive index that is a close match to the refractive index of anuran embryos (Klymkowsky and Hanken, 1991). Embryos were then photographed in 2:1 BBBA.
RESULTS

Gut Morphogenesis in the Carnivore *Lepidobatrachus laevis*

Although the anatomy and histology of the larval gut from the carnivore *Lepidobatrachus laevis* have been described (Ruibal and Thomas, 1988), the morphogenetic events that occur during the embryogenesis of the primitive gut tube of a carnivore are unknown. We therefore examined gut morphogenesis in *Lepidobatrachus* from early tailbud through the feeding stage (G19 through G27). In *Lepidobatrachus* embryos, the left-sided concavity of the foregut which produces the gastroduodenal (GD) loop is generated. But, when compared to the digestive anatomy of the omnivorous *Xenopus laevis* tadpoles, the relative position of the concavity is shifted posteriorly along the axis of the gut tube with respect to the prospective stomach and accessory organs (compare Figure 1 A, B to Figure 1 D, E). This shift results in the formation of a more elongated GD loop (Figure 1 D-F), with the stomach positioned in a more transverse orientation. There is also no visible pancreatic rudiment situated within the curvature of the GD loop (Figure 1D, E). As embryogenesis proceeds, a larger segment of the gut tube is positioned anterior to the GD loop in *Lepidobatrachus* than in *Xenopus* (compare Figure 1E, F to Figure 1B, C). In an omnivorous tadpole such as *Xenopus*, the portion of the gut tube posterior to the GD loop (the intestine) expands and elongates, but the stomach remains rudimentary (Figure 1C). In contrast, during development of the gut in the carnivorous tadpole, the prospective intestine does not significantly elongate, but the stomach expands dramatically (Figure 1F).
Thus, during gut development in carnivorous *Lepidobatrachus* tadpoles, the initially straight gut tube loops to the left and then rotates to the right to form a C-shaped GD loop, but the formation of the concavity is shifted posteriorly along the anterior-posterior axis of the gut tube. In addition, the proportion of gastrointestinal tissue contributing to the stomach and intestine is different in each species. In omnivorous *Xenopus* tadpoles, more tissue is allocated to the long, coiled intestine whereas in the carnivorous *Lepidobatrachus* embryo more tissue is allocated to the stomach.
Figure 1. Gut morphogenesis is dramatically different in *Xenopus laevis* and *Lepidobatrachus laevis*. (A-C) Successive phases of *Xenopus laevis* gut development. *Xenopus* tadpoles have a long, coiled gut tube with a short, anterior gastroduodenal (GD) loop, with a relatively large pancreas situated in the middle of the GD loop. (A) N&F stage 41  (B) N&F stage 44  (C) N&F stage 45  (D-F) Comparable phases of *Lepidobatrachus* gut development. *Lepidobatrachus* tadpoles have a shortened, relatively uncoiled gut tube, with a very large, compartmentalized stomach (st), an elongated, more posterior forming GD loop, and no visible pancreas (*) situated in the middle of the GD loop. (D) G20 (E) G23 (F) G27.
Small Molecule-Mediated Chemical Genetics Reveals a Role for Retinoic Acid Signaling in Gastroduodenal Looping Morphogenesis

As part of a phenotypic screen to identify genetic and biochemical pathways underlying morphogenesis of the gastrointestinal tract in *Xenopus laevis* tadpoles, 21 known and unknown small molecule chemical compounds were tested (Table 1). Upon treatment from late tailbud stages (N&F stage 28) through gastrointestinal looping morphogenesis (N&F stage 46), three compounds induced phenotypic changes in *Xenopus* embryos such that their foregut anatomy resembled the foregut anatomy of *Lepidobatrachus* embryos: diethylaminobenzaldehyde (DEAB, Sigma), Ro-41-5253 (Biomol International), and Cyclopamine (Invitrogen) (Table 1). We chose to focus on two of the compounds, DEAB and Ro-41-5253, because they can be used to manipulate the same pathway. DEAB inhibits the synthesis of retinoic acid (RA) and Ro-41-5253 is a soluble antagonist of RA Receptor alpha (RARα; Keidel *et al.*, 1994; for results of the Cyclopamine treatment see Appendix 1).

*Xenopus laevis* embryos exposed to DEAB during early tadpole stages developed an exaggerated GD loop that forms much more posteriorly along the anterior-posterior axis of the gut tube (Figure 2). This topological conformation is strikingly reminiscent of what is seen in a normal or untreated *Lepidobatrachus* embryo and can be induced in a concentration-dependent manner (Figure 3, results pooled for six separate experiments). In 90% of embryos (n=84) treated with 0.7 mM DEAB the topological conformation of the GD loop resembled that of a *Lepidobatrachus* embryo. Whereas treatment with 0.4 mM DEAB elicited the same
phenotype in only 62% of embryos (n=45). Concentration-dependent alterations in GD phenotype were also observed upon treatment with Ro-41-5253 (Figure 3, results pooled from 3 separate experiments). These results indicate that decreased RA synthesis and/or signaling in an omnivorous anuran embryo results in the formation of a posteriorly shifted GD loop resembling that of a carnivore.
Figure 2. Treatment of *Xenopus* embryos with an inhibitor of retinoic acid synthesis transforms the digestive organ morphology so that it resembles a carnivore. *Xenopus* laevis embryos were subjected to an acute chemical treatment of (A) solvent control or (B) 0.70 mM diethylaminobenzaldehyde (DEAB). (C) An untreated *Lepidobatrachus* gut is shown for comparison. The GD loop forms more posteriorly relative to the position of the stomach (st) in the DEAB-treated *Xenopus* embryo (B), which is strikingly similar to the morphology of the carnivorous *Lepidobatrachus* embryo (C).
Figure 3. Transformation of digestive organ morphology upon inhibition of retinoic acid synthesis and signaling is concentration dependent. There is a concentration-dependent effect on gut morphogenesis in omnivorous *Xenopus laevis* embryos after exposure to small molecule retinoic acid synthesis (DEAB) and signaling (Ro-415253) inhibitors. The percentage of embryos with a more posterior forming GD loop, resembling that of a carnivore is indicated for the different concentrations of each small molecule. In order for the foregut digestive anatomy to be scored as carnivore-like the anatomical conformation of the GD loop of the treated *Xenopus* embryo had to resemble the foregut anatomy of the untreated *Lepidobatrachus* embryo, including an elongated GD loop and displacement of the pancreas. Results from the DEAB small molecule chemical treatments were pooled from six different experiments. Results from the Ro-415253 small molecule chemical treatments were pooled from three different experiments. The n values for each group are shown. Embryos exhibiting severely disrupted development (e.g., massive edema, tail curvature), or abnormal, uninterpretable phenotypes are classified as having suffered teratogenic effects from the chemical treatments.
To determine if endogenously decreased RA signaling underlies the posterior shift in GD loop formation in a carnivorous anuran, we amplified RA signaling in *Lepidobatrachus* embryos by subjecting them to an acute treatment of ectopic RA. RA-treated *Lepidobatrachus laevis* embryos formed a more anterior, shortened GD loop (Figure 4). Thus, RA-treated *Lepidobatrachus* foregut anatomy strikingly resembles what is seen in normal or untreated *Xenopus* embryos and can be induced at a high frequency over multiple doses and stages (Figure 5, results pooled from two separate experiments). *Lepidobatrachus* embryos were treated during three slightly different development windows (G16-19, G17-19, G18-19). Embryos treated from stage G16-19 and G17-19 require lower doses of RA to effectively transform the shape of the GD loop so that it more closely resembles what is seen in omnivorous tadpoles. In 60% of embryos (n=5) treated with 0.1 μM RA from stage G16 the topological conformation of the GD loop resembles that of a *Xenopus* embryo, while embryos treated from stage G17 (n=10) exhibit a similar phenotype 90% of the time at the same dose. Embryos treated from stage G18 exhibit a similar phenotype 92% of the time (n=13) when treated with 0.5 μM RA or 1.0 μM RA. These results suggest that increased RA signaling in a carnivorous anuran embryo can result in the formation of a GD loop resembling that of an omnivore. Furthermore, different threshold levels of RA may be required at different points in development to affect placement of the GD loop. Taken together, the findings from the chemical alteration of RA synthesis and signaling in both *Xenopus laevis* embryos and *Lepidobatrachus laevis* embryos imply that altering RA
signaling in the anuran embryo can influence the position of the GD loop along the anterior-posterior axis of the gut tube.
Figure 4. Treatment of *Lepidobatrachus* embryos with ectopic retinoic acid transforms the digestive organ morphology so that it resembles an omnivore. *Lepidobatrachus laevis* embryos were subjected to an acute chemical treatment of (A) solvent control or (B) 0.05 μM RA. (C) An untreated *Xenopus* gut is shown for comparison. The GD loop forms more anteriorly relative to the position of the stomach (st) in the RA-treated *Lepidobatrachus* embryos (B), which is very similar to the morphology of the omnivorous *Xenopus* embryo (C).
Figure 5. Transformation of digestive organ morphology upon exposure to retinoic acid is stage dependent. Exposure to retinoic acid (RA) elicits a stage dependent effect on concentration in carnivorous *Lepidobatrachus laevis* embryos that were exposed to RA at successively later developmental stages. The percentage of embryos with a more anterior forming GD loop is indicated for each concentration of RA. Results were pooled from two different experiments. The n values for each group are shown. Embryos exhibiting severely disrupted development (e.g., massive edema, tail curvature), or abnormal, uninterpretable phenotypes are classified as having suffered teratogenic effects from the chemical treatments.
Final Placement of Pancreatic Tissue is Dependent upon Location of the Gastroduodenal Loop

In addition to GD looping morphogenesis, manipulation of RA signaling also affected placement of pancreatic tissue in both species. In *Xenopus* embryos, the pancreas is situated within the curvature of the GD loop (Figure 6A, B). However, after exposure to DEAB during early tadpole stages the pancreas is displaced from the center of the GD loop and is positioned more dorsally (Figure 6C). There are no visible pancreatic rudiments situated within the curvature of the GD loop in *Lepidobatrachus* embryos (Figure 6D). Instead, the pancreas is positioned dorsal to the stomach and duodenum and can be detected when the digestive tract is bisected (Figure 6E). Treating *Lepidobatrachus* embryos with ectopic RA results in the arrangement of a ventrally positioned pancreas situated within the concavity of the GD loop (Figure 6F).

RA is known to affect the specification and differentiation of pancreatic tissue (Stafford and Prince, 2002; Stafford *et al*., 2004; Martin *et al*., 2005; Lipscomb *et al*., 2006; Pan *et al*., 2007; Bayha *et al*., 2009). To determine whether the specification of the pancreatic tissue was affected in treated embryos, *insulin* gene expression was analyzed by in situ hybridization (ISH). We found that *insulin* is expressed in a slightly more posterior position in *Lepidobatrachus* embryos (Figure 7D) than in *Xenopus* embryos (Figure 7A). However, in *Xenopus* embryos treated with multiple concentrations of DEAB, neither the level nor the anterior-posterior expression domain of *insulin* was altered. Likewise, in *Lepidobatrachus* embryos
treated with multiple concentrations of RA, neither the level nor the anterior-posterior expression domain of *insulin* was altered. These results suggest that the pancreatic tissue is not being re-patterned or re-specified, but that the anatomical shift in the location of the GD loop is what is influencing the final placement of the pancreas.
Figure 6. Modulation of retinoic acid signaling results in abnormal placement of pancreatic tissue in both omnivores and carnivores. (A) In omnivorous Xenopus laevis embryos the pancreas is situated within the curvature of the gastroduodenal (GD) loop and can be characterized by expression of the hormone insulin (B). (C) Treatment of Xenopus with DEAB, an inhibitor of retinoic acid synthesis, causes lateral displacement of the pancreas so that it is no longer situated within the curvature of the GD loop. (D) In carnivorous Lepidobatrachus laevis embryos there is no visible pancreas (*) situated within the curvature of the GD loop. (E) The pancreas is located dorsal to the stomach and duodenum and can be seen in this posterior view of a bisected gut tube, designated by expression of insulin. (F) Treatment of Lepidobatrachus with ectopic RA induces a shift in the location of the pancreas so that it is situated within the curvature of the GD loop.
Figure 7. Pancreas specification is not affected by variations in retinoic acid synthesis and signaling during gut morphogenesis. (A-C) In *Xenopus laevis* embryos subjected to an acute chemical treatment of either (A) solvent control, (B) 0.4 mM DEAB, or (C) 0.7 mM DEAB the expression domain of *insulin*, a marker for pancreas development, does not vary. (D-F) In *Lepidobatrachus laevis* embryos subjected to an acute chemical treatment of either (D) solvent control, (E) 0.1 μM RA, or (F) 0.5 μM RA from stage G16-19 the expression domain of insulin does not vary.
Alterations in Retinoic Acid Synthesis and Signaling Results in a Shift in the Location of the Gastroduodenal Loop Along the Anterior-Posterior Axis of the Gut Tube

The position of the GD loop in *Lepidobatrachus* embryos could be the result of an anterior-posterior shift in the morphogenetic events that control asymmetric gut looping, or could be due to re-patterning of the foregut in carnivorous embryos. To distinguish between these possibilities, we assessed molecular markers of gut patterning in both omnivore (*Xenopus*) and carnivore (*Lepidobatrachus*) embryos, in the context of both normal and altered RA signaling. In omnivores, GD looping occurs in concurrence with the expression domain of *Nkx2.5*, a homeobox transcription factor and conserved marker for the boundary between the stomach and duodenum (Smith *et al.*, 2000). In contrast, in carnivorous *Lepidobatrachus* embryos, the GD loop forms more posteriorly relative to the *Nkx2.5* expression domain (compare Figure 8A and 8C).

In DEAB-treated *Xenopus* embryos, the GD loop forms more posteriorly in relation to the expression of Nkx2.5, similar to *Lepidobatrachus* (compare Figure 8B and 8C). Likewise, the GD loop forms more anteriorly in relation to the expression of Nkx2.5 in RA-treated *Lepidobatrachus* embryos (compare Figure 8D and 8A). These results illustrate that DEAB-treated *Xenopus* embryos phenocopy *Lepidobatrachus* embryos, and that RA-treated *Lepidobatrachus* embryos phenocopy *Xenopus* embryos, not only at the morphological (GD loop) level, but also at the molecular patterning (*Nkx2.5*) level. Thus, it appears that, in the
carnivorous gut, the position of the GD loop along the anterior-posterior axis of the gut tube is altered, but general gut patterning is preserved.
Figure 8. Alterations in retinoic acid synthesis and signaling repositions the gastroduodenal loop but does not affect molecular patterning. The boundary between the stomach and duodenum is indicated by expression of Nkx2.5. (A) In control Xenopus embryos, the GD loop (red arrowhead) is located adjacent to the Nkx2.5 expression domain (black arrowhead). Upon treatment with DEAB, the GD loop is shifted posteriorly with respect to the Nkx2.5 expression domain and resembles what is seen in untreated or control Lepidobatrachus embryos (C). In control Lepidobatrachus embryos (C) the GD loop is located much more posteriorly with respect to the Nkx2.5 expression domain when compared to control Xenopus embryos (A). (D) After treatment with RA, the GD loop is shifted anteriorly and is adjacent to the Nkx2.5 expression domain, similar to what is seen in control Xenopus embryos.
The Expression of a Marker for Left-Right Asymmetric Patterning Influences the Position of the Gastroduodenal Loop

Anatomical asymmetries in internal organs are shaped by left-right asymmetric gene expression in the embryo. Abnormal RA signaling is known to affect left-right asymmetric gene expression in the embryos of multiple vertebrate species, causing bilateral or abolished expression (Wasiak and Lohnes, 1999; Pitera et al., 2001; Niederreither et al., 2001; Lipscomb et al., 2006; Rubin, 2007). Pitx2, a bicoid-related homeodomain transcription factor, is a downstream effector in the signaling pathway leading to the left-right asymmetries of visceral organs, such as the GD loop, and its expression and function are conserved in Xenopus, chick, and mouse (Ryan et al., 1998). Like several other members of the left-right asymmetry signaling pathway, Pitx2 is only expressed on the left side of the embryo in the lateral plate mesoderm (LPM). However, unlike other members of the left-right asymmetric signaling pathway that are transiently expressed prior to the initiation of organogenesis, Pitx2 also exhibits expression in the organs that become lateralized (Muller et al., 2003). In Xenopus embryos, Pitx2 is expressed in the left LPM beginning at Nieuwkoop and Faber (N&F, 1994) stage 24 and continues to be expressed on the left side of the intestine at N&F stage 42 (Ryan et al., 1998), after initiation of the curvature of the GD loop. It has been established that the tissue where the early expression domain of Pitx2 is located is fated to give rise to the GD loop and anterior portion of the midgut (Muller et al., 2003).
To determine whether a shift in the Pitx2 expression domain correlates with the position of the GD loop in Lepidobatrachus, we analyzed Pitx2 expression by ISH. We found that Pitx2 was asymmetrically expressed on the left side of Lepidobatrachus embryos (Figure 9), as in other vertebrates, but Pitx2 is expressed much more posteriorly in untreated Lepidobatrachus embryos than in untreated Xenopus embryos.
Figure 9. The homeodomain transcription factor *Pitx2* is expressed asymmetrically in *Lepidobatrachus* embryos prior to gut looping morphogenesis. (A-B) In *Lepidobatrachus*, *Pitx2* is only expressed on the left side of the embryo, similar to what is seen in *Xenopus* embryos (C-D, black arrowhead). However, the expression domain of *Pitx2* is much more posterior in *Lepidobatrachus* embryos (compare A to C).
To determine whether altered Pitx2 expression could explain the shift in the position of the GD loop, Pitx2 expression was examined in Xenopus embryos treated with DEAB and Lepidobatrachus embryos treated with RA. Treatment of Xenopus embryos with DEAB resulted in the posterior expansion of the Pitx2 expression domain (Figure 9B), correlating with the more posterior curvature of the GD loop in DEAB treated embryos (Figure 2B). Treatment of Lepidobatrachus embryos with RA results in an anterior shift in the expression domain of Pitx2 (Figure 9D), correlating with the more anterior position of the GD loop in RA treated embryos (Figure 4B). These results suggest that RA influences the distribution of the Pitx2 expression domain, which influences the position of the GD loop along the anterior-posterior axis of the gut tube.
Figure 10. Modification of retinoic acid synthesis and signaling affects the expression of Pitx2, a marker for left-right asymmetric patterning. (A) In untreated Xenopus embryos, the Pitx2 expression domain is restricted to a narrow region abutting the branchial arches (white arrow). (B) Upon treatment with DEAB, the expression domain of Pitx2 expands posteriorly, correlating with the more posterior curvature of the GD loop in DEAB-treated embryos (see Figure 2). (C) In untreated Lepidobatrachus embryos, Pitx2 expression is more posterior than in Xenopus embryos (compare to A). (D) RA treatment elicited a dramatic shift in the anterior border of Pitx2 expression, correlating with the more anterior curvature of the GD loop in RA-treated embryos (see Figure 4).
The Position of the Gastroduodenal Loop is Independent of the Domain of Retinoic Acid Synthesis

To begin to address a possible mechanism by which RA signaling might be altered in *Lepidobatrachus*, we looked at the expression pattern of retinaldehyde dehydrogenase (*RALDH2*) in the carnivorous embryo. RALDH2 is the primary enzyme responsible for RA synthesis during vertebrate embryonic development (Niederreither et al., 2001; Chen et al., 2001). In *Xenopus* embryos, *RALDH2* is known to be expressed in the anterior lateral plate mesoderm (LPM), near the region of the prospective gut tube that will give rise to the GD loop (Chen et al., 2001). To determine whether a shift in the pattern or level of *RALDH2* expression occurred during *Lepidobatrachus* evolution to produce a change in the position or levels of endogenous RA and a subsequent alteration in *Pitx2* expression, we examined *RALDH2* expression by ISH in *Lepidobatrachus* embryos. *RALDH2* expression is similar in *Xenopus* and *Lepidobatrachus* embryos (Figure 11A, C). Moreover, treating *Xenopus* embryos with DEAB had no effect on the spatial expression of *RALDH2* in the anterior lateral plate mesoderm (Figure 11B). Treating *Lepidobatrachus* embryos with RA also had no effect on the spatial expression of *RALDH2* mRNA in the anterior LPM (Figure 11D), similar to what has been reported with RA treatment in *Xenopus* embryos (Chen et al., 2001). These results suggest that although the shift in *Pitx2* expression is contingent upon RA, it is independent of the endogenous domain of RA synthesis.
Figure 11. RALDH2 expression is not altered upon modification of RA signaling. (A) In untreated *Xenopus* embryos Retinaldehyde Dehydrogenase 2, *RALDH2*, is expressed in the pronephic anlage and in the anterior lateral plate mesoderm abutting the branchial arches. (B) An acute chemical treatment with 0.7 mM DEAB has no effect on the *RALDH2* expression domain. (C) *RALDH2* is expressed in a similar expression domain in untreated *Lepidobatrachus* embryos. (D) An acute chemical treatment with 0.5 μM RA has no effect on the *RALDH2* expression domain.
DISCUSSION

The goal of this study was to determine the developmental mechanisms underlying the evolution of the specialized digestive organ morphology seen in *Lepidobatrachus* larvae. It was hypothesized that the evolution of the novel digestive anatomy in *Lepidobatrachus laevis* embryos was the result of an alteration in an existing signaling pathway and that a small molecule-mediated chemical genetic screen could be used to identify the modified signaling pathway(s). We have demonstrated that alterations in late embryonic RA signaling may have contributed to the evolution of the novel morphology of the *Lepidobatrachus* foregut.

An examination of the development of the derived digestive anatomy of the carnivore *Lepidobatrachus laevis* has shown that the symmetry of the initially straight gut tube is broken when the curvature of the GD loop is generated, similar to what occurs in primarily herbivorous *Xenopus laevis* embryo. However, the position at which the GD loop forms along the anterior-posterior axis of the gut tube is shifted posteriorly in *Lepidobatrachus* embryos. A pharmacological increase in retinoic (RA) levels, and presumably signaling, altered GD morphogenesis such that the position at which the loop formed was moved anteriorly. The reciprocal experiment in *Xenopus laevis* embryos, where small molecules were used to reduce levels of RA, altered GD morphogenesis such that the position at which the loop formed was shifted posteriorly.
The results of these chemical genetic analyses are consistent with a recently published study in chick embryos examining the effects of RA signaling on the position of organs along the anterior-posterior axis of the embryo (Bayha et al., 2009). Furthermore, investigations into the role of RA signaling during heart morphogenesis have shown that excess RA truncates the anterior portion of the primitive heart tube and enlarges the posterior segment of the heart tube (Niederreither et al., 2001) which is similar to what was seen in the gut tube of *Lepidobatrachus* embryos exposed to ectopic RA. The amount of tissue anterior to the *Nkx2.5* expression domain in RA-treated *Lepidobatrachus* embryos was considerably less than the amount of tissue anterior to the *Nkx2.5* expression domain in untreated embryos.

Retinoic Acid, the active metabolite of vitamin A, is a diffusible signaling molecule that has essential roles in cell growth, differentiation, and tissue patterning during embryogenesis (Blomhoff and Blomhoff, 2006; Niederreither and Dolle, 2008). RA is created in a two-step reaction whereby Vitamin A or retinol is oxidized to retinal by alcohol dehydrogenases (ALDHs). In the second step of the reaction retinal is oxidized to retinoic acid by retinal dehydrogenases (RALDHs; Ross et al., 2000). There are three RALDH enzymes; RALDH2 is the primary enzyme responsible for the synthesis of RA during vertebrate embryonic development (Niederreither et al., 2001; Chen et al., 2001). RALDH2 is expressed in distinct patterns in the embryo during development and the requirement for RA throughout embryonic development can be substantiated by the early lethality of RALDH2 knockout mutants (Niederreither et al., 2001).
We have demonstrated that the disparity in RA signaling in *Lepidobatrachus* is not mediated by a posterior shift in the domain of endogenous RA synthesis by RALDH2 in the lateral plate mesoderm (LPM), implicating more global variations in retinoid availability, or in the reception or transduction of retinoid signaling, as the more likely source of morphogenetic alteration in the carnivorous species.

RA functions through the activation of retinoic acid receptors (RARs), members of the nuclear steroid hormone superfamily. The three RARs (RARα, RARβ, RARγ) act as inducible transcription factors which regulate gene expression as heterodimers in combination with the retinoid X receptors (RXRα, RXRβ, RXRγ). RAR-RXR heterodimers bind to DNA sequences known as Retinoic Acid Response Elements (RAREs) located within the promoters of target genes (Niederreither and Dolle, 2008). The RARs and RXRs are differentially expressed throughout embryogenesis in spatiotemporally restricted patterns which is believed to play an important role in regional RA activity (Pan *et al.*, 2007). Comparing the expression patterns of retinoic acid receptors and retinoid X receptors in *Xenopus* and *Lepidobatrachus* embryos may provide clarification as to how a reduction of RA signaling during embryogenesis may occur in *Lepidobatrachus* larvae.

Retinoic Acid is essential for diverse developmental processes. During embryogenesis, RA excess and deficiency both have severe teratogenic effects on multiple organ systems (Wasiak and Lohnes, 1999; Zile *et al.*, 2000b; Niederreither *et al.*, 2001; Zile, 2001; Blomhoff and Blomhoff, 2006; Niederreither and Dolle, 2008; Bayha *et al.*, 2009), including the digestive system (Plateroti *et al.*, 2008).
For example, RA signaling has been shown to be required for proper pancreas specification in *Xenopus*, mice, chick, and zebrafish (Stafford and Prince, 2002; Kumar *et al*., 2003; Stafford *et al*., 2004; Chen *et al*., 2004; Martin *et al*., 2005). Pharmalogical perturbation of RA in both *Xenopus* and *Lepidobatrachus* embryos resulted in an anatomical shift in the placement of the pancreas. However, the small molecule chemical treatments had no effect on the early expression domain of the pancreas maker *insulin*. These results suggest that the late-stage treatments initiated in these experiments do not have an effect on pancreas patterning or specification but that the shift in the position of the GD loop affects the final placement of the pancreas.

RA has also been shown to perturb gut elongation and intestinal coiling in *Xenopus* and mouse models (Zeynali and Dixon, 1998; Pitera *et al*., 2001; Lipscomb *et al*., 2006). The malformations generated in these studies with RA excess and deficiency depended on the dosage and the developmental stage at which RA signaling was perturbed (Pitera *et al*., 2001), similar to what was seen in *Lepidobatrachus* embryos treated with exogenous RA. When embryos were treated during earlier stages of development, a lower concentration of RA was required to transform the digestive anatomy so that it more closely resembled what was seen in *Xenopus* embryos. When embryos were treated at successively later stages of development higher doses of RA were required to convert the foregut anatomy so that it more closely resembled that of *Xenopus* embryos. These results imply that
different threshold levels of RA may be required at different points during development to affect placement of the GD loop and pancreatic tissue.

The target genes of RA signaling are unclear, however misexpression of genes in the left-right asymmetry pathway has been observed in embryos exposed to excess RA and in embryos with RA deficiencies (Wasiak and Lohnes, 1999; Pitera et al., 2001; Niederreither et al., 2001; Lipscomb et al., 2006; Rubin, 2007). Abnormal RA signaling is known to affect left-right asymmetric gene expression in the embryos of multiple vertebrate species, causing bilateral or abolished expression (Wasiak and Lohnes, 1999; Pitera et al., 2001; Niederreither et al., 2001; Lipscomb et al., 2006; Rubin, 2007). However it is not known whether modulation of RA signaling affects expression of genes involved in the left-right asymmetry pathway during development of the gastrointestinal system, specifically the GD loop.

Pitx2, a bicoid-related homeodomain transcription factor, is a downstream effector in the signaling pathway leading to the left-right asymmetries of visceral organs, such as the GD loop, and its expression and function are conserved in Xenopus, chick, and mouse (Ryan et al., 1998). Like several other members of the left-right asymmetry signaling pathway, Pitx2 is only expressed on the left side of the embryo in the LPM. However, unlike other members of the left-right asymmetric signaling pathway that are transiently expressed prior to the initiation of organogenesis, Pitx2 also exhibits expression in the organs that become lateralized (Muller et al., 2003). In Xenopus embryos, Pitx2 is expressed in the left
LPM beginning at N&F stage 24 and continues to be expressed on the left side of the intestine at N&F stage 42 (Ryan et al., 1998), after initiation of the curvature of the GD loop. It has been established that the tissue where the early expression domain of Pitx2 is located is fated to give rise to the GD loop and anterior portion of the midgut (Muller et al., 2003). Misexpression of Pitx2 mRNA on the right side of the embryo induces the formation of ectopic concavities on the right side of the gut tube (Appendix 2, Muller et al., 2003). In addition, ectopic expression of Pitx2 mRNA posterior to the endogenous expression domain on the left side of the embryo induces the formation of an elongated GD loop, similar to what is seen in Lepidobatrachus embryos (Appendix 2), suggesting Pitx2 is likely responsible for inducing formation of the concavity on the left side of the gut tube that will give rise to the gastroduodenal loop.

Pitx2 expression is left-sided in the Lepidobatrachus LPM, but the expression domain is shifted posteriorly compared to the expression domain in Xenopus embryos. This is consistent with the observed posterior shift in the position of the GD loop in the carnivorous embryos. Exposing Lepidobatrachus embryos to ectopic RA shifts the Pitx2 expression domain anteriorly, while small molecule-mediated inhibition of RA synthesis in Xenopus embryos expands the Pitx2 domain posteriorly. Thus, during evolution decreased RA signaling in the Lepidobatrachus embryo may have posteriorized Pitx2 expression facilitating the transition to larval carnivory in this species.
Retinoic acid excess, as well as, retinoic acid deficiency, are known to affect expression of Pitx2 in the heart (Wasiak and Lohnes, 1999; Zile et al., 2000a). Furthermore, Pitx2 expression has been shown to be responsive to RA signaling in other organs, such as the eye (Matt et al., 2005; Matt et al., 2008), illustrating that subtle shifts in left-right asymmetric gene expression domains could generate variation in amenable asymmetric organs, such as those of the gastrointestinal, cardiovascular and nervous systems.

In addition to derived digestive anatomy, Lepidobatrachus larvae also exhibit striking craniofacial features adapted for megalophagy. Lepidobatrachus possess a massive, laterally expanded head with a wide mouth. In addition, the young tadpoles display aggressive foraging behavior at the onset of feeding (Ruibal and Thomas, 1988; Fabrezi and Quinzio, 2008). Given that both RA and Pitx2 govern craniofacial and pituitary development (Cohen et al. 1999, Liu et al. 2003), and retinoids are capable of causing neurological defects (Gardiner et al., 2003). Global alterations in RA responsiveness could underlie multiple changes in trophic morphology and behavior required for the capture, ingestion and processing of a novel meat-based diet.

The gross morphology of the Lepidobatrachus gut has been described as functionally and structurally adult-like with a glandular fundic portion, a muscular pyloric region with a sphincter (Ruibal and Thomas, 1988) and the production and secretion of digestive enzymes as soon as feeding commences (Carroll et al., 1991). Many typical larval features never form (Carroll et al., 1991; Hanken, 1992) and as
a result the derived features of *Lepidobatrachus* have been recognized as the result of peramorphosis or precocious metamorphosis (Hanken, 1992; Fabrezi, 2006; Fabrezi and Quinzio, 2008). The digestive tract undergoes few changes during metamorphosis with no extensive reorganization which is similar to what is seen in higher vertebrate embryos (chick and mouse) that immediately develop compartmentalized stomachs and are carnivorous at birth (Smith et al., 2000). The retinoid X receptors necessary for heterodimerization with the retinoic acid receptors are also required for dimerization with other nuclear hormone receptors, including the thyroid hormone receptor (Sachs et al., 2000; Niederreither and Dolle, 2008). Elevated expression of thyroid hormone receptor, as might accompany the accelerated metamorphosis of *Lepidobatrachus*, leads to precocious metamorphosis (Sachs et al., 2000) and decreases RA responsiveness in anuran embryos (Banker and Eisenman, 1993). Variation in steroid-hormone signaling dynamics, including RA and thyroid hormone signaling, may have been one mechanism of generating integrated suites of novel, complex phenotypes during amphibian evolution.

**Future Studies**

The results of this work suggest that decreased levels of RA signaling in *Lepidobatrachus* embryos contributes to the specialized digestive anatomy of these larvae. This is not due to differences in the domain of RA synthesis, but may occur through other processes, such as increased degradation of RA. Enzymes from the Cytochrome P450 26A family are required to metabolize RA and like RALDH2, they are expressed in distinct, tissue specific patterns in vertebrate embryos throughout
development (Ross et al., 2000; Niederreither and Dolle, 2008). In *Xenopus* embryos RALDH2 and CYP26A are expressed in non-overlapping, complimentary patterns in multiple organs (Chen et al., 2001; Niederreither and Dolle, 2008), including the gut (Lipscomb et al., 2006). The complimentary expression of the enzymes responsible for RA generation and metabolism are believed to generate localized gradients of RA in defined regions to pattern tissues, such as the gastrointestinal system. Defining the expression pattern of CYP26A enzymes in *Lepidobatrachus* embryos may indicate whether a reduction in RA signaling during embryogenesis could be attributed to increased RA metabolism. Altered levels of RA signaling may also be brought about through less efficient synthesis of RA from endogenous precursors. Quantification of RA levels in *Xenopus* and *Lepidobatrachus* embryos at different developmental stages using specialized high-performance liquid chromatography procedures (McCaffery et al., 2002) may indicate whether this is a possibility.

Previous work has demonstrated a role for RA in left-right patterning and specification during embryogenesis (Wasiak and Lohnes, 1999; Zile et al., 2000b), however it is unclear how this actually occurs. There is no indication of asymmetric expression of RA generating or metabolizing enzymes (e.g., RALDHs or CYP26As). Evidence of cross-talk between RA and other signaling pathways, such as the Fibroblast Growth Factor (FGF) and Transforming Growth Factor β (TGFβ) pathways has been established (Wang et al., 2006; Niederreither and Dolle, 2008; Bayha et al., 2009). Thus, retinoic acid signaling may be involved in the up-regulation of other asymmetrically expressed mediators of the left-right asymmetry
pathway or other signaling pathways. Interestingly, members of both the FGF family (Zile et al., 2000b) and the TGFβ superfamily (Shiratori et al., 2001) are known to be expressed asymmetrically during organogenesis and to be required for asymmetric development.

It would be of great interest to determine the mechanism through which RA regulates left-right asymmetric morphogenesis. Unfortunately, there is limited knowledge of direct transcriptional targets of RA (Niederreither and Dolle, 2008). Elucidating direct and indirect targets of RA transactivation may lead to clarification of the molecular mechanisms leading to left-right asymmetric patterning of the gut.

Preliminary analysis of the epithelial architecture of the digestive tract in *Xenopus* and *Lepidobatrachus* embryos (Appendix 3) indicates that there is an asymmetry in tissue thickness on the left and right sides of the gut tube in the foregut. If RA is involved in early asymmetric patterning of the digestive tract, finding direct and indirect targets of RA signaling would help resolve the mechanism by which it acts. In addition, establishment of RA targets may lead to clarification of the molecular mechanisms leading to aberration in left-right asymmetric patterning of the gut and other asymmetrically positioned organs affected by abnormal RA signaling, such as the heart.
Reference List


APPENDICES
APPENDIX 1.

Inhibition of Sonic Hedgehog Signaling in Omnivorous Anuran Embryos Results in the Formation of Carnivore-Like Foregut Anatomy
Objective

*Xenopus laevis* embryos were exposed to the hedgehog signaling inhibitor Cyclopamine to determine whether hedgehog signaling is required for gastrointestinal morphogenesis.

Experimental Method

*Xenopus laevis* embryos were treated with 1 x 10^{-7} M, 2 x 10^{-7} M, and 4 x 10^{-7} M Cyclopamine (Sigma). Working concentrations of Cyclopamine were diluted in 0.1X MMR from a 5 mM stock solution prepared in 95% ethanol. *Xenopus* embryos were cultured in 10 ml of each solution from N&F stage 28 through N&F stage 46. Sibling controls were cultured in an appropriate dilution of ethanol to control for any teratogenic effect of the solvent. At N&F stage 46 embryos were fixed in MEMFA and gut morphology was evaluated.

Results and Discussion

*Xenopus laevis* embryos exposed to Cyclopamine during gastrointestinal morphogenesis developed an exaggerated GD loop and displacement of the pancreas from within the curvature of the GD so that it was positioned more dorsally (Figure A1-1). The foregut anatomy of *Xenopus* embryos treated with Cyclopamine shares similarities with the foregut anatomy of carnivorous *Lepidobatrachus* embryos. These results indicate that hedgehog signaling may required for morphogenesis of the GD loop and that hedgehog signaling may have
been perturbed during the evolution of the novel digestive anatomy in carnivorous Lepidobatrachus embryos.
Figure A1-1. Treatment of *Xenopus* embryos with an inhibitor of hedgehog signaling perturbs development of foregut anatomy. *Xenopus laevis* embryos were subjected to a chemical treatment of (A) solvent control or (B) 20 μM Cyclopamine. (C) An untreated *Lepidobatrachus* gut is shown for comparison. In Cyclopamine treated embryos the GD loop forms more posteriorly relative to the position of the stomach (st) which is similar to the GD morphology of the carnivorous *Lepidobatrachus* embryo. Cyclopamine treatment also results in displacement of the pancreas (*, B) from within the curvature of the GD loop (A).
APPENDIX 2.

Misexpression of Pitx2 mRNA Induces the Formation of Ectopic Concavities
**Objective**

*Xenopus laevis* embryos were injected with *Pitx2* mRNA during early cleavage stages to analyze the effect of ectopic expression of *Pitx2* during gastrointestinal organogenesis.

**Experimental Methods**

Figure 1:

*Pitx2* mRNA was prepared using the mMessage Machine Kit (Ambion) from a linearized plasmid. *Pitx2* was cloned into pBluescript II KS and the plasmid was linearized with *NotI* and transcribed with SP6 (Muller et al., 2003). Full-length, capped mRNA was mixed in a 4:1 solution with fluorescein-conjugated dextran as a lineage tracer. A pressure microinjector was used to deliver 0.56 ng of *Pitx2* mRNA to the vegetal dorsal right blastomere at the 8-cell stage.

Figure 2:

An inducible *Pitx2* mRNA construct was prepared using the mMessage Machine Kit (Ambion) from a linearized plasmid. The inducible construct was composed of the full-length Pitx2 gene conjugated to the N-terminal region of the glucocorticoid receptor (GR) hormone binding domain and was cloned into pCS2. The plasmid was linearized with *XbaI* and transcribed with SP6. Full-length capped mRNA was mixed in a 2:1 solution with fluorescein-conjugated dextran as a lineage tracer. A pressure microinjector was used to deliver 1.7 ng of *Pitx2-GR*
mRNA to the vegetal dorsal left blastomere at the 8-cell stage. Pitx2 expression was induced at N&F stage 22 by addition of dexamethasone to the tadpole culture media so that the final concentration of dexamethasone was 10 μM. Tadpole media was changed daily and supplemented with dexamethasone to a final concentration of 10 μM. Control embryos were not subjected to treatment with dexamethasone, preventing induction of ectopic Pitx2 expression.

Results and Discussion

When Pitx2 mRNA is injected into the tissue fated to give rise to the right side of the foregut ectopic concavities form on the right side of the gut tube, where the ectopic Pitx2 is expressed as evidenced by the presence of fluorescein dextran (Figure A2-1C, D). However, when water is injected into the same blastomere, the gastroduodenal cavity forms on the left side of the gut tube, in the proper location (Figure A2-1A, B). Studies using inducible Pitx2 mRNA constructs demonstrate that ectopic expression of Pitx2 on the left side of the embryo in a region posterior to the endogenous Pitx2 expression domain results in the gastroduodenal (GD) loop forming more posteriorly along the anterior-posterior axis of the gut tube (Figure A2-2 C, D). In embryos where the ectopic expression of Pitx2 was not induced, GD looping morphogenesis occurs normally (Figure A2-2 A, B).

These results demonstrate that Pitx2 is an important factor for initiating formation of the concavity that will give rise to the gastroduodenal loop on the left side of the embryo. However, the cellular and molecular mechanisms downstream of Pitx2 that lead to the formation of the gastroduodenal loop are unknown. In
addition, there is limited knowledge of the direct transcriptional targets of Pitx2. Establishing transcriptional targets of Pitx2 will aid in the understanding of how Pitx2 induces the formation of the gastroduodenal loop.
Figure A2-1. Misexpression of Pitx2 mRNA on the right side of the embryo induces the formation of ectopic concavities on the right side of the gut tube. *Xenopus laevis* embryos were injected with a solution containing water (A, B) or 0.56 ng Pitx2 mRNA (C, D). Fluorescein-conjugated dextran was co-injected with the control (water) or mRNA to track the solution throughout development (red fluorescence in B, D). In control embryos, the injected solution is localized to the outer curvature of the GD loop (A, B). In Pitx2-injected embryos, the injected solution is localized to the ectopic concavity on the right side of the embryo (C, D). L marks the left side of the embryo and R marks the right side of the embryo. Photographs were taken from a ventral view.
Figure A2-2. Misexpression of Pitx2 mRNA more posteriorly on the left side of the embryo induces the formation of an elongated gastroduodenal loop. *Xenopus laevis* embryos were injected with a solution containing 1.7 ng Pitx2 mRNA (A, B, C, D). Fluorescein-conjugated dextran was co-injected with the mRNA to track the solution throughout development (red fluorescence in B, D). Ectopic Pitx2 expression was not induced in control embryos and GD looping morphogenesis occurs normally (A, B). In embryos where ectopic Pitx2 expression was induced, the gastroduodenal loop forms more posteriorly along the anterior-posterior axis of the gut tube (C, D). L marks the left side of the embryo and R marks the right side of the embryo. Photographs were taken from a ventral view.
APPENDIX 3.

Analysis of Cellular Architecture of *Xenopus laevis* and *Lepidobatrachus laevis* reveals an Asymmetry in the Thickness of the Foregut Epithelium
**Objective**

The cellular architecture of the digestive tract in *Xenopus laevis* and *Lepidobatrachus laevis* embryos was examined.

**Experimental Method**

Untreated N&F stage 33 *Xenopus laevis* and untreated G20 *Lepidobatrachus laevis* embryos were fixed, embedded in Optimal Cutting Temperature Medium (OCT), sectioned, and processed for immunohistochemistry. For a complete description of the protocol see Appendix 4.

**Results and Discussion**

Transverse sections of *Xenopus laevis* and *Lepidobatrachus laevis* embryos frozen in OCT were processed for immunohistochemistry to visualize the outlines of cells in the endoderm of the gut tube (green fluorescence), the mesoderm of the gut tube (red fluorescence), and the nuclei (blue fluorescence). Examination of the epithelial tissue in both species indicated a consistent asymmetry in the thickness of the tissue on the left and right sides of the gut tube in the foregut region. The thickness on the epithelium on the left side of the foregut is reduced compared to the thickness of the epithelium on the right side of the foregut. The difference in tissue thickness on either side of the lumen may contribute to the formation of the curvature of the gastroduodenal loop on the left side of the embryo.
The left side of the foregut epithelium in *Xenopus laevis* and *Lepidobatrachus laevis* larvae is thinner than the epithelium on the right side of the foregut. *Xenopus* and *Lepidobatrachus* embryos were processed for IHC. *Lepidobatrachus laevis* embryos were stained to visualize the outlines of cells in the epithelium (β-catenin, green), the mesoderm surrounding the epithelium (smooth muscle actin, red), and nuclei (DAPI, blue). *Xenopus laevis* embryos were stained to visualize the outlines of cells (E-cadherin, green), the mesoderm surrounding the epithelium (laminin, red), and nuclei (DAPI, blue). The thickness of the epithelium on either side of the gut tube is indicated with a white arrow. Note the difference in thickness on the left and right sides of the gut tube in both species.
APPENDIX 4.

Immunohistochemistry Protocol
**Fixation**

Embryos were transferred to glass vials. All or most of the 0.1x MMR was removed and replaced with ice cold Dent’s fixative (20% Dimethyl Sulfoxide/80% methanol). Contents of the glass vial were mixed by inversion. The Dent’s fixative was replaced at least 5 times over 20 minutes. The vials were then placed at -20°C overnight.

**Embedding**

The glass vials were removed from -20°C and the embryos were allowed to warm to room temperature. The Dent’s fixative was aspirated from the vial and replaced with phosphate buffered saline (PBS). Embryos were rinsed 3 times with PBS, 5 minutes per wash. Once the washes were completed the PBS was removed and replaced with 15% sucrose/15% gelatin in PBS. The vials were placed on a nutator for ~5 minutes and then set at room temperature overnight. The following day the 15% sucrose/15% gelatin in PBS was removed and replaced with 15% sucrose/7.5% gelatin in PBS. The vials were placed on a nutator for ~5 minutes and then set at room temperature overnight.

Each embryo was transferred to a plastic mold. Most of the 15% sucrose/7.5% gelatin in PBS was removed. A small amount of OCT (Optimal Cutting Temperature tissue freezing medium) was applied to each embryo and then removed. The mold was then filled with OCT. Embryos were positioned within the mold and the mold was transferred to dry ice to freeze. Once the blocks were frozen, they were either sectioned immediately or stored at -80°C until sectioning.
Sectioning

The temperature of the cryostat chamber (Leica CM 1850) was set to -20°C (+/-2°C). Blocks were placed in the cryostat chamber to equilibrate to the temperature of the chamber for 2-3 hours prior to sectioning. Several drops of OCT were placed on a specimen disk and the block was positioned onto the disk. The specimen disk and frozen sample were placed under the heat extractor for several minutes. The specimen disk was mounted into the chuck and the section thickness was set to 12 μm. Sections were cut slowly by moving the blade across the block surface with the anti-roll plate down. Sections were collected onto Fisherbrand Superfrost Plus Microscope Slides. Slides were processed immediately after sectioning.

Staining

Once slides were warmed to room temperature they were incubated in acetone for 2 minutes, removed, and allowed to dry thoroughly. Slides were transferred to a coplin jar containing room temperature blocking buffer (2.5 g Casein Hammerstein Grade (MP Biomedicals), 25 mL 2M Tris pH 7.4, 15 mL 5M NaCl, 250 μl Tween 20, 25 mL Lamb Serum, water to a total volume of 500 mL) and incubated for at least 30 minutes. Primary antibodies were diluted into blocking buffer; 3 mL of blocking buffer was prepared for each slide. E-cadherin (mouse monoclonal antibody) and laminin (rabbit polyclonal antibody) were diluted 1:200. Smooth muscle actin (mouse monoclonal antibody) and β-catenin (rabbit polyclonal antibody) were diluted 1:1000. Three mL of primary antibody solution
was pipetted into Antibody Amplifier chambers (IHC World). Slides were paced into each chamber and slides were incubated in primary antibody solution overnight at 4°C on a nutator. The following day, the Antibody Amplifier was removed from 4°C and warmed to room temperature on a rocker.

Slides were washed 2 times in 1X phosphate buffered saline (PBS) + 0.5% Tween 20 (PTw) for 3 minutes each time. Secondary antibody solutions were prepared by diluting the secondary antibodies (goat-anti-rabbit Alexa 555 and goat-anti-mouse Alexa 488)1:2000 in blocking buffer. Secondary antibody solutions were applied to the chambers containing slides and the slides were incubated in secondary antibody solution for 1 hour at room temperature. Slides were washed 2 times in PTw 20 for 3 minutes each time. Slides were washed 2 times in 1X PBS for 3 minutes each time. Slides were washed in Eriochrome Black (0.1% Eriochrome powder in 1X PBS) for 30 minutes. Slides were then washed in Sudan Black (0.1% Sudan Black in 70% ethanol/30% 1X PBS) for 30 minutes. Slides were jetwashed with 1X PBS and washed in PBS for 10 minutes. Slides were blotted dry with Kim wipes and mounted with Prolong Gold Antifade Reagent with DAPI nucleic acid stain (Invitrogen) and 24 mm x 55 mm coverslips. Slides were allowed to cure overnight in the dark at room temperature.

The following day the edges of each slide were sealed with clear nail polish (Sally Hansen Hard as Nails). After polish dried sections were photographed using a Lexica DM5000B fluorescent microscope. Slides were stored at -80°C.