MODELING HUMAN INTESTINAL DISEASE: ONTOGENY OF POSTEMBRYONIC
ZEBRAFISH INTESTINAL MORPHOLOGY

A Thesis
by
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Abstract

MODELING HUMAN INTESTINAL DISEASE: ONTOGENY OF POSTEMBRYONIC
ZEBRAFISH INTESTINAL MORPHOLOGY

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The goal of this study was to establish zebrafish as a model organism for

intestinal motility disorders in humans. Zebrafish provide a high throughput model

that allows for the examination of the intestine throughout the life of an organism.

While the zebrafish intestine shares a high degree of morphological homology with

the human intestine, little is known about the maturation process. To begin to

understand the maturation of the intestine, I characterized its appearance throughout

the larval period and into metamorphosis. I found that the onset of metamorphosis

coincides with a minimum standard length that ranges from 4.4 to 5.2 mm. Using a

stage range of larval and metamorphic specimens, I used histological methods to

follow the distribution of goblet cells, the mucin-producing secretory cell type of the

intestinal epithelium. I found that goblet cells differentiate in a step-wise manner

over the larval and metamorphic periods. I also determined the timing of gut looping

for the intestine, a key morphological difference in the larval and adult intestine. I

found that gut loops appear a short time after metamorphosis begins. In future

studies, we seek to establish an assay for gut motility for studying mutant lines that

have altered intestinal profiles. The zebrafish intestine may provide a robust model

for understanding human intestinal physiology and disease.

Acknowledgments

I would like to thank everyone that contributed to this research study, especially my advisor, committee, and the students of the Kinkel lab for all of their support, contribution, and hard work: Dr. Mary D. Kinkel, Dr. Andrew Bellemer, Dr. Ted Zerucha, Rachel Krizek, Savanna Sheridan, Alena Norton, Latanya Gordon, and Tierney Daw. I thank my fellow graduate students for their support, guidance, and solidarity throughout my experience. I would also like to thank students and faculty in the Zebrafish Core Facility for providing animal care and zebrafish to use in this study. All procedures were approved by the Institutional Animal Care and Use Committee at Appalachian State University.

Dedication

I dedicate this thesis to the memory of Angelina Noluthando Franse, my beloved grandmother, who passed February 2017 along the journey of this endeavor. Born January 18, 1951, she was the embodiment of love, understanding, and care that I hope to continue spread as I move forward in my life. I would also like to dedicate this to the memory of Max Franse, our family's golden doodle, who also passed in July 2017, born January 17, 2004, who provided simple, angelic, unconditional love everyday he was here.

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Introduction

The zebrafish has become a widely used animal model for a variety of different diseases. Traditionally, the zebrafish was used as a developmental model for its transparency and ease of manipulation in early embryonic stages (Kimmel et al., 1995). The embryo is transparent and therefore ideal for genetic and developmental studies following cell fates within a living system in vertebrates. Transparency is a feature the zebrafish maintains in early postembryonic development as well. Studies using the embryonic zebrafish intestine have uncovered a high degree of homology both in cell types and organization of the zebrafish intestine to the human intestine (Figure 1).

Gut motility disorders include a myriad of diseases affecting normal passage of food through the intestine and colon. Motility is a key factor in digestion, energy balance, and homeostasis (Murphy and Bloom, 2006). One way to measure motility is using a gut transit assay. A gut transit assay quantifies the time it takes for food to pass through the entire gastrointestinal (GI) tract. However, to clearly establish normal physiology, the intestine in and of itself needs to be understood. The intestine is a complex structure defined primarily as an epithelial layer of cells interfacing with multiple body systems. In embryogenesis, it begins as a homogenous strip of endodermal tissue, then differentiates into multiple regions and organs. Along the GI tract, there is an epithelial layer lining the luminal side of the intestinal wall, which comes in contact with intestinal contents, i.e. food. The human and zebrafish intestinal epithelium has three primary cell types: enterocytes, goblet cells, and enteroendocrine cells. Motility involves these cell types, but also incorporates communication with enteric neurons, smooth muscle, and vasculature (Cummings and

Overduin, 2007). Therefore, a clear understanding of the functionality and development of the intestinal epithelium provides essential information for studying motility.
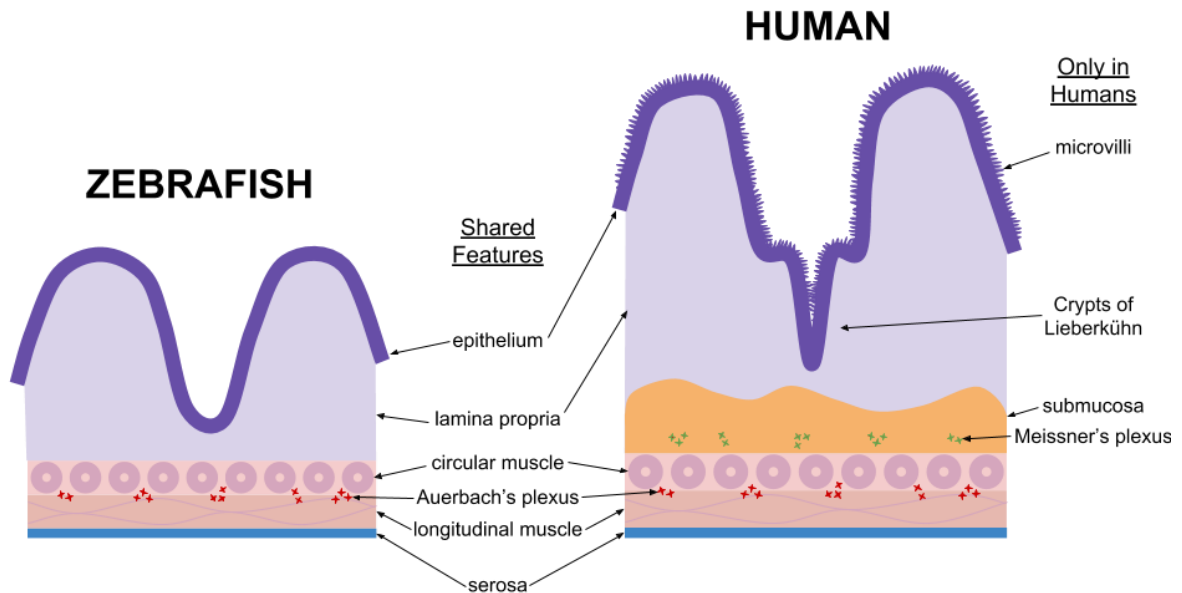
Figure 1. Zebrafish vs. human intestine. High degree of homology between the human and zebrafish intestine. They share major structures and cell types.

The advantages of larval zebrafish for gut function studies include a transparent body wall that allows imaging of the gut contents in live animals. A previous study reported a transit assay that used young larval zebrafish (Field et al., 2009). I propose to build on this work by examining intestinal morphology and the composition of the epithelium that lines the intestinal lumen at specific life stages in wild-type zebrafish. This information will help us to interpret defects that arise in mutant zebrafish with respect to morphology and function of the gut. A gut transit assay that takes into account the specific morphology and epithelial composition of the gut at specific life stages would be much more powerful and informative than simply relying on the young larval stage.

The zebrafish life span is relatively short, ranging between 2-4 years in the laboratory, making it an ideal model for long term disease study. It has four major stages in its life cycle: embryonic, larval, juvenile, and adult (Schilling, 2002). Development is measured in hours post fertilization (hpf) for embryos or days post fertilization (dpf) for young larvae. Major developmental events can be timed early in life, where embryos follow a predictable pattern of growth from fertilization onward. However, this only applies reliably during the embryonic and early larval stages, between 0 hpf and 5 dpf. Once the fish deplete their yolk at 5 dpf and begin feeding externally, they start to show variable growth rates, reaching developmental milestones at different times. This variability in growth rate can complicate studies if fish are sorted by age (Armstrong, 1973; McMenamin et al., 2016; Parichy et al., 2009). Thus, instead of using age, I will use standard length (SL) to track development in postembryonic fish. Standard length is measured from the tip of the snout to the caudal peduncle, the fleshy base of the tail. This method of tracking fish growth is more reliable than calendar age for post-embryonic development (Parichy et al., 2009). Therefore, this experiment will analyze the efficacy of this method for intestinal development tracking.

Metamorphosis is a developmental event marked by rapid changes in the morphology of the entire zebrafish, changing from a larval appearance to a juvenile appearance. This period of transformation is marked by ossification of the primarily cartilaginous skeleton into bone, development of bony adult fins, and the reorganization of major body systems (Parichy et al., 2009). Currently, there is no knowledge of the changes occurring in the intestine during this time. I hypothesize that maturational events in the intestine coincides with metamorphosis. For example, the larval intestine is a straight, relatively simple tube while the adult intestine is more complex and has two loops. Presumably, the morphological

restructuring happens early within the metamorphic period. I would like to figure out the timing and progression of these changes.

In the zebrafish intestine, there are key differences between regions of the intestine apparent early in development, described in more detail below. These regions are not distinguished in the same way as the mammalian GI tract, because the zebrafish intestine lacks sphincters. The region that corresponds with the area of the GI tract from the stomach to the anus in humans is subdivided into three main sections in the zebrafish. There is an anterior section known as the intestinal bulb, similar functionally to a stomach; a middle intestine that contains the loops in adulthood; and finally, the posterior intestine, where food is eliminated. A key difference in the zebrafish intestine is a lack of sphincters clearly subdividing each of these sections (Wang et al., 2010). Nonetheless, there are regional differences that make the anterior, middle, and posterior intestinal regions distinct from one another.

It is generally accepted that there are three distinct regions in the intestine of the larval and adult zebrafish: intestinal bulb, middle intestine, and posterior intestine (Lickwar et al., 2017; Ng et al., 2005; Wallace et al., 2005). This convention is used in describing both the larval and adult intestines, which differ greatly in size and morphology. Grossly, the larval intestine is a straight tube while the adult intestine has two loops (Figure 2A-B). For both life stages, the intestine is open from the esophagus to the anus, with no sphincters separating the three aforementioned regions. By contrast, the mammalian GI tract has four sphincters and one valve separating the oral cavity–esophagus (sphincter), esophagus–stomach (sphincter), stomach–duodenum (sphincter), the ileum–cecum (valve), and the anal canal–external environment (sphincter) (Goyal and Paterson, 1989). At the cellular level, the

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zebrafish intestine has three of the four major cell types present in the mammalian intestine: enterocytes, goblet cells, and enteroendocrine cells (Menke et al., 2011). Paneth cells, the fourth mammalian cell type, are not present in the zebrafish intestine, at least at early stages (Ng et al., 2005). Major details regarding the cell types will be explained later in the literature review.

At the molecular level, the functionality and regionalization of the intestine are clarified in multiple studies of the adult intestine, but not the larval intestine. A diagram of the molecular regionalization illuminates the major conclusions drawn by the primary investigators of the epithelium (Figure 2C). However, given a specimen, both anatomists and cell/molecular biologists would come to different conclusions regarding how to define the boundaries of the regions of the gut. To address these discrepancies, a summary of each largely accepted body of work will be given, then an analysis to determine the presence or absence of a consensus will be conducted.
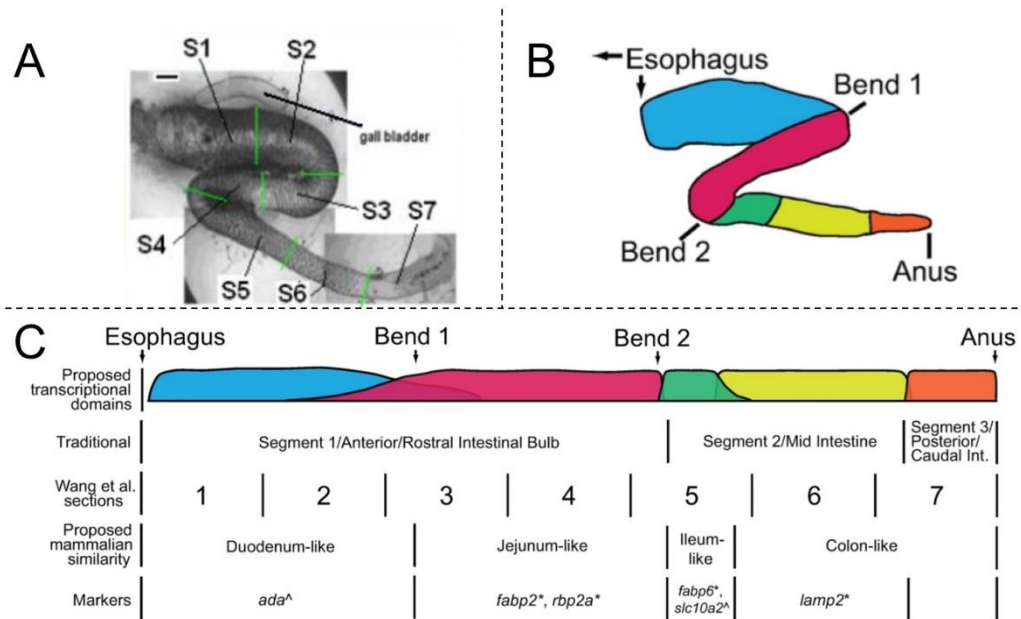
Figure 2. Regionalization studies for the adult intestine. Comparison of regionalization of the adult intestine across multiple studies **(A)** Segments S1-S7 used to divide the intestine for morphological and histological analysis of the organization of the intestine. S1-S5: intestinal bulb, S6: middle intestine, S7: posterior intestine. **(B)** 5 intestinal regions defined for similar transcriptional analysis. **(C)** Overlay of the two systems of demarcation, showing the analogue to the traditional segments, the mammalian small and large intestine, and prominent markers expressed in each region (Lickwar et al., 2017).

In the anterior region, the intestine is behind the heart, nestled within the lobes of the liver and interspersed with pancreatic tissue (Eames-Nalle et al., 2017). The contents of the esophagus are emptied into the intestinal bulb, the widest portion of the intestine. As the lumen begins to narrow, the food moves into the middle intestine and the posterior intestine. The posterior intestine is the last region before waste exits through the vent.

Throughout the life of the zebrafish, the intestine is located ventrally. In larval zebrafish, the intestine is a relatively straight tube from the esophagus to the anus, with a slight bilateral asymmetry (Ji et al., 2016), as shown in Figure 3A. As the zebrafish develops into adulthood, the intestine increases markedly in length and develops two loops in the mid-

intestine region (Ng et al., 2005), as shown in Figure 3B. Neither the timing nor the

morphological progression of this gut looping and epithelial maturation in the post-

embryonic zebrafish has been examined carefully.

Esophagus   Anus
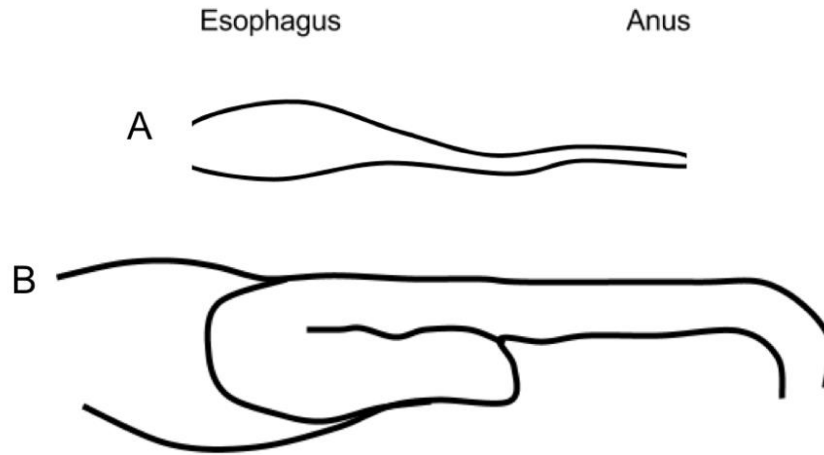
Figure 3. Larval vs. adult intestinal morphology. General morphology of the intestine in
the zebrafish at the (A) larval stage and (B) adult stage.

The intestinal bulb is the anterior-most section of the zebrafish intestine, directly after

the esophagus. It is the region with the widest circumference. Most importantly, zebrafish

have no discernable stomach, a characteristic of the Cyprinid family (Crosnier et al., 2005).

Primarily, the lack of gastric glands and expression of pepsinogens (Kurokawa et al., 2005)

coupled with the expression patterns of fatty acid binding proteins (*fabp*) and lipase F gastric

type (*lipf*) further substantiate the lack of a stomach in zebrafish (Wang et al., 2010). In

Figure 2C, *fabp2* and *fabp6*, known small intestine markers in other vertebrates, are

expressed in the middle intestine of zebrafish but also in the intestinal bulb (Lickwar et al.,

2017). Further, not shown in the figure, *lipf* is a gastric peptide that is ubiquitously expressed

in the enterocytes along the entire length of the zebrafish intestine (Wang et al., 2010). This

nonspecific expression in zebrafish serves to show that although the zebrafish intestine bulb

may hold some functionality of stomach, a specialized stomach is not present.

To distinguish between the middle and posterior intestine requires a microscopic

examination of important structures similar in shape to mammalian villi: villar ridges (Wang

et al., 2010). Villar ridges, also referred to as intestinal folds, serve to increase the surface

area of the intestinal epithelium. They are raised portions of the intestinal wall that are

randomly oriented relative to the anterior-posterior axis. In the intestinal bulb the villar

ridges are tall and pronounced. They have a randomized orientation that consists of both

longitudinal and latitudinal extensions of some ridges (Wang et al., 2010). In the middle

intestine, they shorten and condense, forming large, cuboidal villar ridges along the narrowed

lumen (Wang et al., 2010). In the posterior intestine, they are longitudinally oriented,

specifically running from anterior to posterior, and are much shorter than the villar ridges of

the intestinal bulb and middle intestine (Wang et al., 2010). This pattern is consistent in both

the larval and adult zebrafish (Ng et al., 2005; Wang et al., 2010).

Histological and cellular data support the convention of three distinct intestinal

regions. As previously mentioned, the epithelium has three primary cell types, enterocytes,

goblet cells, and enteroendocrine cells, each of which serve to distinguish the regions. These

cell types have been studied histologically in the larval stage (Ng et al., 2005; Wallace et al.,

2005) and molecularly in the adult stage (Lickwar et al., 2017; Wang et al., 2010).

Enterocytes are the absorptive cells of the intestinal epithelium. They are present

across all three segments of the intestinal epithelium. In the intestinal bulb and middle

intestine, there is an abundance of a specialized enterocyte identified by the presence of a

large vacuole on the apical side of the nucleus by 74 hpf (Wallace et al., 2005). This mirrors

the distribution in the adult intestine, indicating the cell type has an established patterning early in development that is maintained through adulthood (Wallace et al., 2005). These cell types are characterized by the abundance of an apical sodium phosphate (NaPi) transporter and large supranuclear vacuoles containing luminal contents (Wallace et al., 2005).

Goblet cells are a secretory epithelial cell type that produce mucins which protect and lubricate the intestinal epithelium (Kim and Ho, 2010). Early in development, the middle intestine is the only site of goblet cell differentiation (Ng et al., 2005; Wallace et al., 2005). Goblet cells secrete mucins onto the epithelial lining and have a role in innate immunity (Ng et al., 2005). By 14 dpf, goblet cells are differentiated throughout the intestinal epithelium, giving rise to sparse neutral and acidic mucin producing cells in the intestinal bulb and acidic mucin producing goblet cells throughout the intestine. One set of goblet cells produces only acidic mucins and another set produce acidic and neutral mucins from a single cell, but the distinction is not well studied (Ng et al., 2005).

Proliferation rates of epithelial cells are a key feature of the intestine. Stem cells are constantly replenishing the lining of the intestine by differentiating into the cell types present. The intestinal bulb has large villar ridges with proliferation primarily sequestered in the inter-villus spaces between the ridges. Compared to mammalian intestines, zebrafish show a markedly reduced level of surface area due to the lack of microvilli and Crypts of Lieberkühn. Thus, proliferating cells are more exposed to the intestinal lumen. Functionally, the inter-villus spaces demonstrate similar but markedly inferior protection for the differentiating cell types (Kaiko et al., 2016). Similar to Crypts of Lieberkühn, the crypt-like inter-villus spaces protect the stem cells from mechanical forces acting on the epithelium by keeping the stem cells from being fully exposed to luminal contents (shown in Figure 1).

However, the inferiority is in the lack of specialized crypts, which prevents the inter-villus spaces by facilitating strong concentration gradients of luminal contents to protect the stem cells. These are spatial concentration gradients where the crypts prevent adequate diffusion of harmful substances all the way to the base of the crypts, which protects the stem cells. This was observed in an experiment by Kaiko et al., (2016) who demonstrated that exposure to luminal butyrate caused injury to colonic stem cells. The Crypts of Lieberkühn in a murine model provided adequate protection to support proliferation, while the zebrafish intestine showed increased damage and decreased proliferation of the stem cells (Kaiko et al., 2016).

The layers of the intestinal wall also provide insights into functional differences that may be present in each region. The tissue layers of the zebrafish intestine share similarities with the mammalian intestine in that there is a mucosa, muscularis, myenteric plexus (Auerbach's plexus), and serosa. In contrast to the mammalian intestine, the zebrafish intestine lacks a submucosa and submucosal ganglia (Meissner's plexus), shown in Figure 1 (Wallace et al., 2005). The mucosa supports the intestinal epithelium, with the aforementioned cell types, and includes a layer called the lamina propria. The lamina propria contains cells from many converging body systems including the vasculature, lymphatic capillaries, immune cells, connective tissue, etc. The muscularis consists of a layer of circular smooth muscle and a layer of longitudinal smooth muscle. In-between the two muscle layers is the myenteric plexus.

With a basic understanding of the composition of the intestine established above, the physiology follows. Of importance in motility studies, the response to feeding by the intestinal epithelium in humans provides a basis of study for the investigation in zebrafish.

Then, the contribution of the neuronal and muscular systems in the intestine, in conjunction with the intestine, can provide strong observations for motility. The following section will look at the relationship between feeding, function, and effectors of the natural motility in zebrafish.

Basic Intestinal Physiology

During and after feeding, an intricate network of stimulation, positive and negative feedback, and inhibition are responsible for the body's response to food. From ingestion to excretion, each major portion of the digestive tract plays an important role (Cummings and Overduin, 2007). The vertebrate GI tract uses motility for nutrient and water absorption (Malagelada and Malagelada, 2017). Intestinal contents are moved along the length of the intestine via a coordinated system of muscular contraction and relaxation of the intestinal wall (Benson and Gunstream, 1981). These types of movements coupled with the folding of the intestinal wall maximize the interaction of the luminal contents with the intestinal epithelium. Thus, the intestine has a great capacity to respond to and process digested materials because the mechanical force exerted on the food by contraction of the intestinal wall increases the interaction between the intestinal epithelium and the gut contents.

Enterocytes, the absorptive cell types lining the lumen, transport nutrients into the bloodstream. Microbiota are also responsible for processing intestinal contents, and subsequent byproducts may be transported into the body or excreted as waste (El‑Salhy et al., 2016).

In the mammalian GI tract, stretch receptors respond to food as it enters the stomach. These stretch receptors respond to the distention of the stomach by causing release of 5-hydroxytryptamine (serotonin) from enterochromaffin cells (EC cells), a type of

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enteroendocrine cell in the intestinal epithelium (Gershon, 2013). Serotonin plays a major role in the sensation and response to luminal contents. The EC cells, containing roughly 95% of the body's serotonin, provide endocrine and paracrine signals throughout the process of digestion (Shajib and Khan, 2015). Serotonin can, however, also be released by enteric/peripheral neurons and EC cells of the intestine. Intestinal (enteric) serotonin (synthesized by *tryptophan hydroxylase 1*) can produce peristaltic reflexes, while neuronal serotonin (synthesized by *tryptophan hydroxylase 2*) regulates GI motility as a whole (Gershon, 2013). Thus, enteric serotonin is primarily produced for metabolism, while production of neuronal serotonin affects digestion motility. Other endocrine signals begin to coordinate the movement of food from the stomach into the intestine.

In mammals, as a result of a pressure increase in the stomach, chyme moves from the stomach through the pyloric sphincter into the duodenum, the first part of the small intestine. The acidity of the chyme stimulates two pathways in the duodenum. One pathway is mediated by the release of cholecystokinin (CCK) via I cells, which stimulates contraction of the gallbladder (Jolliffe, 2009). Bile emulsifies the fat in the chyme (Gibbs et al., 1973). The second pathway is mediated by the release of secretin from S cells. Secretin is released in response to the acidity of chyme entering the duodenum. Secretin slows gastric emptying, increases the alkalinity of the chyme, and inhibits gastrin release (Jolliffe, 2009). Brunner's cells in the submucosa of the mammalian duodenum release bicarbonate to neutralize the acidity of the chyme. At this point, chyme is in the small intestine. Little is known about the complex interactions between the epithelial lining and its response to chyme outside of rudimentary elements of nutrient absorption. Receptors along the intestinal surface coordinate this absorption and a wide variety of responses, hormonal, neuronal, and

mechanical, ensue (Norris and Carr, 2013). The final destination of the intestinal contents is the colon. The colon accounts for over 70% of the microbial activity in digestion that provides further signals, such as metabolites and byproducts of interaction with luminal contents, for the epithelium to respond to. Here, the contents are dehydrated heavily via aquaporin channels (Kaiko et al., 2016). The resulting waste is stored until excretion.

Between meals, the intestine is primarily governed by a cyclic activity that spreads contractions, known as the migrating motor complex (MMC). Unlike peristalsis and digestion, this system describes passive, self-regulating intestinal movements and impulses. This process involves four phases of contractions that are responsible for clearing the intestine of residues after feeding (Deloose et al., 2012). This process, along with those described above, is not currently understood in the zebrafish model or any other model organism at the moment. Due to the overall rarity of stomach-less vertebrates, understanding the dynamics of chemical and mechanical responses to feeding is a necessary step in creating a model system using zebrafish.

Two important systems that intersect in the intestine are the nervous and muscular systems. These two systems work together for the sensory and mechanical responses to luminal contents. In intestinal studies, the need for immobilizing specimens with anesthetics that alter the nervous and muscular systems may have unintended effects on the physiological readouts. In the following section, considerations of the effects of anesthetics on motility, sensory, and mechanical function will be evaluated.

Effects of Anesthetics on Intestinal Function

Muscle contraction in the smooth muscle of the intestinal wall is the result of two major cell types: pacemaker cells called interstitial cells of Cajal (ICC) and the traditional acetylcholine-mediated neurogenic contraction (Rich et al., 2013). The enteric nervous system (ENS) is a network of neurons with axonal varicosities laying along the circular and longitudinal muscle layers (Uribe and Bronner, 2015). There is a network of ENS cells between the muscle layers, known as Auerbach's (myenteric) plexus, and in higher vertebrates another network of ENS cells, known as Meissner's (submucosal) plexus underlies the submucosa. Auerbach's plexus lies between the longitudinal and circular smooth muscle layers of the intestinal wall. When acetylcholine is released from the neuronal varicosities, it stimulates calcium uptake by the smooth muscle cells, activating calmodulin, resulting in the contraction of the smooth muscle cells (Lyford and Farrugia, 2003). The ICC bypass this mechanism by maintaining a rhythmic supply of calcium influx into neighboring smooth muscle cells that allow for a contraction independent of nervous stimulation (Lyford and Farrugia, 2003). It is hypothesized that ICC cells share a common syncytium with neighboring muscle layer cells (Dickens et al., 1999). ICC cells have mechanosensitive L-type Ca^{2+} channels, which allow for a stretch-activated response to intestinal contents (Lyford and Farrugia, 2003). How ICC and enteric neurons might work together to coordinate gut motility is currently unknown.

To date, it is known that three types of ion channels, calcium (Ca^{2+}), sodium (Na^+), and potassium (K^+) channels, and two neurotransmitters, serotonin and acetylcholine, are primarily responsible for the coordination of neuronal and muscular action potentials in the GI tract. A wide variety of anesthetics target these channels/neurotransmitters in their

mechanism of action to inhibit muscular contractions of the smooth muscle. For example, clinically, Nifedipine is used to reduce high blood pressure by relaxing smooth muscle via a blockage of L-type calcium channels on smooth muscle. Tetrodotoxin (PubChem: CID 11174599), isolated from the puffer fish, results in paralysis by blocking the action of acetylcholine (Kim et al., 2016). A combination of two anesthetics used together, methysergide (PubChem: CID 9681) and atropine (PubChem: CID 174174), antagonizes the effect of serotonin in the blood and GI smooth muscle and affect parasympathetic stimulation of smooth muscle, respectively (Kim et al., 2016).

Tricaine is an ester-type synthetic local anesthetic that can be used for aquatic organisms when dissolved into their water. Tricaine enters through the gills, goes into the bloodstream and is rapidly metabolized before it exits through the gills once again. It is effective in the blood for approximately 1.5-4 hours and is fully undetectable in the blood between 8-24 hours after administration (Carter et al., 2011). The generally accepted concentration of tricaine is 0.02%, which needs to be buffered because tricaine is known to acidify the water. Experiments have been conducted to test whether using a tricaine isomer known as benzocaine can produce longer-lasting anesthesia. Benzocaine has the benefit of requiring a much smaller concentration of 0.0035%, and it also maintains a neutral pH and has seemingly potent results (Wynd et al., 2017). Tricaine primarily works along the axonal membrane by blocking sodium channels to, reduce the excitability of the nerve. Tricaine partially reduces the permeability of sodium, effectively reducing the number of action potentials but does not completely prevent them. Tricaine and benzocaine are lipid-soluble , which allows them to bind the sodium channels directly (Burka et al., 1997; Butterworth and Strichartz, 1990; Carmichael, 1985).

In the case of anesthetics, the question remains if using tricaine, a commonly used anesthetic in zebrafish, would have a major effect on motility of the intestinal wall. As previously stated, the mechanism of action for tricaine is binding and thus blocking voltage-gated sodium channels. I hypothesize that, under anesthesia, the neurogenic contractions of smooth muscle are inhibited, but the rhythmic contractions mediated by the ICC cells are maintained. Because contraction of smooth muscle involves both the ENS and the ICC, it is possible for the calcium-dependent ICC cells to maintain some degree of motility in the presence of tricaine, although functionality may be reduced.

Vertebrate Models for Intestinal Studies

Clinical Motility Studies

Several clinical approaches are currently used to monitor GI function in patients. These findings can loosely correlate function with a variety of metrics, including muscular contractility, transit time, and excretory and microbial byproducts of digestion. These approaches focus primarily on grading symptoms rather than diagnosing patients. However, increased analysis of the clinical data generated from these methods is moving toward better diagnostics for more generalized visceral pathologies. For example, a clinician can use these protocols to understand if intestinal dysfunction is a result of myopathy, neuropathy, or occlusion (Camilleri and Linden, 2016; Malagelada and Malagelada, 2017).

One of the longest standing methods employs a manometry catheter, a device which is inserted through the mouth into the stomach or the small intestine and measures both the amplitude and duration of pressure waves. The catheter has several manometers placed at regular intervals that can detect the movement of peristaltic waves down the GI tract. Recordings are taken for approximately 3 hours during fasting and for 2 hours after eating.

The recorded wave patterns of hospitalized patients are then compared to the baseline data collected from healthy volunteers and those with known motility dysfunction (Malagelada and Malagelada, 2017). As a result, there are three characterized patterns that can indicate dysfunction from myopathy, neuropathy, or bowel obstruction based on a manometer reading (Malagelada and Malagelada, 2017). Recently, manometry has moved toward higher resolution and fiber optic catheters, which has a higher sensitivity to contractions and smaller intervals between sensors. These methods, however, are primarily clinical observations that have yet to studied in a published body of work (Malagelada and Malagelada, 2017).

Lactulose Breath tests are used to measure hydrogen levels in the breath that can be an indicator of small bowel motility. When a person ingests lactulose, an indigestible carbohydrate, gut microbiota in the small intestine produce hydrogen gas as a byproduct (Miller et al., 1997). This peak in hydrogen can then be measured because it increases exponentially in parts per million as lactulose enters the intestine. However, lactulose is known to increase gastric emptying thus, biasing the test results (Camilleri and Linden, 2016).

Gastroenterocolonic scintigraphy uses gamma rays to capture intestinal contents in transit through the body wall (Krevsky et al., 1986). A subject is given a radiopaque meal that can then be photographed at regular time intervals to follow the progression of the meal through the body wall. This can be used at the whole gut, gastric, enteric, and colonic levels. Potential drawbacks include differential transit rates for food. The results of this test are much closer to an unbiased transit time. The primary drawback is a potential lag in the radiolabeled particles in digestion (Camilleri and Linden, 2016).

In a study with 10 patients taking a simultaneous lactulose breath test and gastroenterocolonic scintigraphy readings, results were consistent for both tests (Miller et al., 1997). However, when lactulose was removed from the meal, gastric emptying was significantly slowed, but other solids and radiopaque gut contents emptied into the intestine sooner. This serves to confirm the bias that lactulose increases gastric emptying. The results also give credit to these two tests in assessing motility, but applications for these tests have remained for use in understanding symptoms of dysfunction more clearly rather than for diagnostic purposes (Camilleri and Linden, 2016).

Using these clinical methods for better evaluation of symptoms will add value to a formal diagnosis. However, because of the intestinal behavior and physiology is usually peripherally involved in the full disease manifestation, pure diagnosis using these methods is unlikely. Diseases that are characterized as bowel dysfunction often involve other pathologies. For example, Hirschsprung's disease results in megacolon, but the cause is a neuronal deficit (Amiel and Lyonnet, 2001). Another example is Crohn's disease, which is primarily an immune system defect that causes colonic ulceration (Torres et al., 2017). A stronger characterization of symptoms of a disease can lead to more precise diagnoses.

Guinea Pig Antrum Strips:

A challenge for physiologists and cellular biologists is differentiating the functions of different cell types that have similar behavior patterns or work in highly coordinated networks with other cell types. In a study using the guinea pig intestine for ex vivo intestinal physiology, researchers explored cells of the intestinal wall sharing electrical syncytia to understand the nature of the pacemaker cells, Interstitial Cells of Cajal (ICC), as driving cells for muscular contractions of the intestinal wall. The experiment was conducted using guinea

pig stomach-antrum strips. They were able to differentiate between the longitudinal smooth muscle, circular smooth muscle, and ICC using electrical activity and immunohistochemistry (Dickens et al., 1999).

To conduct this experiment, they stunned and exsanguinated the guinea pig, and then dissected the stomach out. Keeping the tissue intact and alive using an organ bath of oxygenated physiological saline at 35°C, they attached the strips of tissue to traditional microelectrodes. Electrophysiological recordings came from two groups of tissue. The first group was also attached to a force transducer for muscle contraction, while microelectrodes were attached for simultaneous readings of amplitude and frequency of contractions. The second group had microelectrodes filled with either potassium chloride with neurobiotin or lithium chloride with Lucifer Yellow that pierced individual cells, whose recordings were used to track the flow of current corresponding with the stimulation. Their saline was also washed with standard nifedipine to reduce the amplitude of the waves or variable concentrations of caffeine to observe effects on myogenic waves. Reducing the intensity of waves also prevented too much diffusion of the dyes, thus allowing an observer to follow the flow of current for the cell being measured (Dickens et al., 1999). The second group of cells was also fixed and processed for antibody reactivity or direct observation.

ICC were characterized as the driving cells of the muscular slow wave pattern evidenced by their common syncytium with cells of a particular muscle layer (Dickens et al., 1999). They were able to determine that the ICC were different from the myenteric neural cells based on their electrical activity. Myenteric neurons exhibit the traditional short burst electrical activity of an action potential, while ICC cells have a long-standing depolarizing current that moves through the syncytia to neighboring circular and longitudinal smooth

muscle cells (Dickens et al., 1999). So albeit having a cell body with several processes stretching across the muscle layers similar to the enteric nervous system, ICC have a direct electrical connection to both circular and longitudinal smooth muscle layers. Muscle cells share an electrical syncytium with ICC that allow for coordinated movements. The ICC provide a rapid and consistent depolarizing current which drives the slow waves of the circular smooth muscle

Motilin and Intestinal Strips

Motilin (MOT) and its receptor is a hormone that has been found in the GI tracts of humans, pigs, cows, rabbits, cats, sheep, and horses. MOT and its receptor are not present in murine GI tracts (Kitazawa et al., 2017). Generally, MOT regulates contractions during fasting, particularly relevant in Phase III of the migrating motor complex. It also stimulates GI motility in mammals and birds. MOT-producing enteroendocrine cells are present in the duodenal mucosa of humans. In zebrafish, mRNAs for a motilin-like peptide (zfMOTLP) and its receptor were identified (Kitazawa et al., 2017).

Kitazawa et al. (2017) tested the response of tissue to exogenous zfMOTLP in the following experiment. The approach was to use tissue from vertebrates known to have motilin receptors and measure their response to exogenous motilin. Specimens were anesthetized, euthanized, and then tissues were collected from rabbit duodenum and longitudinal smooth muscle, mouse ileal segments (15mm), chick ileal strips, and zebrafish intestinal bulb strips. Samples were attached to an isometric force transducer, submerged in a 5 ml organ bath, and equilibrated for 1 hour in Krebs solution or fish physiological salt solution that was oxygenated and temperature controlled at 37°C or 23°C, respectively. After that, 3 to 4 washes with acetylcholine ensured consistent and reproducible contractions

before measurements were recorded. To ensure that exogenous MOT was binding the

motilin receptor, tissues were washed with GM109, an MOT receptor antagonist, which

prevented contraction.

zfMOTLP was only able to produce low amplitude contractions on non-zebrafish

tissues at high concentrations (1-10 µM). Rabbit duodenal strips and chick ileum had the

highest amplitude contractions produced by zfMOTLP. However, when human MOT (100

nM) was applied to those same tissues, the force of the contraction was 3000 times stronger

than that produced by zfMOTLP (Kitazawa et al., 2017). Reactivity of zfMOTLP (3-100

nM) and its receptor was tested using transgenic HEK293 cell cultures that expressed the

zfMOTLP receptor. Treatment with zfMOTLP increased intracellular calcium levels, while

human MOT (100 nM) had low affinity and reactivity with the zfMOTLP receptor at the

same concentration.

Interestingly, the exogenous zfMOTLP was only able to produce weak contractions in

zebrafish intestinal strips at high concentrations. Further investigation of the expression

pattern of the zfMOTLP receptor showed a high prevalence in the middle intestine, but

compared to expression levels found in the chick digestive tract, had a very low expression of

the MOT receptor overall (see Figure 4 below). This may suggest that zebrafish have a

motilin system that is not necessarily significant for GI motility (Kitazawa et al., 2017).

Unfortunately, this experiment required the use of a wide range of anesthetics.

Pentobarbital sodium was used on the rabbit, isoflurane was used on the chick, and eugenol

was used on zebrafish (Kitazawa et al., 2017). Pentobarbital sodium (PubChem: CID

23676152) is a barbiturate that is used to induce depressive effects in the central nervous

system but can also affect inhibitory pathways in the peripheral nervous system (Kim et al.,

2016). Eugenol (PubChem: CID 3314) is a hepatotoxic derivative of clove oil that does not

seem to have direct effects on the intestine (Kim et al., 2016). Isoflurane (PubChem: CID

3763), however, is known to increase the time that gap junctions close and decrease the time

gap junctions are open, increase calcium storage in the sarcoplasmic reticulum via activation

of calcium-dependent ATPase, and can bind to the GABA receptor (Kim et al., 2016). This

can have effects on intestinal motility at the muscular and neuronal levels (Dickens et al.,

1999). Some of these mechanisms of action could have contributed noise to the readouts of

the contractions.
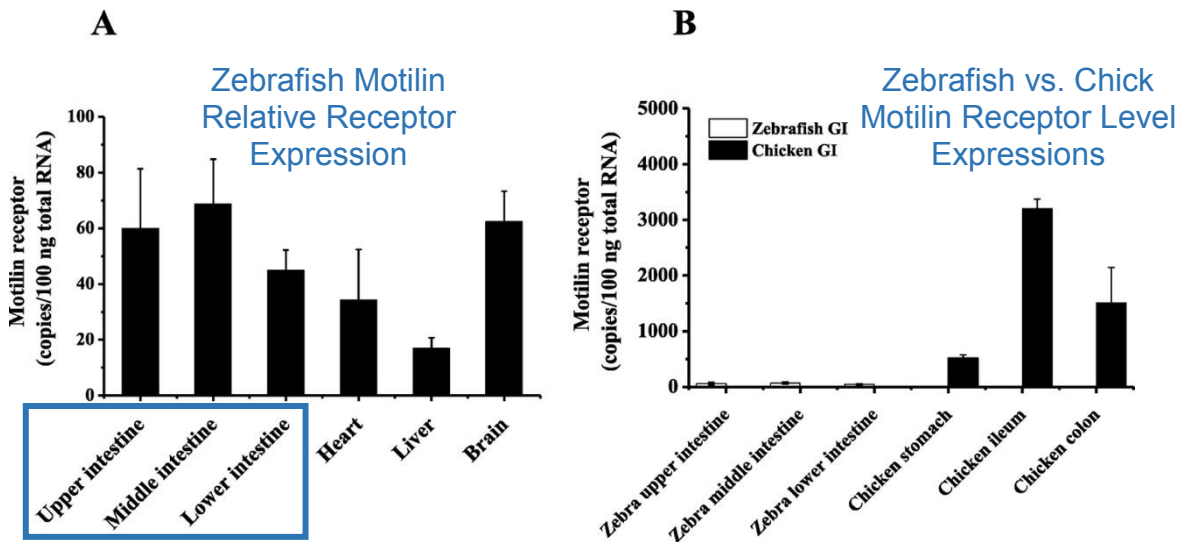
Figure 4. Expression of MOT receptor mRNA in various tissues of the zebrafish. (A)
The columns show the expression levels of zebrafish MOT receptor mRNA in the upper,
middle and lower intestines and in the heart, liver, and brain. Blue box shows intestine,
relative expression levels in anterior, middle, and posterior intestine. (B) Comparison of
mRNA expression levels of the MOT receptor in the GI tracts of zebrafish and chickens.
Values are means ± S.E.M. of 4 individual experiments. Data for chickens were from
Kitazawa et al. (2013). Modified from Kitazawa et al., (2017).

Improving in vivo studies

In summary, most intestinal studies require removing portions of tissue from the organism. The tissues are sustained in physiological solutions for force and electrical readings, while the cells are identified primarily through histology. All studies highlighted above require a complete sacrifice of the animal, using anesthetics and euthanasia. Due to its composite anatomy of multiple organ systems and multilayered functioning, experimentation on the isolated intestine is prone to uncontrollable variables. Additionally, as described above, many anesthetics have negative residual effects on neuronal and muscular activity, thus disrupting normal intestinal motility.

Due to the potential confounding factors of *ex vivo* studies, live imaging of intestinal motility in live specimen presents opportunities for novel discoveries. A number of labs have taken advantage of the transparency of larval zebrafish to image smooth muscle contractions through the body wall in a live specimen (Heanue et al., 2016; Holmberg et al., 2007; Njagi et al., 2010). Some labs have even used larvae to track intestinal motility and transit times (Brady et al., 2017; Field et al., 2009). However, all of these studies required Tricaine to anesthetize and immobilize the fish for imaging. This is why establishing a live model for a gut transit assay that reduces the use of chemical anesthetics will provide invaluable information for better understanding natural intestinal motility.

In the following section, the actual experimental design of intestinal studies will be evaluated for feasibility and form. The intestine can be studied using *in vivo*, *ex vivo*, and live imaging. Physiological studies, however, need access to the intestine, thus, ex vivo studies involving excision of the intestine will be explored in greater detail. Lastly, *in vivo* live imaging, the most likely candidate for the eventual goal of motility studies in the

zebrafish model, will be considered in its present use. All three of these methods will be summarized and evaluated for feasibility in intestinal studies in the next section.

Experimental Design for Intestinal Studies

Peristalsis is a coordinated pattern of muscular contraction and relaxation of the intestinal wall that moves food along the GI tract (Benson and Gunstream, 1981). There are two muscular layers that line the intestine, the outer longitudinal muscle layer which is coated by the serosa and the inner circular muscle layer which is closer to the lumen. These muscle layers primarily respond to input from Auerbach's plexus, the enteric neurons between the two layers of muscle (Lyford and Farrugia, 2003). Since the intestinal wall is innervated intrinsically by enteric neurons, peristalsis and other gut movements can occur without input from the central nervous system. Thus, the intestine can be excised and observed *ex vivo* while still maintaining motility.

In addition to being studied *ex vivo*, intestinal motility is also studied using indirect methods of observation. The vertebrate intestine is difficult to directly observe because of its location in the body. Additionally, due to genetic, developmental, and nutritional variation between individuals, understanding the physiology of the intestine requires sophisticated analysis. In most situations, described in the next two paragraphs, the readouts from ex vivo monitoring or indirect observations have such poor correlation with clinically important indices and contain such high variability that they serve little purpose in characterization of distinct patterns or physiology. As a result, broad-stroke and indirect measurements from both *ex vivo* and indirect analyses can be easily confounded by these variables. Thus, fine-tuned, direct methods for observation of intact intestines are being developed to improve the quality of information.

In a hospital setting, motility is observed as part of inpatient and perioperative care. Clinicians want to keep track of intestinal function, which includes muscular and nervous function involved in intestinal motility. There are various indirect methods that are used to track intestinal motility. One such method uses radiolabeled markers and time-lapse gamma-ray images to track the progression of a meal from injection to excretion (Krevsky et al., 1986). Another approach uses the health and strength of the two muscular layers as an indicator of disease. This is done by using a manometer, which, as described earlier, is a catheter that contains an array of pressure sensors that record muscular contractions through reading the force applied to the lumen by the muscles of the intestine. The resulting pattern of contraction generated by the sensors can be used to detect irregular or weak peristaltic movements (Malagelada and Malagelada, 2017). Finally, byproducts of digestion such as microbial metabolites and excreted waste can be measured to ascertain proper intestinal function (Miller et al., 1997). This approach assesses the metabolic activity of the colonic flora and its impact on intestinal mucosal absorption and secretion as an important aspect of colonic health. However, all of these methods result in extremely wide ranges of variability between individual patients and are easily confounded because of the multilevel organization of the intestine (Camilleri and Linden, 2016). For example, irregular manometer readings can result from problems with muscular cells, pacemaker cells, endocrine cells, neuronal cells, vasculature, etc. Therefore, while there are multiple methods already in existence for assessing motility, it is clear that indirect measurements lack the resolution for extrapolation of data for clinical significance.

Laboratory methods for observing the intestine have more flexibility in terms of gaining direct access to the intestine. Mammalian studies in research labs have also used

approaches similar to those found in clinics. *Ex vivo* experiments, such as tissue preparations of intestinal segments, intestinal rings that maintain the lumen, and strips of the intestine, can be used to observe motility patterns, both peristaltic and otherwise. However, *in vivo* studies would be ideal for achieving strong, reliable data that can be used to clearly interpret intestinal dysfunction and dysmotility.

In zebrafish, the intestine has been studied for genetic characterization of motility disorders and phenotypic variation. Most commonly, motility studies have utilized mutants with phenotypes showing reduced number or reduced spatial distribution of enteric neurons (Field et al., 2009; Heanue et al., 2016; Kuhlman and Eisen, 2007). Fortunately, the zebrafish larva is transparent throughout early post-embryonic development, allowing direct imaging of the gut without surgical manipulation (Wallace et al., 2005). As a result, live imaging of peristalsis in the intact animal is a strong *in vivo* method for creating spatiotemporal maps, understanding contractility with normal physiological signals, and correlating visual and quantifiable data to clinical indices. Examples of these will be summarized and analyzed below.

Despite the availability of genetic models of zebrafish with intestinal defects that affect motility, little work has been conducted on the physiology and function of these models. Currently, there are several assays developed for gut transit in early larval stages that track the progression of food through the digestive tract (Abrams et al., 2016; Brady et al., 2017; Field et al., 2009). There is also a study on the force of contraction for adult zebrafish tissue preparations that measures the amplitude and frequency of contraction (Kitazawa et al., 2017) Developing novel methods for quantifying force of contraction in

both *in vivo* and *ex vivo* zebrafish models could provide new insights into human intestinal

diseases .

Ex vivo studies in mammalian models using organ bath

Because the intestine is deep within the body cavity, most studies require an *ex vivo*

approach. Therefore, studying the physiology of the intestine requires excising it from the

animal. As previously stated, this works primarily because the enteric nervous system is self-

contained with its own sensory neurons, motor neurons, and interneurons. This means that

the intestine can continue contraction and relaxation in isolation of the body without the need

for input from the central nervous system. This is done using organ bath studies, which can

maintain excised organs outside the organism. Figure 5 provides a schematic for a general

organ bath study. Researchers have taken this opportunity to look at a range of effectors to
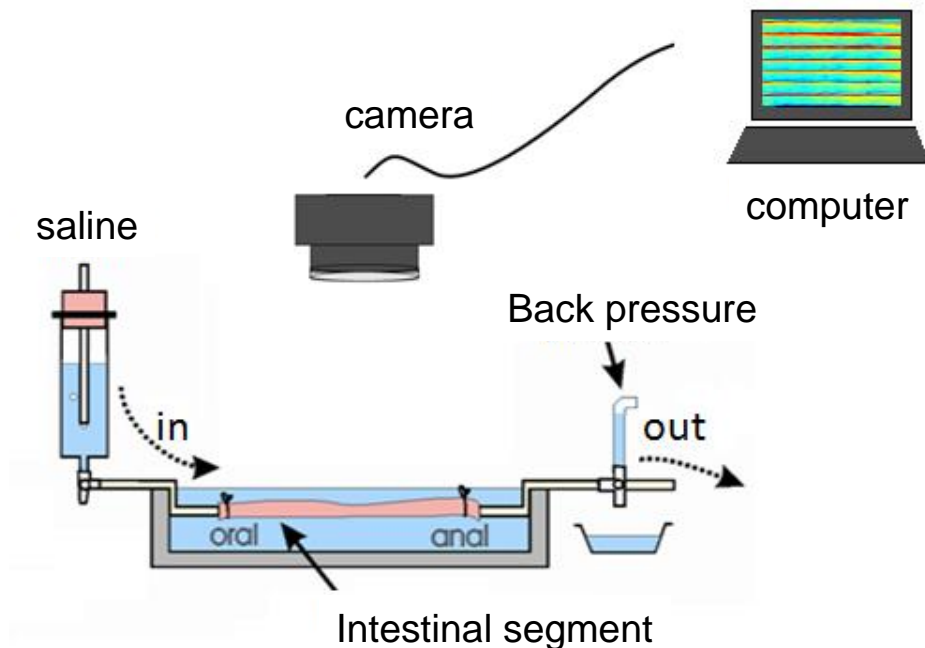
see how the intestine responds.

Figure 5. General set-up of organ bath studies. A schematic of a flow-through organ bath, where saline is the physiological solution and the organ is submerged saline and cannulated to the inflow and outflow of saline. Modified from (Ellis, 2018).

Essentially, the organ bath provides a favorable and nutritive environment to sustain the organ outside the body by providing electrolytes, glucose, aeration, and a temperature that mimics *in vivo* conditions. A variety of attachments for the organ can accommodate segments of intestine, strips of intestinal wall, and circumferential rings of intestine in multiple orientations. and for the data acquisition interface allow for a wide range of measurements of force, frequency, and duration of muscular contractions. The organ bath can then be used to understand the effects of exogenous stimulation on the contractility of the organ such as drug treatments and hormones. In general, the organ bath is a versatile tool for studying various physiological aspects of the intestine (Wikswo, 2014).

Once the isolated intestine equilibrates to the physiological solution, experiments can be conducted to measure spontaneous and induced muscular contraction and relaxation patterns. However, an important consideration is the perfusion method used. A perfusion method is the combination of the contents of the physiological solution and the movement of the solution within the organ chamber vessel. An organ bath experiment can be either flow-through or incubating. A flow-through experiment replenishes the organ chamber with fresh physiological solution at a constant rate. An incubation experiment simply anchors to the organ in a static bath of physiological solution with aeration. Typically, a flow-through experiment is used in drug treatment testing by added and removing a drug as desired. On the other hand, an incubation experiment is ideal for force and physiological investigation. Depending on the length of the experiment, the perfusion method, which is the quality and flow rate of the physiological solution, can be an unforeseen confounding factor for the experiment (Schreiber et al., 2014a; Wikswo, 2014).

Maintaining the organ properly in isolation is vital to providing meaningful data that might be useful for developing new disease treatments. For example, Schreiber et al. (2014) tested a variety of perfusion methods and their effects on measurable motility. They characterized motility as moving in the oral, anal, or oro-anal directions. In order to characterize motility, they found that luminal pressure needed to be maintained precisely. They achieved this by maintaining the organ at a 4 to 9 cm depth in the water column, since anything above or below that resulted in decreased contractions. Further, they found that simple solutions, such as Tyrode's, were only feasible for short-term experiments while more complex solutions, containing vitamins, organic and inorganic salts, sustained motility longer (Schreiber et al., 2014b). However, there are limitations to *ex vivo* experimentation. Since

the organ is removed from peripheral inputs, such as blood flow, lymphatics, endocrine signals, and the remainder of the GI tract, it eventually loses its functional ability. The clear division between the luminal and peritoneal sides of the intestine can also be lost during dissection since the boundaries are unclear on a macro-level, which also disrupts the microbiome.

In addition to motility studies, *ex vivo* work is used to investigate the electrical properties of the intestine. For example, Spencer et al. (2003) conducted studies on the electrophysiology of coordinated motility patterns, particularly focused on the propulsion of the bolus in the colon. Their model was the guinea pig. Animals were anesthetized, euthanized, and then 20 mm segments of the distal colon were excised, the lumen was cut open, and the tissue was pinned flat against a coverslip. Simultaneous electrode recordings were taken at the anterior and posterior of the tissue. Importantly, they were able to record separate electrical readouts from the longitudinal and circular smooth muscle layers. A video recording of the tissues on the microscope was created in parallel with a video of the oscilloscope so that they could correlate in real-time the movement of muscle and readouts of electrical activity (Spencer et al., 2003).

In this study, two sets of readings were taken for each muscle layer: a gross anterior and posterior electrode reading and then an anterior and posterior electrode reading from the circular muscle and the longitudinal muscle. The first set of readings demonstrated that there is a simultaneous anterior excitation and posterior inhibition of the stretched tissue on either side of the bolus. Thus, peristalsis creates a coordinated opposition on either side of the bolus which moves the bolus in the correct direction. While the anterior section is contracting, the posterior section is relaxing, resulting in the bolus being pushed toward the

posterior. This study confirmed the classic work of Bayliss and Starling (1899) in which

they used a surgical method to access the intestine in dogs and observed that there is a series

of contractions above the bolus along with simultaneous relaxation below the bolus. They

referred to this pattern of motility as the "law of gut" (Bayliss and Starling, 1899). As shown

by the work of Spencer et al., modern approaches support the conclusions of this early work)

In further work by Spencer et al. (2003), a second set of readings investigated the

circular and longitudinal smooth muscles separately at the anterior and posterior end, for a

total of four readings. This approach uncovered the interplay between the excitatory and

inhibitory pathways of neurons within the myenteric plexus that influence contraction versus

relaxation of the smooth muscle. However, the interactions between the neurons were not

clearly defined. Due to the lack of direct communication between neurons of the two smooth

muscle layers, it was determined that interneurons facilitate the coordination of

communication between the two layers (Spencer et al., 2003).

In motility studies, coordinated movement patterns are of great significance.

Generally, contractions move continuously along the intestine with varying force. This

means that a method that can provide insight on the entirety of the intestine can resolve

motility patterns with greater clarity. Thus, spatiotemporal mapping of contractions is often

combined with *ex vivo* and *in vivo* experiments to better understand intestinal function.

Figure 6 gives an example of how the organ looks and the resulting spatiotemporal map:
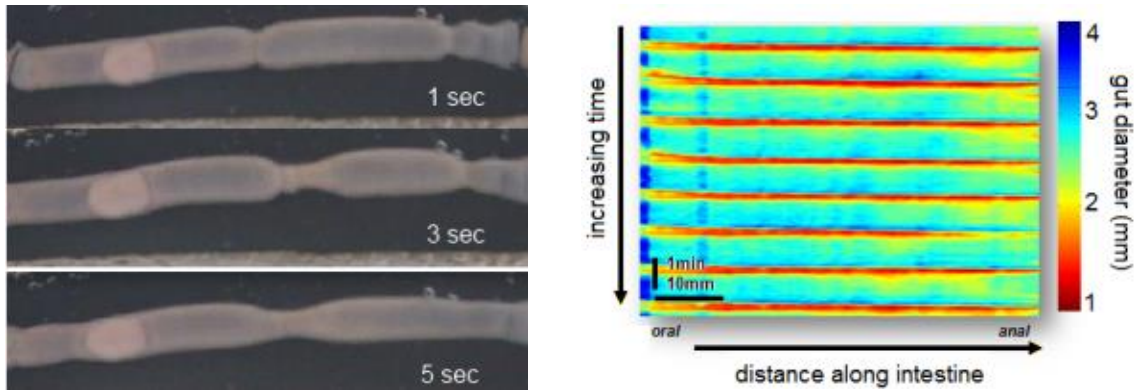
Figure 6. Spatiotemporal mapping from live imaging of an excised intestine. Example of an organ bath set-up for analysis via spatiotemporal mapping. A) Resulting footage laid out frame by frame. Changing the time interval will change the map. B) Resulting spatiotemporal map of the intestine. Modified from Melina Ellis (https://organsa12.imascientist.org.au/profile/melinaellis/printview/)

Spatiotemporal maps (STMaps) of motility are diagrams created by analyzing video images of organs in an *in vivo* or *ex vivo* experimental set up. The video captures the changing diameter of the lumen along the entire length of the organ as a result of contraction and relaxation. STMaps use luminal diameter from video recordings to track motility. The benefit of this technique is an ability to analyze whole segment function simultaneously and understand how motility works locally and globally in the organ. An advantage of video imaging is that it can be adjusted for speed of playback and multiple analyses can come from a single organ bath study (Kendig et al., 2016; Swaminathan et al., 2016).

To continue with guinea pig experiments, 8 cm segments of the colon were excised and submerged in physiological solution (Kendig et al., 2016). Luminal pressures were varied by injecting fluid into the intestine and clamping the ends to distend the segment. Then video recordings were taken during the distension and after clamps were removed to return to rest. The same procedure was performed with perfusion media that was varied in

nutrient, agonists/antagonists, and drugs, but the specific methodology is not clearly articulated (Kendig et al., 2016). The purpose of this study was to provide a method for creating the spatiotemporal maps of motility to understand the various motility patterns that arise from specific mechanical and chemical conditions. Ultimately, the resulting spatiotemporal maps allowed investigators to quantify frequency, velocity, duration, and direction of motility (Kendig et al., 2016).

Ex vivo approaches are also useful for testing the effects of drugs on gut function. For example, Swaminathan et al. (2016) provided a method for analyzing motility in 5-7 cm segments of colon and the effects of pharmacological substances in a murine intestine. The procedure was a total of 4 hours, with 30 minutes of equilibration time and 4 15-minute videos with varying perfusion media. For representative results, they used Tropisetron, a serotonin agonist, on wild-type and neuroligin-3 mutants to see the effects on colonic migrating motor complexes. Individuals with autism spectrum disorders (ASD) have been shown to have defects in intestinal motility and neuroligin-3 has been found to be mutated in ASD individuals. The antagonists had a greater effect on the mutant mice, significantly decreased rate of contraction compared to the wild-type mice (Swaminathan et al., 2016).

This same experiment was performed in goldfish, a member of the Cyprinid family along with zebrafish. In this experiment, 4-5 mm segments of intestine were excised to test the effects of Galanin (GAL) on force of contraction. GAL is known to be expressed in peripheral tissues of both humans and fish. A flow-through organ bath system allowed for the addition and removal of pharmacological effectors in physiological solution. Three conditions were tested: acetylcholine (ACh), GAL, and ACh + GAL. Since ACh stimulates muscle contraction, increased spontaneous contractions were observed and this condition was

used as a positive control for subsequent experiments. When GAL alone was added, the spontaneous contractions were significantly reduced in amplitude but not in number. However, when GAL was added 2 minutes after ACh, the amplitude of the spontaneous contractions increased significantly. This demonstrates the modulatory role of GAL, with the ability to excite and inhibit motility (Mensah et al., 2018; Olsson and Holmgren, 2001).

In our lab, I am concerned with the feasibility of organ bath studies in zebrafish. An important detail to note is that the zebrafish is significantly different in size compared to the mammalian models outlined above and to the goldfish. The total length of the adult zebrafish intestine is approximately 10-12 mm in length when it is folded (Kitazawa et al., 2017). A mouse has a 33 cm small intestine and the guinea pig has a 145 cm small intestine (Gabella, 1987). An exhaustive search for goldfish length data provided inconclusive information on sizes for comparison with zebrafish. The Fishbase.org database provides an estimated average length of approximately 10 cm, but also reports maximum lengths of 48 cm (Kottelat et al., 1993). Compared to the consistently 30-50 mm length of an adult zebrafish (Parichy et al., 2009), the goldfish is presumably much larger in size. However, Kitazawa et al. (2017) was able to excise relatively small lengths of intestine that could be used in zebrafish models using an organ bath.

Zebrafish Physiology Techniques

Kitazawa et al. (2017), already described in above sections, used an organ bath in conjunction with a force transducer to determine the response of the intestine of various species, including zebrafish, to motilin. Motilin is an important hormone that is secreted by the proximal small intestine and activates the migrating motor complex to ensure regular contractions move food and other materials through the lumen. The zebrafish intestinal bulb,

was attached to a force transducer. The specific length of the segment was not given, but a third of the intestine is approximately 4 mm, which would encompass the intestinal bulb. Exogenous samples of both zebrafish and human motilin were added to the intestinal segments. Human motilin was unable to produce any contraction while zebrafish motilin, at high enough concentrations, was able to produce contractions (Kitazawa et al., 2017). This pioneering study demonstrates that *ex vivo* experiments are possible in the adult zebrafish model. Figure 7 shows the response of the zebrafish intestine to A) human motilin and B) zebrafish motilin, showing that the intestine is stimulated more readily by zebrafish motilin in a dose dependent manner.

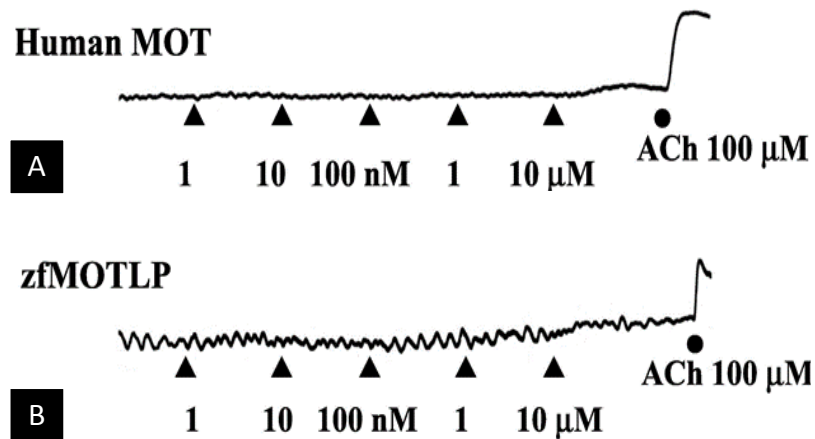**Figure 7. Responses to exogenous motilin in zebrafish tissues.** Example of contraction data that can be collected using *ex vivo* approaches for zebrafish intestine studies (A) Human and (B) Zebrafish motilin (Kitazawa et al., 2017)

Other *in vivo* studies of larval zebrafish reveal the efficacy of using live imaging alone for generating data. Due to the transparency of the zebrafish intestine in early

development, it is possible to observe motility in live, intact larvae. For example, Kuhlman and Eisen (2007) characterized wild-type zebrafish intestinal movements using a camera and imaging analysis. Zebrafish from 3.5 to 4.5 days post fertilization (dpf) were anesthetized and video recordings were made of peristalsis. As the intestine develops, nonspecific focal contractions progress into distinct anterograde waves in the intestinal bulb and retrograde waves in the middle intestine. Thus, the directionality of peristalsis is established for wild-type early larvae (Kuhlman and Eisen, 2007). In turn, mutants with reduced number of enteric neurons showed decreased propagation of these waves (Kuhlman and Eisen, 2007).

Live imaging can be enhanced with fluorescent probes. Shi et al., (2014) used DCFH-DA, a fluorescent probe specific to hydrogen peroxide, in live larvae between 2 and 4.5 dpf. Larvae were embedded in 1% agarose after anesthesia for live imaging. The images generated using the fluorescent probe increased contrast between the lumen and the intestinal wall. Figure 8 is an example of how fluorescence can enhance visualization of the intestine in live fish. It ascertains the normal development of the luteinizing-hormone treated zebrafish, which have decreased peristalsis frequency due to the treatment (Shi et al., 2014).
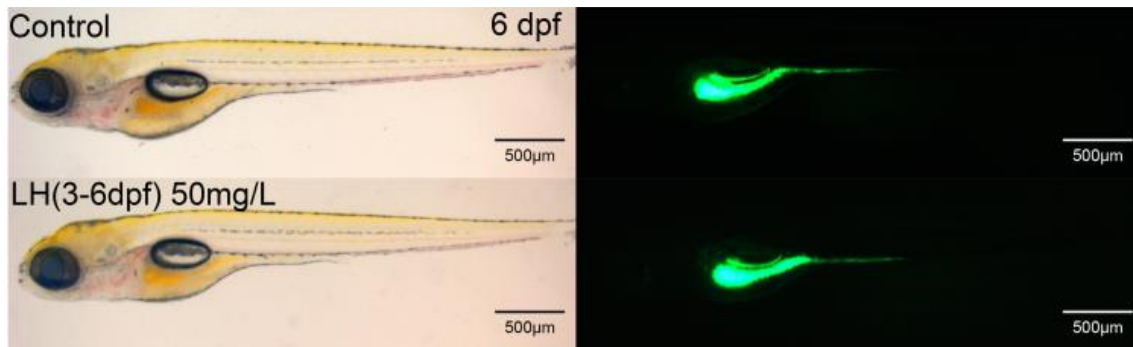
Figure 8. Live imaging of luminal space in 6 dpf zebrafish intestines. Wild type and luteinizing-hormone treated zebrafish showing similar morphology grossly and in the intestine. From (Shi et al., 2014).

Extending the live imaging technique into later ages shows that the motility pattern reported in Kuhlman and Eisen (2007) is maintained. Using 7 dpf larvae, Heanue et al. (2016) compared wild-type zebrafish with heterozygous *ret* mutants that had varying degrees of colonic aganglionosis, which is a lack of enteric neurons in the distal part of the intestine. To compare gut function of the two groups, fish were anesthetized, laid laterally in an agarose trough, and video recorded for 16 minutes. Spatiotemporal maps of the videos were generated and representative movies of peristalsis were created by speeding up playback 20 times (Heanue et al., 2016). Mutants with a more severe aganglionosis showed reduced frequency and amplitude of peristalsis waves. The advantage of this system is that data generated from live imaging and video recording reduces confounding factors associated with excising the intestine.

A further benefit of *in vivo* studies using live imaging is that software can be used to interpret the data in a variety of ways. For example, the time and distance traveled can be

useful in understanding the relative force of contraction even in the absence of attaching a force transducer. Spatiotemporal mapping of changes in the diameter of the intestine allows observation of motility patterns. Such mapping can be used to compile a library of movements. In humans, the gut has a wide range of motility patterns, characterized as phases of the migrating motor complexes, spontaneous contractions, cyclic slow waves, peristalsis, segmentation, pendular movements, etc. In future work, the same sets of motility patterns can potentially be observed in the zebrafish model to enhance our understanding of the overlap between human and zebrafish gut function.

Studies such as those described above can help to determine the main differences and similarities in intestinal activity between the human and zebrafish. This will enhance our ability to translate observations from the zebrafish to humans. For example, the human small intestine, which is divided into three segments, the duodenum, jejunum and ileum, has variable rates of transit depending on which segment of small intestine is considered. The duodenum has the shortest transit time, a matter of minutes. This is because the duodenum has a specialized endocrine system that senses luminal contents which then stimulates the segment to contract to move the bolus along the tract. This quick transit time aligns with its primarily endocrine function of sensing luminal contents and recruiting the proper enteroendocrine signals. Contents move through the jejunum in 1 to 2 hours, while they remain in the ileum for 3 to 6 hours (Connell, 1961). This occurs because most of the digestion and absorptive functions happens in these segments which requires a longer transit time. In the simplified larval zebrafish intestine with its limited compartments, it is currently not known whether there are distinctive transit time patterns for each section of the GI tract. Given the morphological differences of the three segments, it is possible, there may be

different transit times along GI tract. It is possible that in the adult zebrafish, the intestine may follow a pattern of motility similar to humans where luminal contents move through the intestinal bulb, middle, and posterior intestine at variable rates. However, gut transit has not been studied in the adult zebrafish, and studies in larvae have investigated only overall transit rates rather than investigating motility in specific segments.

Objectives of this study

The primary purpose of this study is three fold. First, I would like to determine the standard length at which larvae enter the metamorphic stage. I can then use this information to track the maturational events in the intestinal epithelium. This includes localizing the differentiation of goblet cells through time and determining whether gut looping occurs during metamorphosis. All of the data generated in this study will contribute to the goal of developing a gut transit assay to uncover functional roles of different intestinal genes.

Materials and Methods

Husbandry

Zebrafish were approved for use by the Institutional Animal Care and Use Committee at Appalachian State University (IACUC protocol #16-05). All animals were wild type, obtained from the local pet shop. Adults were raised according to *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish Danio (Brachydanio rerio)* (Westerfield, 2007). This included a 14-hour light cycle with light from 9:00AM to 11:00PM each day. Fish were fed twice daily, once with dry food at the beginning of the light cycle and once in the afternoon with 2-day old brine shrimp (Artemia). Water quality was monitored and maintained daily, with conductivity between 450-600 microsiemens, acidity between pH 6.8-7.2, and water temperatures between 27ºC and 28ºC. Adults used for histology were obtained from the Appalachian State University Zebrafish Core Facility.

Larval zebrafish were raised on tabletop nurseries following the regimen outlined in Norton et al. 2018 (Norton et al., 2018). Embryos for larval studies were obtained from crosses with adults from the Zerucha Lab in the Zebrafish Core Facility. For larval sampling for histology, embryos were collected from the Zebrafish Core Facility and raised in the Kinkel Lab.

Metamorphosis Analysis

Individual fish were pre-screened for larval or metamorphic morphologies and some samples were chosen as representatives of larval and metamorphic for intestinal histology, described below. Larval and metamorphic zebrafish were anesthetized by gradually lowering water temperature to 17.5ºC using ice made of E3 medium (5mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mm $MgSO_4$). Larvae were transferred to a stage micrometer (Peak Glass

Stage 50) and imaged using a Canon Rebel T5i on an Olympus SZX12 stereoscope. Images were analyzed for length using ImageJ (Schneider et al., 2012). Standard length is a measurement of the line from the tip of the snout to the peduncle, the fleshy base of the tail. In larval fish, the peduncle may not be formed or visible, thus the end of the notochord is used instead. Standard length was measured for metamorphic zebrafish while notochord length was measured for larvae. The final measurement is referred to as standard length hereafter for clarity.

A pool of 247 fish at 15, 21, and 22 dpf were scored for metamorphosis. A mix of ages prevented calendar age induced bias. Three researchers scored fish independently and only agreed upon scores were used to analyze metamorphosis onset. A range of metamorphosis was determined; the longest fish with larval morphology was set at the top of the range while the shortest fish that had entered metamorphosis set the bottom of the range.

Length Measurement

Fish were fasted overnight prior to length measurement. Individuals were anesthetized by gradually lowering water temperature to 17.5℃ using ice made of E3 medium. The fish was then immediately transferred to a stage micrometer (Peak Glass Stage 50) using a wide-bore fire-polished Pasteur pipet. The micrometer was held submerged under pre-chilled E3 medium in a petri dish to maintain the fish in an anesthetized state during imaging. The fish was imaged in lateral view against the micrometer using a Canon Rebel T5i camera mounted on an Olympus SZX12 stereomicroscope. The microscope had transmitted light through the base and overhead lighting from a fiber optic illuminator (Cole-Parmer Instrument Company). Images were captured using a 1.0x objective The fish was then immediately transferred to cold fixative as described below.

Sample Preparation

For fixation of larvae, an anesthetized fish was immediately transferred to a glass bottle containing cold 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and rocked gently at 4ºC overnight. Some samples were decapitated prior to submersion into PFA, while others were left intact. This was done to improve fixation of the intestinal epithelium; decapitated specimens had exposed intestines that were infiltrated more quickly by fixative. Bottles were labeled so that individual fish could be matched to their live images. After overnight fixation, specimens were pre-embedded in 1% low melting temperature agarose (VWR International) dissolved in 1X PBS. Vinyl cyromolds (Tissue-Tek®, Torrance, CA), 15x15x5mm, were placed on a chilled metal block, and a layer of agarose was poured, enough to cover the bottom of the mold. The remainder of the agarose was kept on a heating plate at a low setting to maintain a liquid state. Three specimens were transferred carefully, one at a time, using straight forceps (World Precision Instruments) and laid flat on the agarose. Once oriented on their side, agarose was poured to cover the specimens, adjusting specimen with a fishing line loop if necessary, and the chilled block and mold were placed in 4ºC to solidify. The specimens were oriented in the same direction and were tracked in order (top to bottom) for continuity from live imaging to slides. An example is shown in Figure 9.

For fixation of adult tissues, specimens were fasted overnight to clear gut contents. Specimens were dissected under 1X PBS following the en-bloc dissection described in Eames-Nalle 2017 and schematized in Figure 10 (Eames-Nalle et al., 2017). Once the intestine was excised and isolated, specimens were moved directly to fixative overnight.

They were pre-embedded and shipped in the same manner as the larval specimens, detailed above.

After the agarose polymerized, the block was held between two 25x32mm biopsy foam pads (Electron Microscopy Sciences), one on each side, and transferred to a tissue-embedding cassette (Electron Microscopy Sciences). The cassette was then transferred to a specimen jar with 4% PFA and shipped overnight to AML Laboratories (Saint Augustine, Florida) for processing. Samples were paraffin embedded and serially sectioned at 5 µm as described above.
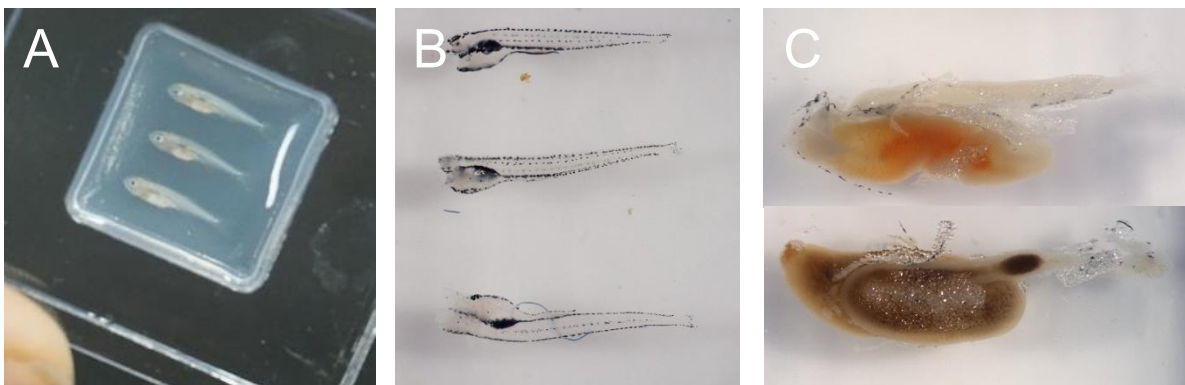

Figure 9. Specimen preparation. Fixed specimen pre-embedded in 1% agarose blocks for subsequent histology. **A.** Whole 23 dpf metamorphic samples. **B.** 15 dpf larval and metamorphic samples decapitated prior to fixation. **C.** Adult intestines excised using en-bloc dissection.
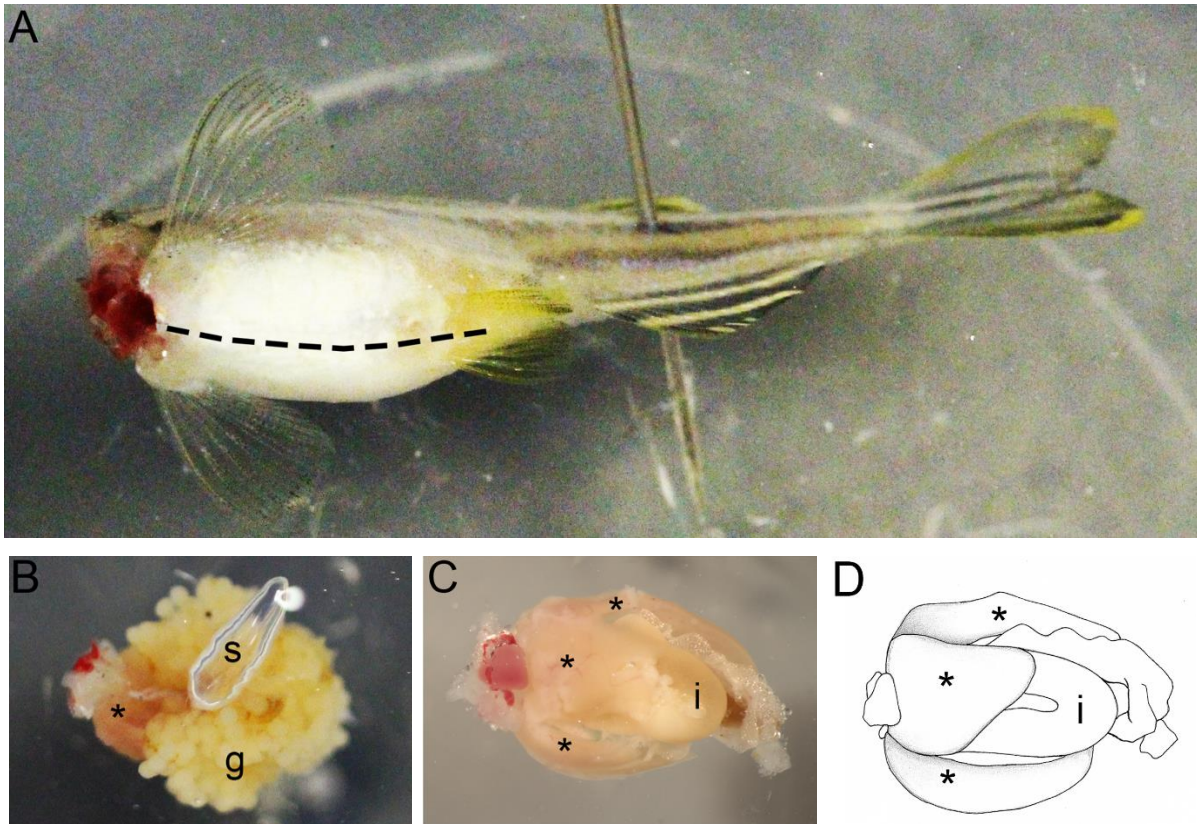
Figure 10. Isolation of the adult intestine. A. Wild-type adult specimen pinned onto a Sylgard dissecting dish for dissection. Dotted line at the ventral midline showing location of the posterior to anterior incision to access the abdominal cavity. **B.** Excised visceral organ mass, including the intestine; *, liver lobe; s, swimbladder; and g, gonads (female). **C.** Gonads and swim bladder removed. The first loop of the intestine is indicated by 'i'. **D.** Drawing of C, outlining three lobes of the liver, indicated by asterisks. The intestine is closely associated with the liver and visceral fat (Eames-Nalle et al., 2017).

Histology

Goblet cells were stained by modifying an established method for Alcian Blue - Periodic Acid Schiff's staining of zebrafish intestinal epithelium (Trotter et al., 2009). The following procedure was completed by slotting slides into a slide rack 12 at a time and following a series of incubations. Each time the slides were immersed in a solution, the solution was fresh.

To clear paraffin wax, slides were immersed in xylene (EMD Millipore) twice for 2 minutes each. Tissues were incubated in anhydrous ethanol three times for 2 minutes each to clear xylene for rehydration. Next, slides were flushed under running tap water for 1.5 minutes to hydrate tissues. Slides were transferred to 3% acetic acid (VWR International) for 15 minutes to equilibrate tissues for goblet cell staining. Then, slides were incubated in 1% Alcian Blue (Alfa Aesar) dissolved in 3% acetic acid for 10 minutes. Once again, slides were flushed under running tap water for 3 minutes, dipped in RO/DI water, before moving to brush border staining.

Once goblet cells were stained, Schiff's reagent (Electron Microscopy Sciences) was used as a brush border stain to outline the intestinal epithelium. Slides were incubated in Schiff's reagent for 13 minutes, rinsed thoroughly for 5 minutes with running tap water, and then dipped in RO/DI water. Mayer's Hematoxylin (Electron Microscopy Sciences) was used as a counterstain for the rest of the tissues. Slides were incubated in Mayer's Hematoxylin for 1 minute, rinsed under running tap water for 1.5 minutes, and then transferred Scott's Tap Water (Electron Microscopy Sciences) for 1 minute.

Tissues were incubated in anhydrous ethanol three times for 2 minutes to be dehydrated again for xylene. Slides were immersed in a series of xylenes twice for 2 minutes each. Once tissues were dehydrated, Permount (Fisher Scientific) mounting media was dropped onto the stained specimen carefully using a pasteur pipette, and then a 24x60mm glass coverslip (Fisher Scientific) was placed gently over the mounting media using coverslip forceps (World Precision Instruments). Excess mounting media was dabbed off with a xylene soaked Kim-Wipe. Completed slides were laid facing up to dry overnight.

Tissue Imaging and Analysis

       Digital images of stained slides were captured at 100x magnification using a Moticam 2.0 (Motic, British Columbia) mounted on a Leica Upright CME light microscope. For each fish, multiple images were taken across the anterior-posterior length of the intestinal tract using Motic Images 3.0 (Motic, British Columbia). Images were used to analyze the appearance of goblet cells, which were easily discernable as large bright blue spots marked by Alcian Blue along the intestinal epithelium, which was marked in fuchsia. The images were then stitched together using Canon Utilities PhotoStitch software (Canon) to reconstruct the intestinal tract prior to analysis. The stitched images were used to assess whether the intestinal tract had developed loops. If an image showed multiple, noncontiguous sections of lumen, this was a clear indication of a looped gut.

Results

To begin this study, I hypothesized that zebrafish undergo many intestinal changes during metamorphosis. Throughout metamorphosis, the zebrafish undergoes a wide range of internal and external changes that take the fish from a larval to adult morphology. For example, several body systems are reorganized, the skeleton ossifies from cartilage to bone, and several pairs of bony fins replace the larval fin fold. It would follow that the intestine undergoes some structural reorganization during metamorphosis as well. I am specifically interested in determining what metamorphosis looks like in relation to the intestine, thus it must be determined when metamorphosis occurs. What are the best strategies for intestinal studies since the larval/metamorphic and adult intestines are very different in size? Isolating the intestine for morphological investigation will look different in larval versus adult tissues.

The intestine starts off relatively simply, with a straight gut tube and limited cell types in the early larval stage. For example, goblet cells are known to be fully expressed along the length of the adult intestine, but are differentiated only in the middle intestine of the larval zebrafish. It continues to develop and mature into a much larger, more complex organ, including the appearance of two loops, and I hypothesize, a variety of specialized regions of differentiated cell types. As part of my exploration of the gut, I needed to answer several questions to gain a better understanding. For instance, when do these transitions in intestinal morphology and composition occur? Is gut looping observed early in metamorphosis, is it gradual, or does it happen quickly? What is the manner of goblet cell differentiation from the early larval stage, sequestered in the middle intestine, into the adult stage, expressed throughout? When during development do these changes occur? These are the driving questions behind this study to better understand the zebrafish intestine, which will ultimately lead to more robust conclusions in human disease modeling.

Metamorphosis Analysis

The analysis first focused on 9 to 22 dpf zebrafish. Each fish was imaged. Standard length was determined from the images. Figure 11 shows that as larval zebrafish fish grow through time, the length disparity increases for fish. This length disparity can still be observed even when conditions such as feeding regimen and care are standardized. Figure 11 is showing representative results from data collected at 4 institutions (Norton et al., 2018).
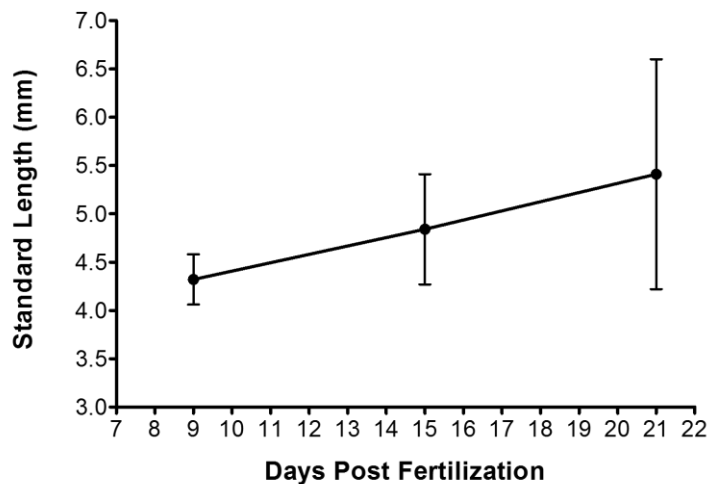
Figure 11. Growth in length through time. Larvae were anesthetized and imaged live. Standard length was measured from digital images. 9 dpf, n = 82; 15 dpf, n = 132; 21 dpf, n = 57; mean and standard deviation is shown.

Next, I determined what the morphology of those fish was, larval or metamorphic (Figure 12), based on external characteristics. These characteristics include condensation of cartilage to form the caudal fin (hypural condensation), flexion of the notochord, and recession of the fin fold. Images taken to measure standard length were also used in a qualitative analysis to determine if the fish was larval or metamorphic based on visible indicators of metamorphosis. If the zebrafish exhibited a cartilaginous caudal fin

accompanied with a high degree of notochord flexion, it was considered metamorphic. If the

zebrafish still had a smooth fin fold and straight notochord, it was considered larval.
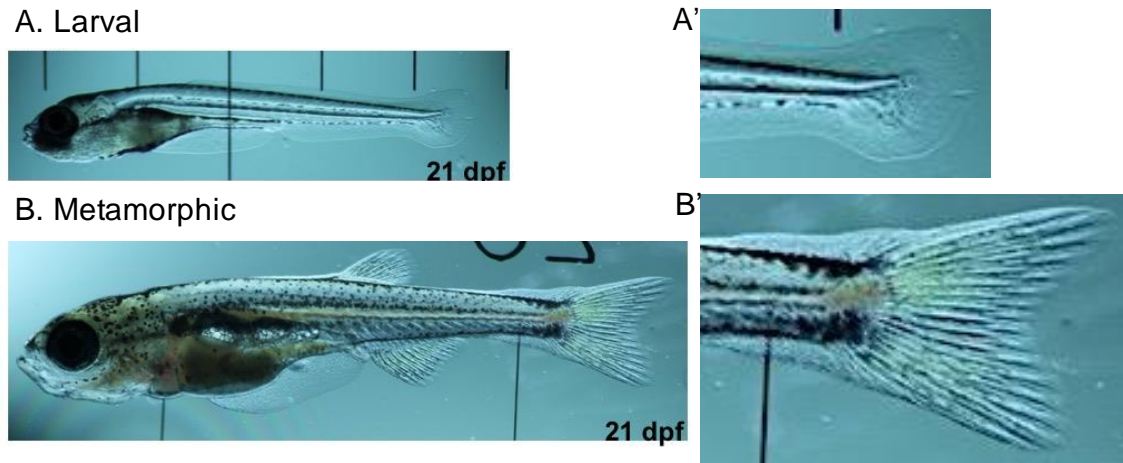

Figure 12. Metamorphosis analysis of postembryonic zebrafish. Fish reared together in the same tank mature at different rates. **A.** Larval fish at 21 dpf, live image. **A'** Higher-magnification view of the caudal fin fold. No bony fin rays are apparent. The notochord is flexed. **B.** Metamorphic fish at 21 dpf. **B'.** Higher-magnification view of the caudal fin. The fin fold has disappeared. Bony rays are apparent in the fin. The caudal peduncle (fleshy base of the fin) is apparent.

Finally, each fish was plotted by the standard length (SL) and scored indicated

whether the fish was larval or metamorphic (Figure 13). This was done to compare the

standard lengths of larval and metamorphic zebrafish. I noticed a distinct length threshold

for metamorphosis in which fish past 5.22 mm SL were metamorphic, while fish below 4.41

mm SL were larval, with a 0.8 mm SL overlap. Thus, I concluded that metamorphosis

begins as early as 4.4 mm SL for some fish and by 5.2 mm SL, all fish have begun
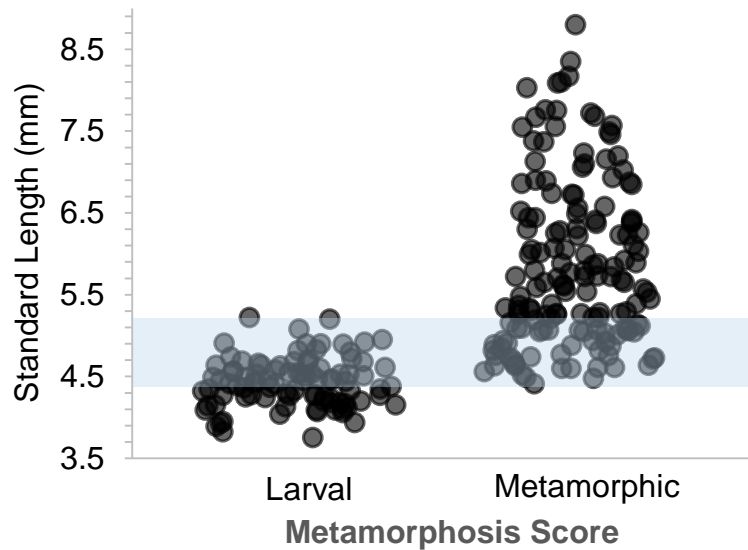
metamorphosis.

Figure 13. Determination of a length threshold for metamorphosis. Live, wildtype zebrafish at 15, 21, and 22 dpf were imaged and analyzed for life stage and standard length (SL). The blue bar indicates the range at which metamorphosis begins. The lower limit of the range is 4.4 mm SL and the upper range is 5.2 mm SL. Each circle represents an individual. n = 247.

Using the data collected from the standard length and metamorphosis scoring, Table 1 and 2 were generated to show, comparatively, the benefit of using standard length instead of calendar age for this type of study. Table 1 examines metamorphosis using the widely accepted measure of days post fertilization (dpf), which is the convention for tracking zebrafish development extended from embryonic studies. Both 15 dpf and 21 dpf fish raised under the same conditions show the same percentage of metamorphic fish, 61%. That moves up to 74% for 22 dpf. These data demonstrate the potential for intestinal studies that involve developmental staging to be confounded by calendar age. Table 2 organizes the data by standard length rather than by age. Here, I observe that there is a length threshold of 4.2 mm SL at which fish begin to enter the metamorphic period. Further, there is a length threshold of 5.2 mm SL at which 100% of fish have begun metamorphosis. For fish within the 4.4 and

5.2 mm SL threshold, there is a 50% chance of either larval or metamorphic morphology. However, outside of either range, there is 100% certainty. Therefore, I concluded that above and below the length threshold for metamorphosis, there is a certainty when judging metamorphosis. Next, I observed if this marked the beginning of key changes in the intestinal epithelium.

Table 1. Metamorphosis analysis using calendar age.

Age	Larval Stage	Metamorphic Stage	Percentage Metamorphic
15 dpf	36	56	61%
21 dpf	51	81	61%
22 dpf	6	17	74%

Table 2. Metamorphosis analysis using standard length. The data shown in Table 1 were re-analyzed.

Standard Length	Larval Stage	Metamorphic Stage	Percentage Metamorphic
< 4.41 mm	43	0	0%
Within Threshold	49	49	50%
> 5.22 mm	0	106	100%

Maturation of the Intestinal Epithelium

Once metamorphosis was generally characterized for the age range I was working in, a histological analysis provides the remaining details for major epithelial cell differentiation events and morphological changes of the intestine. To begin this analysis, I chose goblet cells, a major secretory cell type of the intestinal epithelium. Goblet cells primarily function as mucin-producing cells. Mucins are involved in coating intestinal contents for smoother movement along the intestinal epithelium. Goblet cells also play a role in innate immunity, providing a physical barrier of entry to some pathogens along the intestine. I chose to look at goblet cells because they are a prominent cell type that is known to shift expression patterns along the intestine from the larval to the adult stage. I hypothesized a change over time in the period of metamorphosis for two reasons: 1) metamorphosis is the time when major changes occur in the zebrafish 2) during the larval period, the yolk is depleted and zebrafish begin exogenous feeding, which could contribute to structural changes in the intestinal epithelium.

Goblet cell density is known, in general, to be different in different regions of the intestine during the larval, juvenile (after metamorphosis is completed), and adult stages (Ng et al., 2005). However, these studies do not have clear and accurate staging information necessary for postembryonic zebrafish studies in general. I decided to look at goblet cells first because they are clear cellular indicators of gut differences in the epithelium. Previous studies showed that at 5 dpf, corresponding with approximately 3.90 mm SL, goblet cells were found only in the middle intestine. However, later stages determined (Ng et al., 2005). In my study, metamorphic fish with length close to the metamorphosis threshold determined

in Figure 13 were chosen to see how length coincided in goblet cell composition in the intestinal epithelium.

Our hypothesis was that metamorphosis marked the beginning of major changes in the intestine. I wanted to know if larger larval zebrafish maintained a similar morphological and goblet cell distribution or if the changes from the larval to adult stage occurred earlier than metamorphosis. Four larval specimens were examined ranging from 4.70 mm SL and 5.15 mm SL. In Figure 14, a 4.89 mm SL larval fish was examined for epithelial maturation beyond the data available in the literature. I found that goblet cells were sequestered in the middle intestine and specimens maintained a straight gut tube.

The next portion of the study was twofold: first, to test the metamorphosis threshold for marking major developmental changes in the intestine and second, to see if the intestine underwent any changes during metamorphosis. To test the strength of our threshold standard length for entering metamorphosis for predicting intestinal maturational changes, I examined specimens close to the metamorphosis threshold. A group of 6 fish raised in the same tanks, under the same conditions, with the same calendar age was examined. All fish were 15 dpf. This included a 5.15 mm SL fish and 5.99 mm SL fish, which provide the representative images for the following conclusions.

A 5.15 mm SL larval fish was analyzed via histology (Figure 15). In the group of fish below the metamorphosis threshold, the intestinal epithelium had the same composition as the larval fish in Figure 14. It is important to note that this specimen has all of the external indicators of larval morphology, including an underdeveloped caudal fin and a discernible fin fold. The intestinal epithelium still only showed goblet cell differentiation in the middle

intestine. Thus, this suggests that the chance of intestinal epithelial changes occurring in the

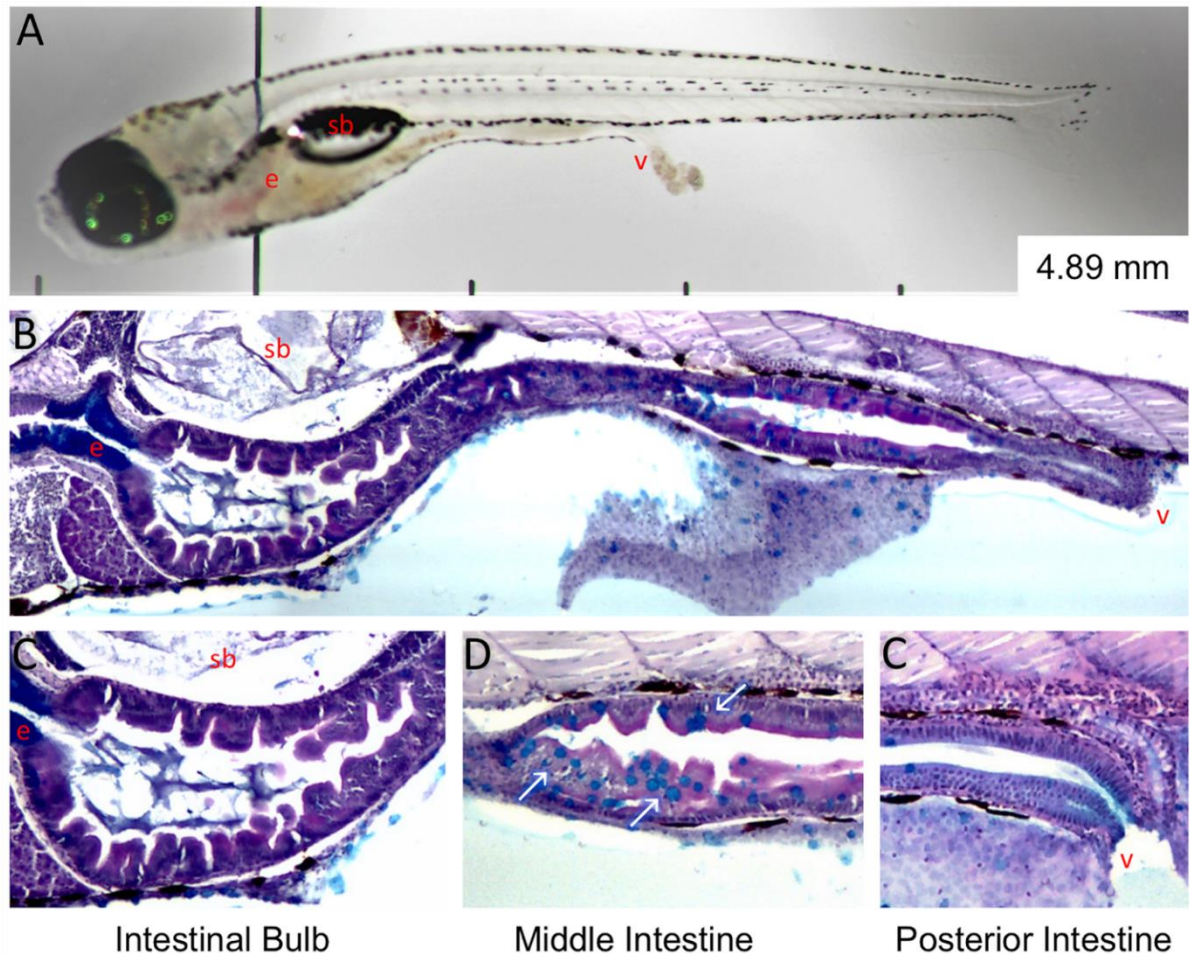larval stage for goblet cells before metamorphosis.

Figure 14. A larval-stage zebrafish specimen at 4.89 mm SL. A. Qualitative analysis of
the live specimen shows a lack of caudal fin development, with limited notochord flexion or
hypural condensation. sb, swimbladder; e, esophagus; v, vent. **B.** Histological analysis of the
gut tube at 40x magnification shows that the intestine is a straight, unlooped gut tube. The
tissue was processed with with Alcian Blue and Periodic Acid Schiff's stains. Counter-
staining used Mayer's hematoxylin. Panels C-E show higher-magnification views of panel B.
C. 100x magnification of the intestinal bulb**.** Staining shows a complete absence of goblet
cells. **D.** 100x magnification of the middle intestine. Staining reveals a high level of goblet
cell differentiation (white arrows). **E.** 100x magnification of the posterior intestine. Staining
shows a complete absence of goblet cells. All panels shown with anterior to the left.

Figure 15. A larval-stage zebrafish specimen at 5.15 mm SL. A. Qualitative analysis of the live specimen shows a lack of caudal fin development, with limited notochord flexion or hypural condensation. sb, swimbladder; e, esophagus; v, vent. **B.** Histological analysis of the gut tube at 40x magnification shows that the intestine is a straight, unlooped gut tube. The tissue was processed with with Alcian Blue and Periodic Acid Schiff's stains. Counter-staining used Mayer's hematoxylin. Panels C-E show higher-magnification views of panel B. **C.** 100x magnification of the intestinal bulb**.** Staining shows a complete absence of goblet cells. **D.** 100x magnification of the middle intestine. Staining reveals a high level of goblet cell differentiation (white arrows). **E.** 100x magnification of the posterior intestine. Staining shows a complete absence of goblet cells. All panels shown with anterior to the left.
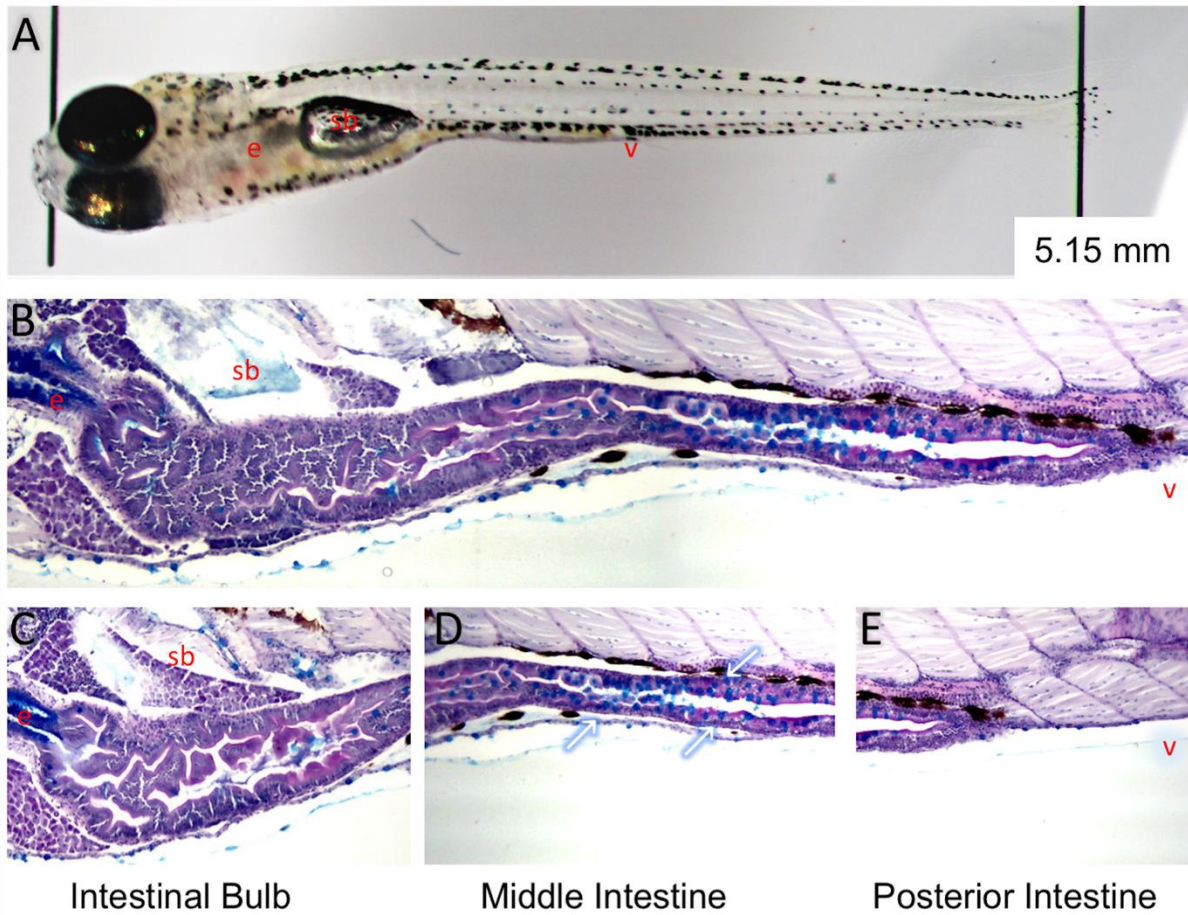
A 5.99 mm SL metamorphic fish was analyzed via histology (Figure 16). This is a fish that is just beyond the metamorphosis threshold and coming from the same group of fish as the Figure 15, prepared for histology the exact same way. It is evident that this specimen has a highly developed caudal fin, with already visible fin rays. Another indicator of metamorphosis is the development of the anterior swim bladder lobe, a maturational event observed after caudal fin development (Parichy et al., 2009). Looking at Figure 16C, it is clear that the intestinal epithelium begins differentiating a set of goblet cells in the anterior-most portion of the intestinal bulb at this stage. An analysis of the additional serial sections of the specimen, not shown, exhibits the stepwise differentiation of a group of goblet cells in the anterior intestinal bulb that are spatially separate from the population in the middle intestine. I ascertained that metamorphosis is a prerequisite to intestinal changes and that the epithelial begins to mature during the period of metamorphosis.

As metamorphic zebrafish grow during the metamorphic stage, goblet cells become more numerous along the entire length of the intestine. I chose further along in metamorphosis to see if any changes to the intestinal epithelium continued. Beyond the range of metamorphosis threshold samples, I observed three specimens between 7.20 mm SL and 7.43 mm SL. Figure 17 provides a representative sample analyzed for the presence of goblet cells. At this stage, the intestinal epithelium is completely populated by goblet cells, as seen in aduls, showing an even distribution from the anterior intestinal bulb through to the end of the posterior intestine at the vent. This means that as metamorphosis continues, the intestinal epithelium also continues to undergo remodeling.

Figure 16. A metamorphic-stage zebrafish specimen at 5.99 mm SL. A. Qualitative analysis of the live specimen shows caudal fin development, with hypural condensation and fin rays. sb^a, anterior swimbladder lobe, sb^p, posterior swimbladder lobe; e, esophagus; v, vent. **B.** Histological analysis of the gut tube at 40x magnification shows that the intestine is a straight, unlooped gut tube. The tissue was processed with Alcian Blue and Periodic Acid Schiff's stains. Counterstaining used Mayer's hematoxylin. Panels C-D show higher-magnification views of panel B. **C.** 100x magnification of the intestinal bulb. Staining shows differentiated goblet cells in the anterior intestinal bulb (white arrows). **D.** 100x magnification of the middle intestine. Staining reveals a high level of goblet cell differentiation (white arrows). Data for the posterior intestine is unavailable. All panels shown with anterior to the left.
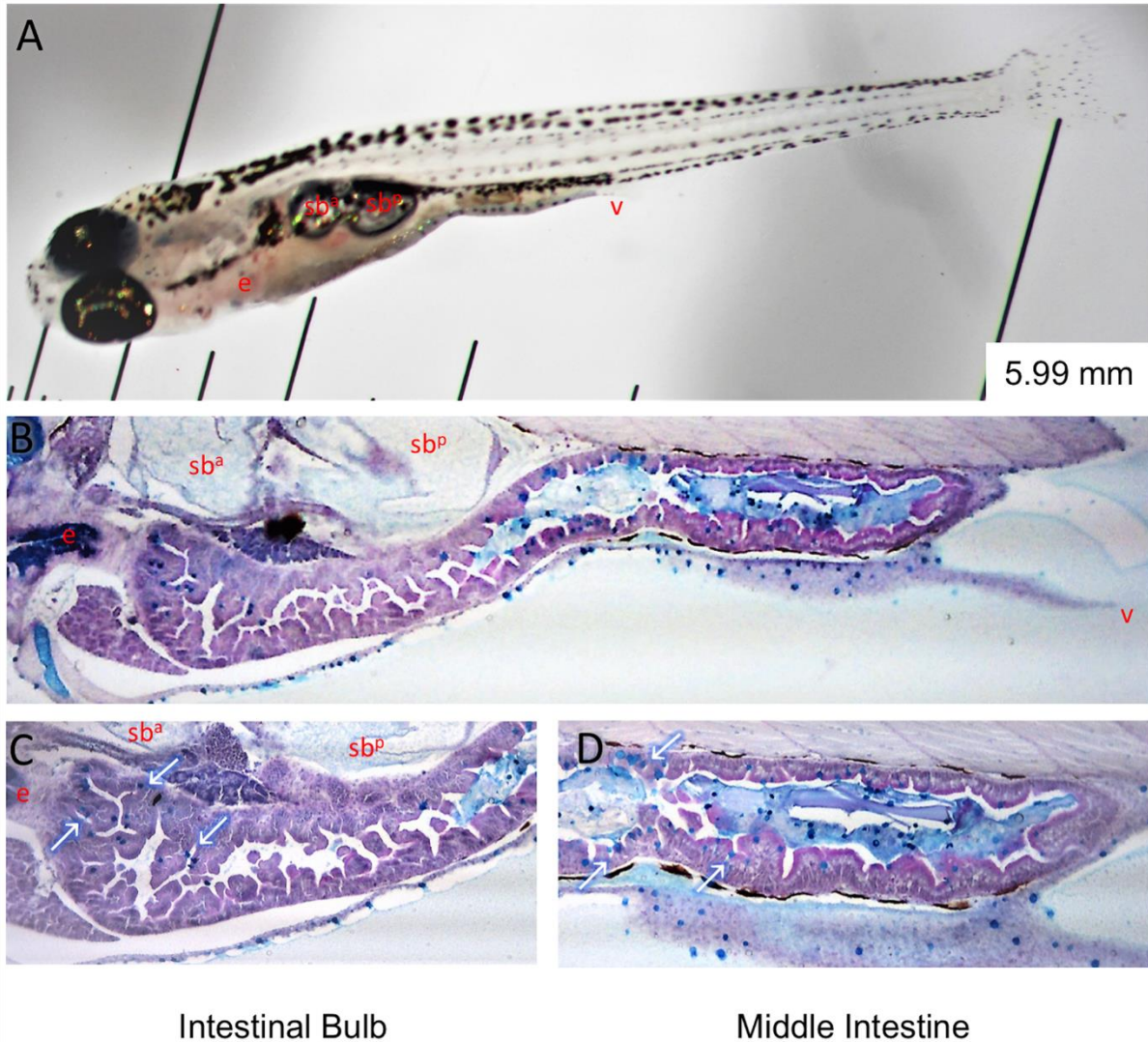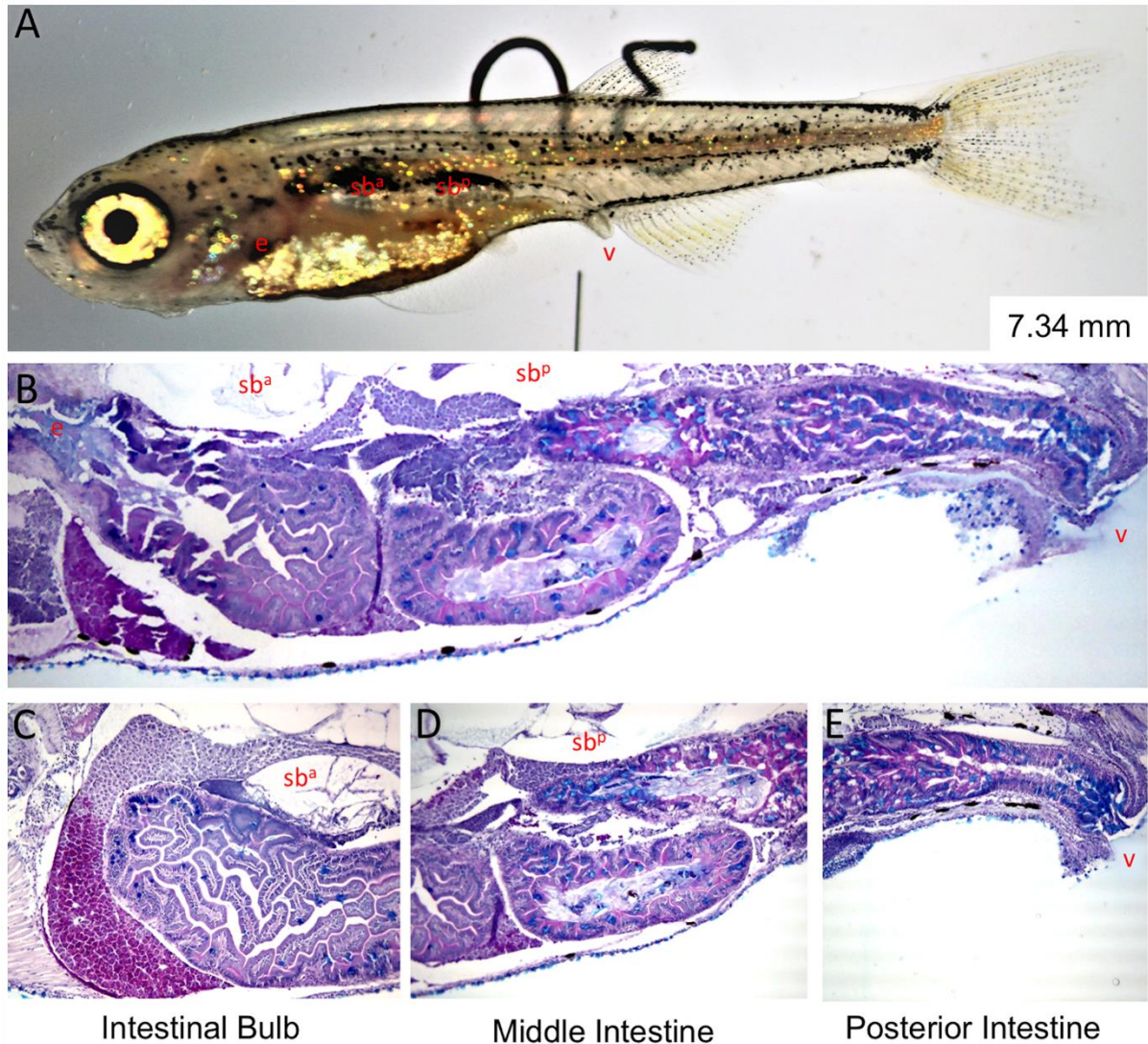
Figure 17. A late metamorphic-stage zebrafish specimen at 7.34 mm SL. A. Qualitative analysis of the live specimen shows a high degree of caudal fin development, appearance of new adult fin structures, and a larger, double-lobed swimbladder. sb^a, anterior swimbladder lobe, sb^p, posterior swimbladder lobe; e, esophagus; v, vent. **B.** Histological analysis of the gut tube at 40x magnification shows the intestine has morphologically remodeled to a looped gut tube. The tissue was processed with Alcian Blue and Periodic Acid Schiff's stains. Counterstaining used Mayer's hematoxylin. Panels C-E show higher-magnification views of panel B. **C.** 100x magnification of the intestinal bulb. Staining shows differentiated goblet cells embedded throughout the intestinal bulb. **D.** 100x magnification of the middle intestine. Staining shows the discontinuous lumen indicative of gut looping, as well as full goblet cell differentiation throughout. **E.** 100x magnification of the posterior intestine. Staining reveals a high level of goblet cell differentiation. All panels shown with anterior to the left.

From the data, it is evident that the zebrafish intestinal epithelium matures during metamorphosis. Using goblet cells, a prominent cell type in the intestinal epithelium of both humans and zebrafish, I was able to track the differentiation from the larval stage into metamorphosis. Using a group of 10 zebrafish from 4.70 mm SL to 7.43 mm SL, I rendered a complete ontogeny of goblet cell differentiation. Goblet cells differentiate in a stepwise manner, starting with a population in the middle intestine, followed by the appearance of goblet cells in the anterior intestinal bulb. Later in metamorphosis, there is a complete population of the intestine by goblet cells, as observed in the adult intestine. It is clear that external indicators of life stage used in the metamorphosis analysis coincide with the maturation of the intestinal epithelium. The same samples were used for the subsequent investigation of gut looping.

Analysis of gut looping

Gut looping is another feature that has a different manifestation in the larval versus adult zebrafish. It is known in larval zebrafish that the intestine is a relatively straight tube from esophagus to vent. In the adult zebrafish, the intestine has two loops in the middle intestine, right after the intestinal bulb. However, it is not known at which point along the life span of the zebrafish this looping occurs.

I wanted to explore two possibilities: 1) the gut loops develop gradually, where looping is not obvious until much later in life or 2) the intestinal loops are evident immediately, defined early in the maturations of the intestine. Again, I hypothesize the second possibility, that metamorphosis is the time when the looped gut is defined morphologically. Using the slides generated from the 10 samples between 4.70 and 7.34 mm SL, a range that covers a large portion of metamorphosis, I set out to determine if the gut

loops during this period. Of the 10 samples, the 3 longest length zebrafish, from 7.20 to 7.43

mm SL exhibited looping in the middle intestine that resembled the adult. The remaining 6

samples had no clear indications of gut looping. Thus, I conclude that the intestine loops

early in metamorphosis and simply continues growing in size as the zebrafish grows.

Adult Intestine

Although not shown in the data, adult intestines were also dissected to see if goblet

cells were differentiated the same way found in the metamorphic intestine. The entire length

of the intestine is fully populated with goblet cells. The gut is also looped, which is known in

the literature. Whether there is a functional difference that arises from gut looping is still

unknown.

Ontogeny of the postembryonic zebrafish intestine

New information regarding the ontogeny of the zebrafish intestine has been presented

in this study. Specifically, I have uncovered details about goblet cell expression and gross

morphology through the metamorphic stage. Albeit a small sample size of just 10 samples,

these novel data provide insight into some major post-embryonic intestinal development

events along with offering a system for tracking the intestinal tract. Essentially, I am able to

provide a way to stage the maturation of the intestine in live specimens using external

indicators while elucidating what some of the gross intestinal changes were. The schematic

in Figure 18 outlines the major findings of this investigation.

First, goblet cells differentiate in a step-wise manner over the period of

metamorphosis. In early larval stages, goblet cells are first differentiated in the middle

intestine (Figure 18A). As the zebrafish grows in length through time and enters

metamorphosis, goblet cells continue differentiating in a step-wise manner. The intestinal

bulb differentiates goblet cells that are distinct and spatially from the population of goblet cells in the middle intestine (Figure 18B). Once the zebrafish is in metamorphosis and continues changing, the distribution of the goblet cells expands across the entire length of the intestine, filling in the remainder of the intestinal bulb and the posterior intestine (Figure 18C). This is also the time when gut looping is first observed in the intestine. Figure 18D shows the known morphology of the adult intestine. The juvenile intestine, the life stage at the completion of metamorphosis, is simply inferred from the presence of loops and distribution of goblet cells found during metamorphosis.
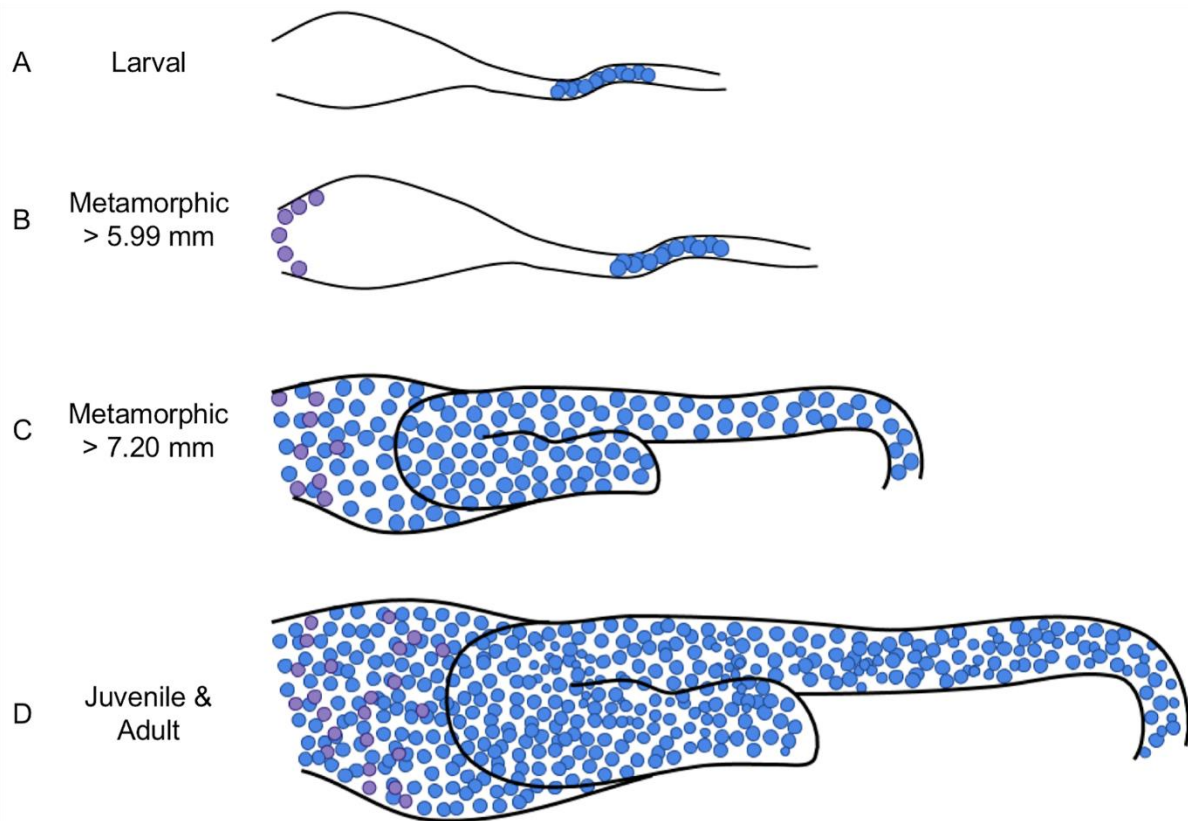
Figure 18. Schematic of goblet cell distribution and gut looping. Blue dots represent acidic mucins, while violet dots represent goblet cells that produce both acidic and neutral mucins. **A.** A larval gut tube with goblet cells sequestered in the middle intestine and a straight morphology. **B.** After metamorphosis begins, goblet cells appear in the anterior intestinal bulb, while the overall morphology is still straight. **C.** Goblet cell expression covers the entire intestinal epithelium and there are two gut loops in the middle intestine. **D.** The results from the dissected adult intestine with known loops and the juvenile intestine, with loops and goblet cell distribution extrapolated from the two adjacent stages. The neutral and acidic mucin-producing goblet cells are sequestered in the intestinal bulb.

<center>**Discussion**</center>

The embryonic development of the gut tube has been well-characterized and multiple studies have demonstrated the ease with which early larvae can be used for in vivo studies of intestinal function, including observations of peristalsis and nutrient absorption. Although the intestine is functional at 5 dpf, it continues to mature for an extended period such that the relatively simple, straight larval gut has developed into a more complex, looped gut by the time adulthood is reached. To begin to understand the maturation of the intestine, I observed several novel characteristics of the zebrafish intestine and also fortified some previously studied hypotheses about metamorphosis with our own data. The progression of gut looping through the larval, metamorphic, and adult stages has been mapped using morphological observations. Early goblet cell distribution has been mapped from early larval stages to the late larval and metamorphic stages.

Metamorphosis Analysis

In conjunction with normal growth patterns characterized by Parichy et al. (2009), it is clear that standard length does inform with more clarity than calendar age the developmental stage of the zebrafish. I was able to determine a metamorphosis threshold from data collected and scoring based on the normal table of development (Parichy et al., 2009). Because the range of sizes also increases with time, a natural disparity from differential growth rates and individual behaviors such as feeding and swimming, I needed a metamorphosis threshold based on a consistent indicator of development like standard length. This is important because metamorphosis marks a period of major anatomical transition from larval morphology to adult morphology. Variations in how individuals progress through metamorphosis can be lost if calendar age is the only consideration for describing the life

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stage. Therefore, unlike the hour-by-hour precision of embryonic development, staging at the postembryonic level requires a different approach.

Parichy et al. (2009) describes the normal postembryonic growth for wild-type zebrafish based on external characteristics and obvious changes in skeletal maturation. Major developmental milestones are described using standard length. Although the introduction of the Parichy et al. (2009) study alludes to an undefined period of metamorphosis, data is provided that describes changes to be expected in metamorphosis. For example, from 4.3 to 9.0 mm SL, the skeleton ossifies from cartilage to bone. Notochord flexion is quantified, changing from 0º to 38º between 4.6 and 6.3 mm SL. The condensation of caudal fin hypurals is observed starting at 4.5 mm SL. These findings in Parichy et al. (2009) are in line with the findings of our metamorphosis analysis, which helps with the validity of defining the beginning of the metamorphic period in this thesis. I was able to solidify a standard length for the beginning of major metamorphic changes that overlapped with the changes reported by Parichy et al. (2009). This suggests that a minimum standard length for the onset of metamorphosis is more likely than a minimum time post-fertilization, as in the embryonic standard (Kimmel et al., 1995; McMenamin et al., 2016; Parichy et al., 2009).

The metamorphosis analysis in my lab revealed that there is a correlation between the developmental benchmark of metamorphosis and standard length, which was previously unidentified in other studies like the ones mentioned above. Before a certain length, determined to be 4.4 mm SL, all zebrafish, regardless of age, are larval. Once they are past this length, there is a period of ambiguity where they can be larval or metamorphic in appearance, but by 5.2 mm, the zebrafish have all reached gross indications of

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metamorphosis. In contrast, tracking with days post fertilization produces ambiguous

metamorphosis conclusions, with a limited certainty of the stage of the fish, thus making

reporting unreliable. Therefore, I conclude that fish below 4.4 mm SL have a larval

morphology, while fish above 5.2 mm SL are in metamorphosis. Thus, when interpreting

findings in the remainder of the study, standard length is the method used to indicate staging.

Histological Analysis

Since metamorphosis corresponds with rapid changes for several body systems, I

chose this time period to observe the intestine. I hypothesized that gut looping would most

likely occur in this window. Thus, larval and metamorphic zebrafish were measured and

fixed for histology. I chose Alcian Blue and Periodic Acid Schiff's staining as this stain

allows observation of goblet cells in the epithelium and gross intestinal morphology,

respectively (Trotter et al., 2009). Therefore, I was able to analyze gut looping and the

distribution of goblet cells using the same tissue sections. Figure 18 provides a composite

schematic that summarizes both gut looping and goblet cell differentiation.

The intestine develops gut loops after metamorphosis has begun

One major difference between larval and adult intestines is the looping of the gut.

Larval fish have a straight intestinal tract. Adult zebrafish have two loops in the mid-

intestine. Since intermediate life-stages have not been previously studied with respect to

intestinal maturation, I examined several different specimens. These specimens ranged from

4.70 to 7.43 mm SL, thus I had a combination of larval and metamorphic specimen. Our

histological analysis shows that gut looping is first observed after the onset of

metamorphosis. Gut looping does not coincide with the onset of metamorphosis. Rather,

after metamorphosis begins, the intestinal epithelium matures, growing in length to

accommodate the subsequent development of loops. I hypothesize that this is when the intestine begins to grow rapidly in length, building to the eventual looping of the gut observed in Figure 18. Thus, gut looping has begun by accommodating the extra intestinal length needed for the loops, but the intestine shifts morphologically later in metamorphosis.

The findings in this thesis shed light on some staging discrepancies of previous studies. For example, in the histological examination of the zebrafish intestine conducted by Ng et al. (2005), a 14 dpf zebrafish with a straight gut tube was classified as a juvenile, the stage after metamorphosis (Ng et al., 2005). However, calendar age data shown in Table 2, there is approximately a 39% chance that the fish is larval and 61% chance of that it is metamorphic. Looking at the fish qualitatively, the fish would be staged as a larval or early metamorphic, rather than a juvenile because of its straight gut tube. Therefore, in agreement with Parichy et al. (2009), I find that it is desirable to use fish size, along with morphological characteristics, to determine the life stage of the zebrafish. By contrast, using calendar age alone can lead to faulty conclusions.

Goblet cells differentiate in a step-wise manner

Next, I examined the maturation of the intestinal epithelium by mapping the goblet cells in a stage series using Alcian Blue staining. The stain attaches to the mucins, which then appear as large blue spots along the intestinal epithelium. Goblet cells are known to be located throughout the intestinal epithelium in adults, but only in the middle intestine in early larvae (Ng et al., 2005; Wang et al., 2010). Consistent with this, I found goblet cells only in the middle intestine in smaller larval specimens. By contrast, in larger metamorphic specimens starting from 5.2 mm SL, goblet cells appeared in the anterior-most region of the intestinal bulb, immediately following the metamorphosis threshold. By the time the gut has

begun to loop no later than 7.20 mm SL, the goblet cells populate the entirety of the intestine to a similar density as the adult zebrafish. This suggests that goblet cells differentiate for an extended period of time and in a step-wise manner. Further, this suggests the hypothesis that other cell types of the intestinal epithelium may also differentiate in an extended and step-wise manner.

An extended period of intestinal maturation would not be surprising, in light of the dramatic changes in life stages that the zebrafish undergoes, from larval, to metamorphic, juvenile, and adult stages. At each life stage, the zebrafish may be exposed to different environments and different diets. It would be significant to characterize the morphological effects of these diets and environments on the zebrafish intestinal maturation.

I found that both gut looping and goblet cell distribution could be mapped histologically. Figure 18 summarizes these findings. In the larval stage, the middle intestine is the only site for goblet cells differentiation. Once metamorphosis begins, goblet cells appear at the anterior of the intestinal bulb. However, the beginning metamorphosis does not follow immediately with gut looping. Later during the metamorphic period, gut looping is observed in conjunction with a full-length expression of goblet cells from the anterior to the posterior of the intestine. In juvenile and adult zebrafish, it then follows that goblet cells are distributed throughout the entire intestine and gut looping is observed (Ng et al., 2005; Wang et al., 2010). In turn, functional aspects of the intestine may undergo an extended period of maturation well beyond early larval stages. In ongoing studies, the Kinkel Lab is expanding the analysis to include additional epithelial cell types and known markers of intestinal function.

Future Directions

Over the past several years, zebrafish have provided a promising model for studying human disease. The similarity to human development as well as the tractability for studying specific phenotypes allows for experimentation in human drug targets, medical intervention, and genetic applications (Crosnier et al., 2005; Wallace et al., 2005). The research conducted in this thesis coupled with findings in previous studies provides a foundation for studying intestinal motility disorders in zebrafish. By creating a link between the larval and adult stages in zebrafish, this opens the door for ontogenetic studies of many of the known congenital disorders that occur in humans. Diseases such as metabolic disorder, various bowel disorders, and intestinal malformations, need experimental approaches that will span the life of an organism. The effects of these diseases require a model system that includes the maturational changes occurring in an individual. The intestine is the gate keeper of many of the environmental interactions that an individual is exposed to. A more complete understanding of the histology and gross morphology of the zebrafish intestine through different life stages will help to interpret the physiological assays performed in wild-types and mutants.

One such application as for studying Hirschsprung's disease, an intestinal disorder marked by colonic aganglionosis resulting in impaired motility. Roughly one in 5000 children are born with the disease and the survival rate is between 90% and 99% into adulthood (Prato et al., 2011). This means that society has a growing population of people who are genetically predisposed to this and other such intestinal disorders. Using zebrafish, scientists can compare normal versus mutant physiology to understand the implications of such phenotypes through the life of an individual. This could potentially aid in removing

confounding factors of morbidity and mortality due to well-established environmental and genetic risk factors. Indeed, several studies have already used gut transit assays in larval zebrafish to reveal new insights into Hirschsprung's diseases (Abrams et al., 2016; Brady et al., 2017; Field et al., 2009). However, these studies had several limitations can be overcome in future studies.

Previous gut transit assays have found transit times for emptying the intestine that were approximately 24 hours (Brady et al., 2017; Field et al., 2009), with fluorescently-labeled plastic beads mixed into food moving from the intestinal bulb to the posterior intestine slowly over the course of this period. Preliminary data in our lab shows that intestinal bulb emptying is gradual, with intestinal contents entering the middle and posterior intestine in small portions. The total transit time for transit studies in our lab shows about 5 hours total. Differences in transit could be due to the unnatural food particles being indigestible in the studies reporting 24-hour transit. Thus, feeding zebrafish natural diets has the potential to greatly improve future transit studies.

The composition of the intestinal epithelium is another research area of interest. In this study, I characterized the step-wise differentiation of goblet cells in the early larval and metamorphic periods. This means other cell types have the potential to differentiate in a similar manner during this period or in other life stages as well. Table 3 provides a list of candidate genes for intestinal cell types that have not been mapped or characterized.

Although little is known about the role of these genes in human and other model organisms, a brief description of each can be provided. Anoctonin 1 (*ano1*) and Kit receptor a (*kita*) are both known to be expressed in Interstitial Cells of Cajal (ICC) in the muscularis. It is also known that *ano1* is expressed in the embryonic, early larval, and adult zebrafish tissues, while *kita* has only been mapped in adult tissues (Rich et al., 2013; Sanders and Ward, 2007; Uyttebroek et al., 2013). Genes for motilin (*mlnr*) and ghrelin (*ghsra*) receptors have been identified in the intestine (Kitazawa et al., 2017; Koven and Schulte, 2012; Olsson et al., 2008). They have both been mapped in the adult tissues, while *ghsra* has also been shown in early larval tissues. Table 3 gives a summary of these findings and shows the areas still remaining to be investigated and characterized. Cells expressing these genes can be identified using in situ hybridization or immunohistochemistry, with multiple antibodies available for each one. The genes have primarily been identified via RT-PCR and RNA sequencing, which does not give a complete picture of expression within tissues, specific tissue layers, or organization and distribution within a tissue.

Table 3. Candidate genes for intestinal mapping and functional studies.

Gene name	Symbol	Location	Zebrafish expression pattern			
			embryonic	early larval	late larval	adult
Anoctomin 1	*ano1*	ICC in muscularis	✓	✓	?	✓
Kit receptor a	*kita*	ICC in muscularis	?	?	?	✓
Motilin receptor	*mlnr*	intestine	?	?	?	✓
Ghrelin receptor	*ghsra*	intestine	?	✓	?	✓

Once localization of these genes is established, functional studies logically follow. These genes can be used in conjunction with our gut transit assay. This would involve knocking down the gene and conducting a gut transit assay to see if transit times are affected in deficient versus wild-type zebrafish. If transit times are slower for the deficient zebrafish, our assay be able to inform whether the deficiency is across the entire length of the intestine or in specific regions. There is a lot of potential for uncovering some of these and other little known functions of genes in the intestine.

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Appendix A

Table 4. Histological specimen details and preparation.

Standard Length (mm)	Decapitation Before Fixation	Calendar Age (days)
4.70	Yes	15
4.87	No	23
4.89	Yes	15
5.15	Yes	15
5.54	Yes	15
5.69	Yes	15
5.99	Yes	15
7.20	No	23
7.34	No	23
7.43	No	23
Adult Female	Dissected	~ 1 year
Adult Male	Dissected	~ 1 year

Vita

Kutala Faith Franse was born in Cape Town, South Africa, to Doris and Eldon Franse. She graduated from the North Carolina School of Science and Mathematics in June 2009. The following autumn, she entered the University of North Carolina at Chapel Hill to study Biology, and in May 2014 she was awarded the Bachelor of Arts in Biology. In the fall of 2016, she accepted a research assistantship in Cellular and Molecular Biology at Appalachian State University and began study toward a Master of Science degree. The M.S. was awarded in December 2018.