THE ROLE OF HORMONE RECEPTORS DURING AND AFTER DIGESTION

A Thesis
by
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Abstract

THE ROLE OF HORMONE RECEPTORS DURING AND AFTER DIGESTION

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The goal of this study is to understand how the ghrelin receptor and motilin receptor regulate normal gut motility. Previous work suggests that the ghrelin receptor is activated before a meal to cause hunger, and during a meal to cause stomach emptying. After a meal, the motilin receptor is activated and causes "sweeping" movements that clear undigested debris from the gut. Many diseases involve delayed stomach emptying or insufficient gut clearing. Therefore, both receptors are being studied in order to develop improved treatments for intestinal diseases. In my studies, I am using zebrafish as a model for human gut motility disorders. I developed and tested an assay that allows the observation of gut movements and emptying in live zebrafish. I found that emptying the intestine after a meal required 4 hours (hrs). By contrast, when fish were maintained at 20^{o}C to slow metabolism, emptying the intestine was delayed and required 7 hrs. Treatment with $MgSO_4$ to speed up gut movements resulted in faster emptying, within 3 hrs. This is proof-of-principle that this assay can detect decreases and increases in gut motility. In ongoing experiments, I am using small molecule agonists and antagonists to target each receptor, as well as an *mlnr* mutant zebrafish line.

This approach will allow myself to demonstrate the roles of these receptors in a genetically-tractable vertebrate model. In future studies, we will build on this work to investigate disorders such as irritable bowel syndrome and chronic constipation.

Acknowledgments

I would like to thank Dr. Mary Kinkel for allowing me to join her lab, and for motivating me throughout this program. I wish to also thank my thesis committee members, Dr. Cort Bouldin and Dr. Darren Seals, and my lab mates, Savanna Sheridan, Daniel Lauffenburger, and Caleb Lombard, for their contributions to this research study. I want to acknowledge undergraduate students, graduate students, and faculty for providing animal care and zebrafish to perform this study. All procedures were approved by the Institutional Animal Care and Use Committee at Appalachian State University. This study was supported by two research awards from the Office of Student Research.

Dedication

I would like to dedicate this thesis to my wonderful family and friends that have supported me throughout this journey. I could never have done this without my loving parents, Shad and Robin Franklin, with their consistent encouragement. I have met some extraordinary people at Appalachian State University, and I will cherish our friendships forever. I also want to dedicate my thesis to my two standard poodles, Aspen and Penelope. They have given unconditional love and have been with me every step of the way.

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Introduction

Gastrointestinal (GI) diseases and disorders such as irritable bowel syndrome (IBS), gastroparesis, ileus, and small intestinal bacterial overgrowth (SIBO) affect many people in the United States (Keller et al., 2018). There is currently no cure or medication that can completely resolve GI diseases and disorders; however, there are medications and dietary changes that can reduce the symptoms slightly. Genes that are implicated in GI disease have been investigated; however, the GI tract is still poorly understood. Zebrafish (*Danio rerio*) is an established model system for developing and testing potential treatments for GI disorders and diseases. Zebrafish are a valuable model organism for numerous reasons, as the embryos and larvae are transparent, so it is easy to analyze internal structures such as the intestinal bulb, mid-, and distal-intestine (Clark and Ekker, 2015). Because the body wall is transparent, the GI transit may be monitored using a gut transit assay, a non-invasive observation of intestinal contents (Field et al., 2009).

The eggs are fertilized and developed externally, so it is relatively easy to study development. Zebrafish have rapid development when compared to other animal models. The embryonic stage exists from 0 days to 3 days, the larval stage from 3 days to approximately 15 days, and the juvenile stage from approximately 15 days to 3 months. By 3 months, zebrafish are typically able to reproduce, and they are then classified as adults. The rapid development can be beneficial when studying a variety of disorders over various life stages.

To study gut motility, the zebrafish intestinal tract needs to be well understood, both in terms of the physical changes it undergoes as well as the genes that control this process. The zebrafish genome has been sequenced, allowing researchers to manipulate and introduce

mutations (James et al., 2019). This method is an excellent way to study GI disorders and diseases affecting humans.

Intestinal Tract of the Zebrafish

Vertebrates have a gastrointestinal tract characterized by different segments that are typically well-defined anatomically and functionally. The segments are the esophagus, stomach, small intestine, and large intestine. The small intestine comprises the duodenum, jejunum, and ileum. The small intestine is where most nutrient absorption occurs, while the large intestine is where water and salt absorption occur.

Among vertebrate model organisms, the zebrafish has the simplest intestinal tract. Despite its simplicity, the zebrafish intestinal tract is not well characterized. During the larval phase, the gut tube is straight, and the body walls are transparent; however, as metamorphosis occurs, the gut tube becomes looped (Figure 1). An outstanding question is how to define the anatomical and functional regions. Most studies have suggested that the zebrafish intestinal tract has three segments: the intestinal bulb (IB), the mid-intestine, and the distal intestine (Wallace et al., 2005; Wang et al., 2010). However, some studies suggest there are 5 segments, including the duodenum, jejunum, ileum, large intestine, and the zebrafish are considered stomach- less, but the intestinal bulb acts as a reservoir for ingested food (Lickwar et al., 2017). A key element as to how to anatomically and molecularly identify the segments based on previous experiments is by the height of epithelial folds (Wallace et al., 2005; Wang et al., 2010), gene expression (Lickwar et al., 2017; Wang et al., 2010), epithelial cell type (Wallace et al., 2005), and transcriptional start sites (TSS) (Lickwar et al., 2017).

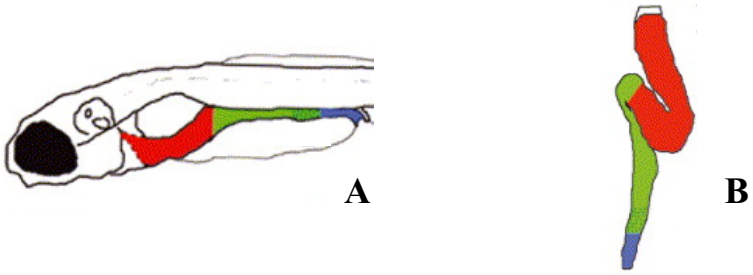

Figure 1. General morphology of the zebrafish intestine. A. Diagram of the straight gut tube present in larval zebrafish before metamorphosis. B. Diagram of the looped gut present in an adult zebrafish after metamorphosis. Red: intestinal bulb, green: mid-intestine, blue: posterior intestine. From Wallace et al. (2005).

Epithelial Folds

The epithelial folds in zebrafish are similar to intestinal villi in mammals in that they both absorb nutrients from food. One difference is that the epithelial folds are proportionally larger in the zebrafish intestine than the intestinal villi in mammals (Wallace et al., 2005). The epithelial folds, also known as villar ridges in zebrafish, increase the surface area of the intestinal epithelium, thus increasing nutrient absorption (Wang et al., 2010). As previously mentioned, the zebrafish intestinal tract could be divided into three anatomical regions or five anatomical regions based on different research articles involving epithelial folds (Wallace et al., 2005; Wang et al., 2010). In the study from Wallace et al. (2005), adult zebrafish intestines were isolated and divided into three sections, as indicated in Figure 1B. Wallace and colleagues described the height of the intestinal folds and found that the height decreased from relatively tall folds in the intestinal bulb, to short folds in the posterior intestine.

In the study from Wang et al. (2010), the epithelial folds were investigated in more detail. First, the adult zebrafish intestine was isolated and cut into seven equal segments, as shown in Figure 2. This study revealed that the epithelial folds are densely-packed in anterior regions corresponding to segments 1-5 (Figure 2B-F). In segment 6 (Figure 2G) the epithelial folds start to diverge, and by segment 7 (Figure 2H) the epithelial folds have almost disappeared completely, leaving a smooth fold-free surface. Wang et al. (2010) concluded that the zebrafish intestinal tract is divided into a small intestine and a large intestine.

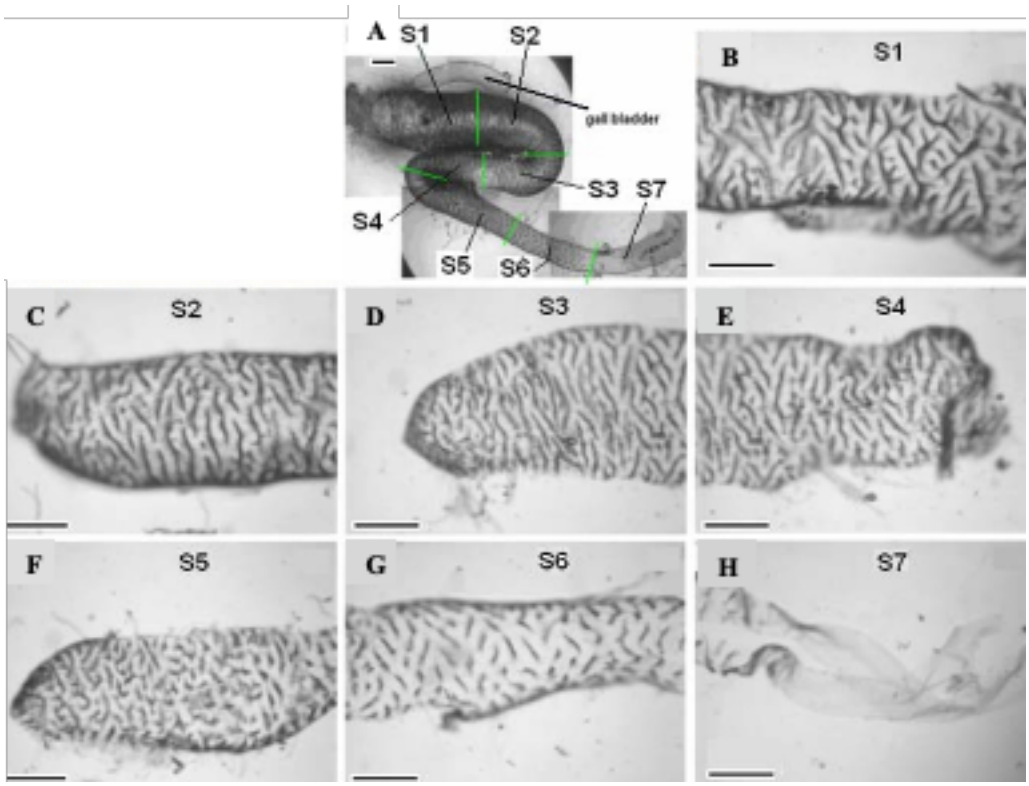

Figure 2. Epithelial folding in the adult zebrafish intestine. A. Composite image of the isolated intestine. A. The green lines indicate the seven cuts that were made through the intestine. B-F. Segment 1 through S5 have a similar density of epithelial folds. G. Epithelial folds are less dense. H. No epithelial folds are present. For all panels, anterior is to the left. From Wang et al. (2010).

To support this argument, Wang et al. (2010) also considered the height of the epithelial folds. Figure 3 shows the height of the epithelial folds in each segment. Segment 1 through segment 6 revealed folds that ranged in height. Segments 1 through segment 5 are very similar in villar ridge height and are the tallest. However, segment 6 drops in epithelial fold height. Segment 7, as mentioned before, has no epithelial folds (Wang et al., 2010).

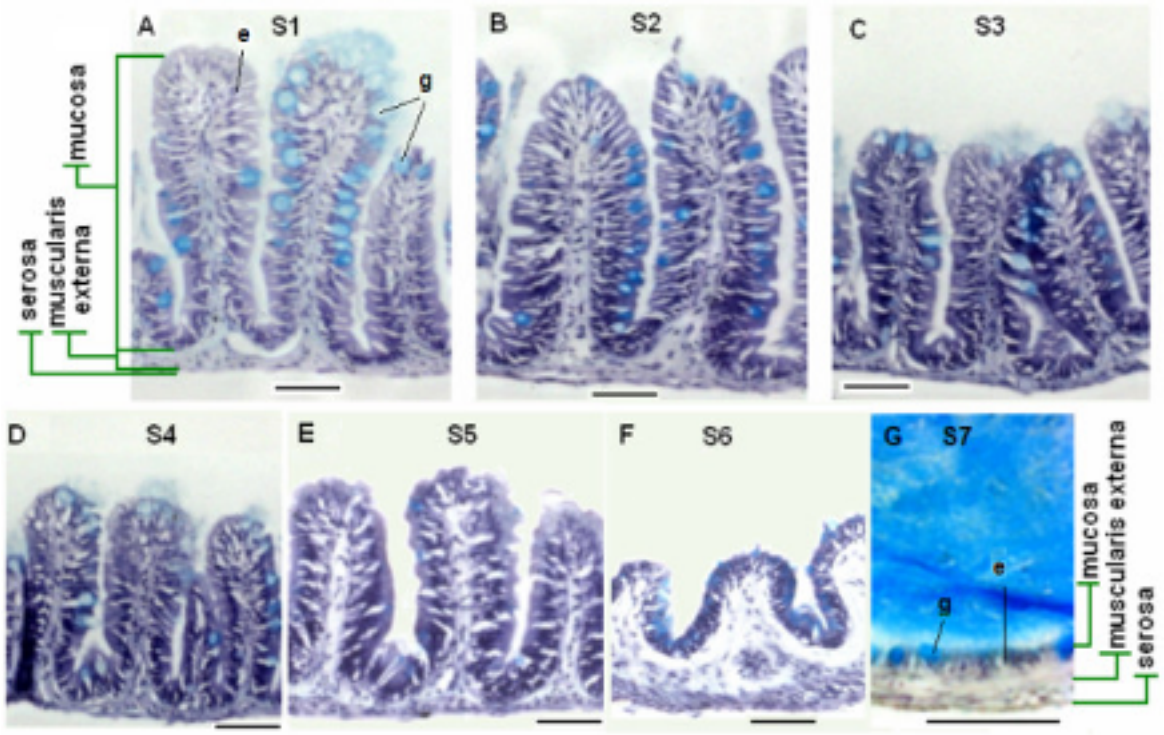

Figure 3. Cross sections of anterior-posterior segments of entire adult zebrafish intestine. Three tissue layers are present: mucosa, muscularis externa, and serosa. A-E. Segments 1-5 are similar in height of the folds. F. Segment 6 has shorter folds. G. Segment 7 has no folds. From Wang et al. (2010).

In the Wang et al. (2010) study, the authors concluded that one way to identify the intestinal bulb is by considering the height of the epithelial folds. The intestinal bulb was defined as segment 1 through segment 5. This intestinal bulb or anterior intestine could

correlate with the small intestine in mammals and other amniotes, as this is where most nutrient absorption occurs. Since segment 6 has more dispersed folds and shorter folds, it could be correlated to the large intestine of mammals and other amniotes. Lastly, segment 7 has no folds, correlating it to the rectum where waste is excreted out. Based on the result of Wallace et al. (2005) and Wang et al. (2010), the intestine can be divided into three anatomical segments based on epithelial fold density and height.

Gene Expression in the Intestine

Since the intestinal regions can be differentiated anatomically, the regions should also differ in function. To address this, Wang et al. (2010) used transcriptome analysis to characterize the regions. Gene expression was compared across the intestinal segments. The intestinal segments were separated based on gene expression (Figure 4). The study found that 2,558 genes are differentitally expressed throughout the zebrafish intestine through an ANOVA analysis. Segment 1 through segment 5 had highly-overlapping gene expression. Segment 6 and segment 7 were largely distinct from the anterior segments. Wang et al. (2010) concluded that there are three molecularly distinct regions in the adult zebrafish intestine.

Next, Wang et al. (2010) analyzed whether the zebrafish intestinal segments could be correlated to either the small or large intestine of the mouse or human. They used well-known molecular markers of the mammalian small intestine including *fabp2*, *vil1l*, *apoa1*, and *apoa4* and found that these four genes were highly expressed in segments 1-5. However, they decided segment 5 could be described as a transitional segment because different genes are expressed throughout segments 6 and 7 (Wang et al., 2010). Two genes that are expressed

in mouse and human large intestine, *cfl1* and *aqp3*, are also expressed in segments 5-7. In segments 6 and 7, *cfl1* and *aqp3* were highly expressed. Overall, the Wang et al., 2010 study suggests that segments 1-4 may be analagous to the mammalian small intestine, segment 5 is a poorly-defined transitional segment, and segment 6 and 7 are analagous to the mammalian large intestine.

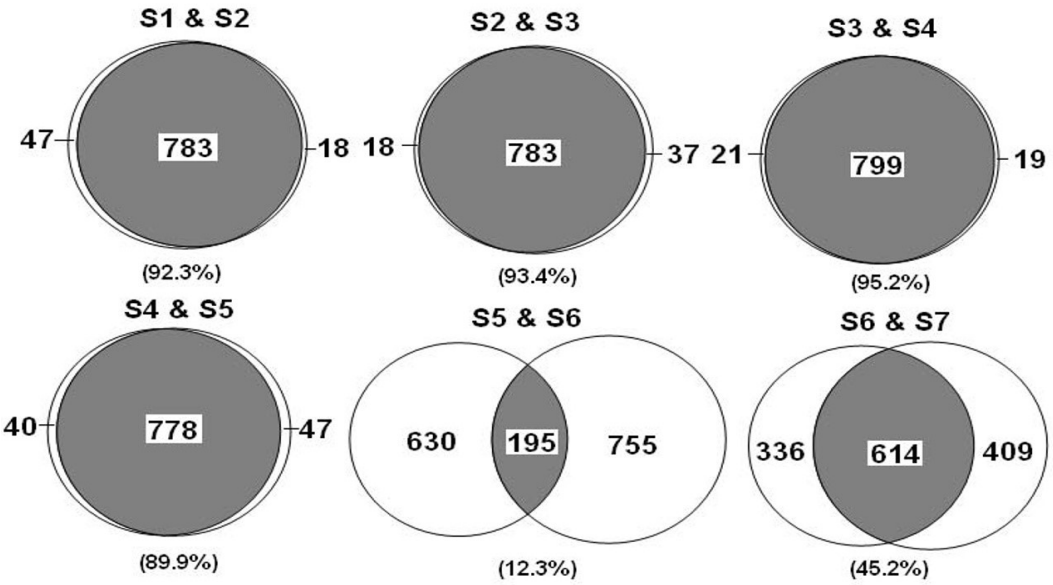

Figure 4. Gene expression of intestinal segments of the adult zebrafish. Significant overlap in upregulated genes is seen in segments 1-5, while segment 6 and segment 7 show less similarity, as indicated by the percentages shown. From Wang et al. (2010).

In a subsequent study by Lickwar et al. (2017), the conservation between genes expressed in adult mouse intestine and adult zebrafish intestine was investigated. To see the similarites between gene expression, the mouse intestine was divided into the duodenum, jejunum, ileum, colon, and cecum. The zebrafish intestine segments were compared to the mouse intestine segments (Figure 5). Adenosine deaminase (*ada*) was highly expressed in the mouse duodenum and when compared to the zebrafish intestine, *ada* was expressed in

segments 1-2. Two more genes, *fabp2* and *enpep*, were highly expressed in the mouse jejunum and ileum and found that these genes were expressed in segments 3-5 of the zebrafish intestine. There were also genes expressed in the colon of mice that were expressed in the distal portion of the zebrafish intestine. While the Wang et al. (2010) study suggested there are three segments that make up the zebrafish intestine, Lickwar et al. (2017) looked closer and suggested that the intestine has five segments.

Transcriptional Start Sites (TSS)

Lickwar et al. (2017) next studied transcriptional start sites (TSS) during larval development and compared the TSS to adult zebrafish intestine stages and found that they remained similar. Next, the zebrafish intestinal segments were compared with mammalian intestinal segments. Lickwar et al. (2017) proposed that there were five TSS domains in zebrafish. Each transcriptional start site correlated to an intestinal segment by a specific gene marker. As seen in Figure 6, specific gene markers defined each TSS. The first segment that is duodenum-like was marked by *ada*. The jejunum-like segment was marked by *fabp2* and *rbp2a*. The ileum-like segment was marked by *fabp6* and *slc10a2*. The colon-like segment was marked by *lamp2* but the expression of *lamp2* abruptly stopped after the 6th cut section of the zebrafish intestine. Overall, Lickwar et al. (2017) determined that the TSS analysis showed five different segments in the zebrafish intestine. These segments consisted of the traditional three parts of the intestine: the intestinal bulb (similar to the mammalian duodenum and jejunum), the middle intestine (similar to the mamalian ileum), and the posterior intestine (similar to the mammalian colon).

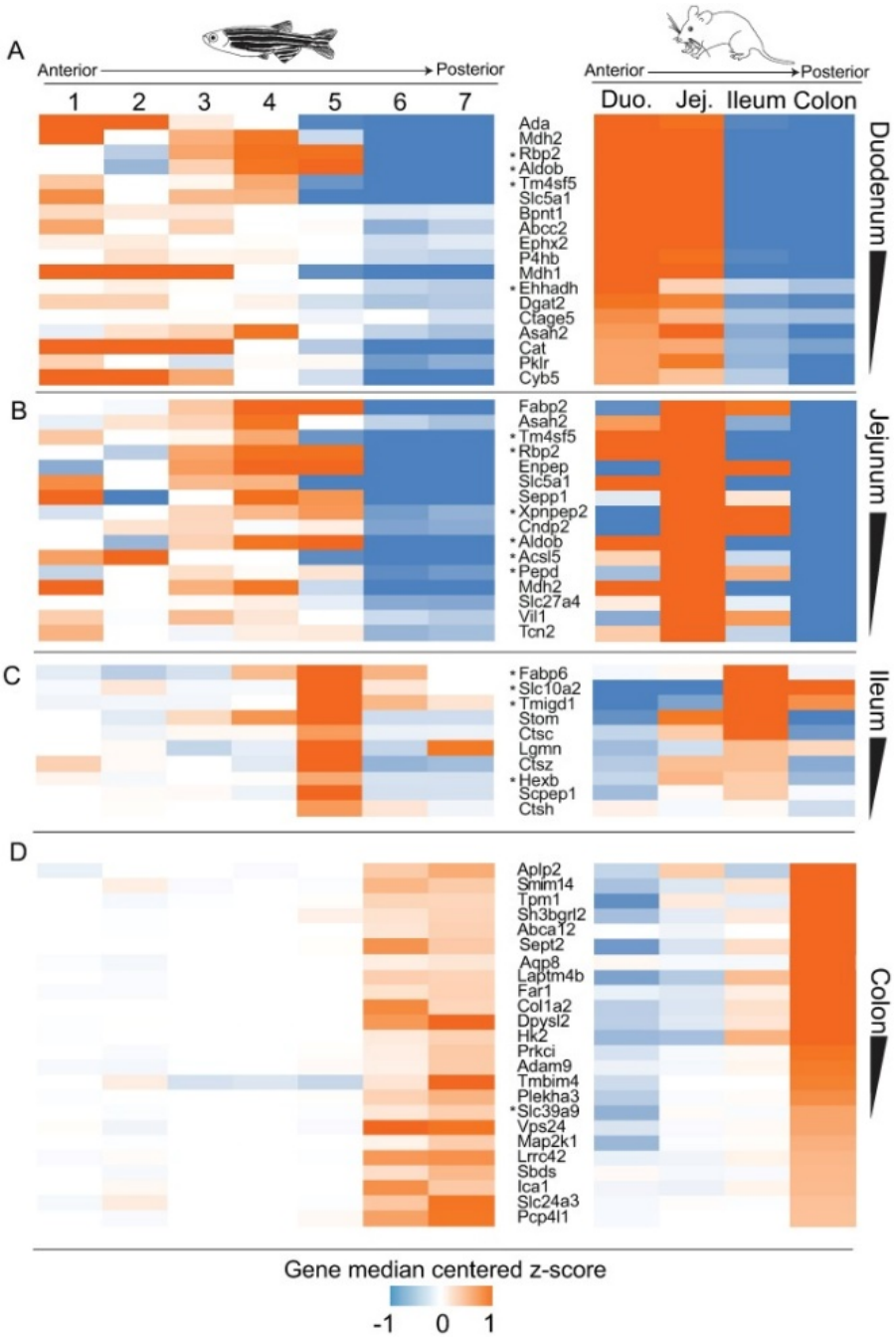

Figure 5. Gene expression similarities between mouse intestine and zebrafish intestine. A heat map shows a comparison of gene orthologs that are rated by a z-score from -1 to 1. A. Zebrafish segments 1-2 are similar to mouse duodenum. B-C. Zebrafish segments 3-5 are similar to mouse jejunum and mouse ileum. D. Zebrafish segments 6-7 are similar to mouse colon. From Lickwar et al. (2017).

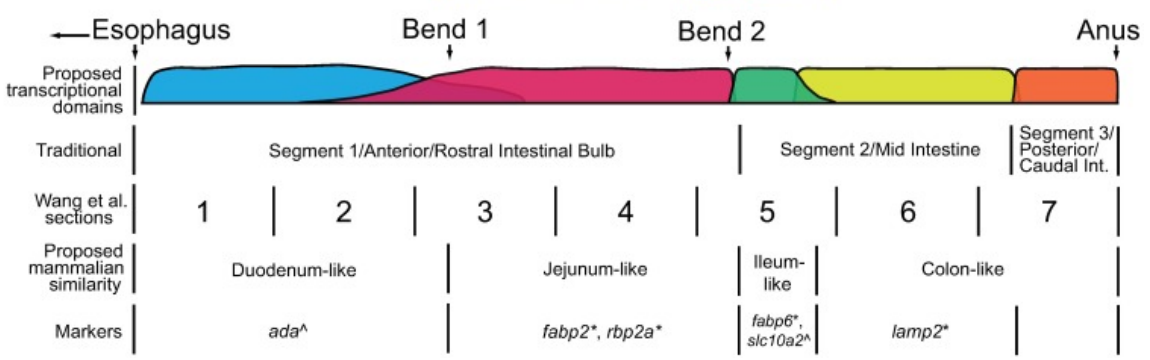

Figure 6. Proposed transcriptional and functional domains. The colors indicate five proposed transcriptional domains. Bend 1 and Bend 2 refer to the intestinal loop. The domains are arranged linearly in parallel with the traditional anatomical segment names. Number 1 through 7 indicates the seven segments utilized by Wang et al. (2010) in their gene expression study. From Lickwar et al. (2017).

Intestinal Epithelial Cell Types

Throughout the zebrafish intestine, there are different specialized cells in the epithelium. The most numerous cell types in the adult intestine are the enterocytes, enteroendocrine cells, and goblet cells (Wallace et al., 2005). In mammals, enterocytes are absorptive cells that line the intestinal lumen in the small intestine. Goblet cells secrete mucus to protect the mucous membranes in the small and large intestine in mammals. Enteroendocrine cells are found in the small intestine in mammals and release a variety of hormones. Lastly, NaPi+ enterocytes are specialized enterocytes that function as antigen-presenting cells. They are similar to M-cells that are involved in the mammalian immune response system (Wallace et al., 2005).

Based on the study of Wallace et al. (2005), enteroendocrine cells were rarely found past the intestinal bulb/anterior segment in zebrafish. Goblet cells were found in all intestinal regions in the adult zebrafish intestine, however, they were detected only in the mid-intestine in larval zebrafish. Paneth cells were not found in any intestinal region because zebrafish,

unlike mammals, lack crypts (Wallace et al., 2005). Enterocytes were not found past the mid-intestine. NaPi+ enterocytes were found in the posterior-most region of the mid-intestine and in the posterior intestine. This study suggested that the zebrafish intestinal segments can be identified histologically by characterizing the specific cell types that are found in the epithelium lining the intestinal lumen.

Intestinal Loop Region

In zebrafish, a functional intestine is developed by 5 days post fertilzation (pdf) and continues to grow for about 4 weeks (Li et al., 2019). The intestinal loop does not form until about 3-4 weeks, depending on the health and size of the zebrafish (Li et al., 2019). To understand gut looping, Li et al. (2019) imaged the zebrafish intestine over a period of 4 weeks. They also measured the epithelial fold width and height. They found that when the zebrafish are 3 weeks old, the intestine starts to create an S shape which then grows anteriorly (Li et al., 2019). In Figure 7, the zebrafish intestine was imaged from 19 dpf to 33 dpf. During 20 dpf to 22 dpf, the loop began developing in the mid-intestine and grew anteriorly, and pulled the posterior intestine anteriorly. From day 20 to 26 dpf, the highest growth rate was observed. By 33 dpf, the intestinal loop grew significantly and took on the adult S-shaped morphology. When the formation of the loop was completed, the posterior intestine barely extended past the loop. Figure 8 illustrates the regions of the adult zebrafish intestine that were sampled for proliferation rates.

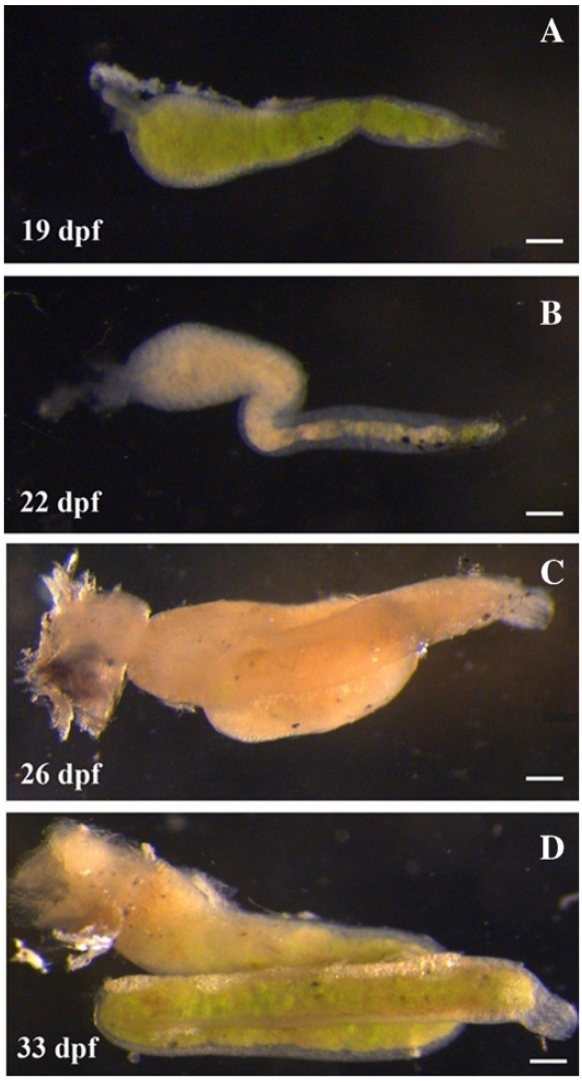

Figure 7. Development of zebrafish intestinal loop occurring over a 4-week period. A. Larval intestine has a straight gut tube. B. The intestinal loop starts to develop. C. The intestinal loop has folded back on itself creating the s shape and continues to grow. D. The loop is developed. From Li et al. (2019).

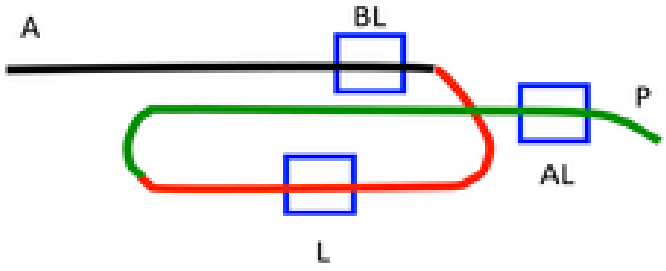

Figure 8. Segments of the adult intestine. Black line and A: anterior intestine (without the loop), red line: loop (anterior region), green line and P: posterior intestine. The blue boxes indicate three different regions that were sampled including BL: before loop, L: loop, AL: after loop. From Li et al. (2019).

Based on this work, there was no solid evidence as to which segment the intestinal loop belongs to. The intestinal loop is a region that should not be lumped in with the anterior or middle intestine based on the Li et al. (2019) study. In previous studies, Wallace et al. (2005) and Wang et al. (2010) were able to differentiate segments based on the height and density of the epithelial folds. I think epithelial fold height, cell types, gene expression, and proliferation rates alone are not enough to confine the loop region to a specific segment of the zebrafish intestinal tract, especially when only based on age. It has been shown that fish body length is better to show developmental progress than age (Parichy et al., 2009). Li et al. (2019) analyzed the intestinal bulb at 26 dpf, when in fact, the loop could have already been developing depending on the size of the fish. It would be more beneficial to use length as a way to track metamorphosis of the intestinal tract when performing experiments in zebrafish. However, because the studies performed by Li et al. 2019 based the metamorphosis experiments on age rather than body length, this could lead to inaccurate results and interpretations.

Gut Motility

As previously mentioned, the zebrafish intestine is comprised of three segments, the intestinal bulb, mid-intestine, and the posterior intestine. Each segment has its own specific function. To study GI disorders the gut motility must be understood as well. The migrating motor complex (MMC) controls gut motility between meals. The MMC itself is controlled by many intestinal receptors.

Migrating Motor Complex

The migrating motor complex (MMC) is an electrical and mechanical mechanism that propels contents through the GI tract (Romanski, 2017). This complex was first discovered in dogs and was termed the "interdigestive motor complex" as it is initiated in-between meals (Vantrappen et al., 1977). The MMC is initially activated when chyme reaches the intestinal mucosa where chemoreceptors and mechanoreceptors are present. It continues during the fasting state and is disrupted only once feeding occurs again. The MMC is controlled by intestinal receptors, intestinal microbiota, and the enteric nervous system. Hormones related to the MMC function are ghrelin, motilin, serotonin, somatostatin, enkephalins, pancreatic polypeptide, neurotensin, cholecystokinin (CCK), and gastrin (Romanski 2017). These systems must work together for proper cycling of the MMC. Without the continuous cycling of the MMC in each region of the GI tract, food and debris could become lodged and contribute to gastrointestinal diseases and disorders, including obesity, anorexia nervosa, and can contribute to small intestinal bacterial overgrowth (SIBO) (Deloose and Tack, 2016).

The MMC occurs in many animals including humans and dogs (Takahashi 2013). The MMC has either three or four phases, depending on the regions of the GI tract and the

species. These phases proceed through each gastrointestinal segment before starting again. The main part of the MMC is the activity front, consisting of rhythmic contractions of the intestinal smooth muscle (Vantrappen et al., 1977). Phase I was described as a nearly complete absence of pressure waves, implying little contraction (Vantrappen et al., 1977). During phase II the pressure waves occur in irregular sequences (Vantrappen et al., 1977). There is a burst of rhythmic contractions in phase III, and by phase IV there is a rapid decrease in both amplitude and regularity of the pressure wave peaks (Vantrappen et al., 1977).

Migrating Motor Complex Phases

Deloose and Tack (2016), in contrast, stated that there were only three phases that make up the MMC. They divided these phases based on a gradual enhancement of contractile activity in humans (Deloose and Tack, 2016; Romanski 2017). In humans, the MMC can begin in the stomach or the small intestine. Studies have shown that the MMC can start in the stomach and extend to the terminal ileum, taking between 1.5-2 hours to complete (Deloose et al., 2019; Romanski 2017).

Vantrappen et al. (1977) and Romanski (2017) described a fourth MMC phase. Phase IV is considered the shortest phase in the MMC cycle and is characterized by a rapid decrease in both amplitude and regularity of the pressure peaks. It resembles the irregular contractions of phase II (Romanski, 2017). Based on current research, no one has tried to disprove Vantrappen's or Romanski's research regarding four MMC phases. They were able to separate the phases by how long they lasted in the gastric section of the human GI tract. Phase I lasted 28 minutes, phase II lasted 42 minutes, phase III lasted 2 minutes, and phase

IV lasted 7.1 minutes (Romanski, 2017). In other sections of the GI tract, there are mainly only three phases of the MMC that can be traced. There is no consensus on the purpose of the absence of the fourth phase.

Overall, some studies have found the MMC consists of three phases, (Deloose and Tack, 2016) while other studies found four phases (Vantrappen et al., 1977; Romanski 2017). In humans, Romanski was able to find four phases of the MMC in the stomach but not in the small intestine. However, the number of phases varied depending on which animal species was studied. In canines four phases were present in both the stomach and small intestine. It seems the number of phases during the MMC cycle is dependent upon species and segmentation of the gastrointestinal tract.

Hormonal Control of the MMC

Motilin exerts considerable control over the digestive process, particularly during the phase III contractions of the MMC are controlled by motilin in humans (Miyano et al., 2013). During phase I of the MMC, the pH is alkaline, and during phase II and phase III the pH drops. The drop in pH increases the release of motilin from the duodenal mucosa. Another factor that controls motilin release is the presence of nutrient uptake (Takahashi 2013). The consumption of food suppresses motilin release, resulting in a decrease of contractions. Janssens et al. (1983) found as plasma levels of motilin increase or decrease, the phase III contractions of the MMC do the same. The motilin-dependent contractions were confined to the human stomach (Deloose et al., 2019; Janssens et al., 1983).

G protein-coupled receptors

Two of the major hormone receptors in humans are the ghrelin receptor (GHS-R1a) and the motilin receptor (MLNR). These receptors are G protein-coupled receptors (GPCRs). GPCRs interact with GTP-binding proteins (G proteins). Many drugs have been created to target GPCRs, as they are the largest family of integral membrane proteins. GPCRs mediate responses to hormones and carry out biological functions such as vision, olfaction, and taste (Fredriksson et al., 2003).

There are around 800 GPCRs distributed among the Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin subfamilies (Fredriksson et al., 2003 and Zhang et al., 2006). When a ligand binds to a GPCR, it triggers a conformational change that activates downstream signaling networks (Gether, 2000). The two main signal transduction pathways are the cAMP signaling pathway and the phosphatidylinositol signaling pathway. After the pathways are activated, β-arrestin will desensitize the GPCR by blocking G-protein signaling. There are four members that comprise the β-arrestin family, visual arrestins (arrestin1 and arrestin4) and the non-visual arrestins (β-arrestin1 and β-arrestin2) (Chutkow et al., 2010). The visual arrestins are responsible for rhodopsin desensitization.

The largest subfamily of GPCRs is the Rhodopsin subfamily, which includes the ghrelin receptor and the motilin receptor (Fredriksson et al., 2003). Receptors in this subfamily have a short N-terminal domain, and 701 receptors have been identified in mammals (Fredriksson et al., 2003). The receptors within the rhodopsin family are the most diverse. They are involved in regulation of metabolism, reproduction, and neural function such as taste and smell. The receptors that belong to the Rhodopsin family are divided into four subgroups: $\alpha, \beta, \gamma,$ and δ. Most of the receptors that are classified under the β-group are

involved in the regulation of feed intake. The GHS-R family, including the motilin receptor and the ghrelin receptor, are classified under the β-group (Fredriksson et al., 2003). The motilin receptor and ghrelin receptor have multiple roles in regulating metabolism.

GHS-R Family

The ghrelin receptor and motilin receptor are present in the GI tract of mammals such as humans, dogs, and rabbits (Feighner et al., 1999; Folwaczny et al., 2001; Holst et al., 2004; Howard et al., 1996). Both receptors belong to the growth hormone secretagogue receptor (GHS-R) family of receptors. The GHS-R family contains seven members: ghrelin receptor (gene symbol GHSR), motilin receptor (MLNR), neurotensin receptor 1 and 2 (NTSR1, NTSR2), neuromedin-U receptor 1 and 2 (NMU1, NMU2), and GPR39 (Holst et al., 2004). The structure of the ghrelin receptor and motilin receptor are shown in Figure 9. The first reports for human ghrelin receptor and motilin receptor found that they share 52% amino acid identity and 86% transmembrane domain identity (Feighner et al., 1999; McKee et al., 1997).

Figure 10 shows the phylogenetic relationships of the GHS-R family members. The ghrelin receptor, Neurotensin Receptor 2, and GPR39 have a high degree of constitutive activity. The ghrelin receptor, neurotensin receptor 2, and GPR39 can generate a cellular response of up to 50% of its maximal efficacy without the binding of its peptide ligand (Holst et al., 2004). Constitutive activity is the ability for a receptor to become active without the binding of an agonist.

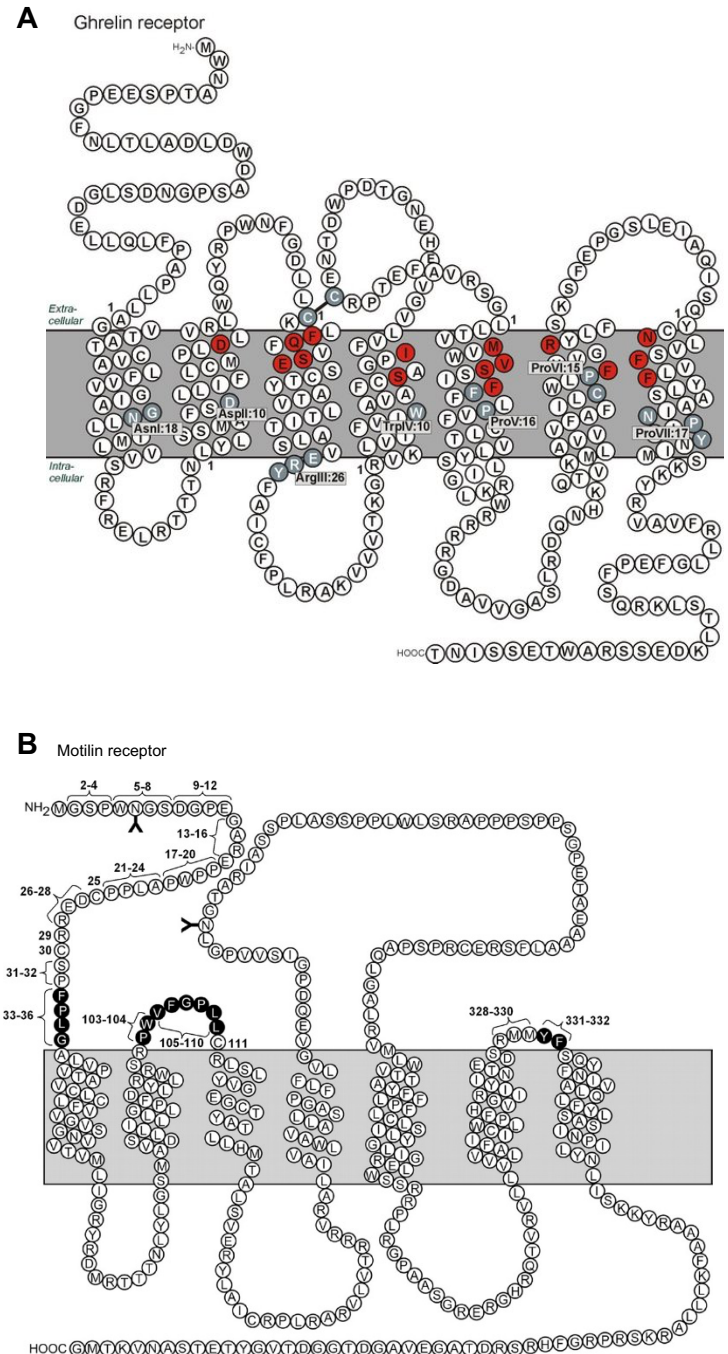

Figure 9. Structures of the human ghrelin receptor and motilin receptor. The amino acid sequences (1-letter codes) are indicated for each receptor. A. Ghrelin receptor. White letters on dark circles indicates highly conserved residues. Black letters on red circles indicate mutations. B. Motilin receptor. Brackets indicate deletions. White letters on black circles are alanine replacement constructs. Panel A modified from Holst et al. (2007); panel B modified from Matsuura et al. (2006).

Figure 10. The growth hormone secretagogue receptor family. A phylogenic tree showing the relationships of the GHS-R receptors. The *black dots* indicate the three receptors that have been demonstrated to display a high degree of constitutive signaling activity. The *white dots* indicate receptors that do not show constitutive signaling activity. From Holst et al. (2004).

Ghrelin Receptor

The ghrelin receptor was discovered in 1996 during the search for an additional receptor that controlled the secretion of growth hormone (GH) (Howard et al., 1996). Previous studies suggested that GH secretion was only controlled by the growth hormone-releasing hormone (GHRH); however, there was an additional receptor involved because of the use of a different signaling pathway (Howard et al., 1996). The ghrelin receptor (GHS-R1a) is a 366-amino acid protein with seven transmembrane domains. It has been found in the hypothalamus, pituitary gland, and the GI tract (Dass and Munonyara et al., 2003; Gnanapavan et al., 2002; Holst et al., 2004; Howard et al., 1996). There is a splice variant of the ghrelin receptor known as GHS-R1b that is truncated (Leung et al., 2007; Howard et al., 1996). GHS-R1b consists of 289 amino acids with a five-TM domain. The major difference between these two receptors is that ghrelin does not bind to GHS-R1b; it only binds to GHS-R1a to carry out biological functions (Howard et al., 1996; Leung et al., 2007).

In the GI tract of both rodents and humans, the ghrelin receptor is located on neuroendocrine cells (Dass and Munonyara et al., 2003; Holst et al., 2004). It is also located in the central nervous system (Cong et al., 2010). In the GI tract, the ghrelin receptor controls gastric motility during a meal (Asakawa et al., 2001). GHS-R1a has broader functions as well, such as GH release, appetite stimulus, regulation of energy balance, enhancement of dopamine signaling, and cognitive improvement (Figure 11). These different functions have their basis in differing intracellular signaling pathways and the tissues in which the receptor exists.

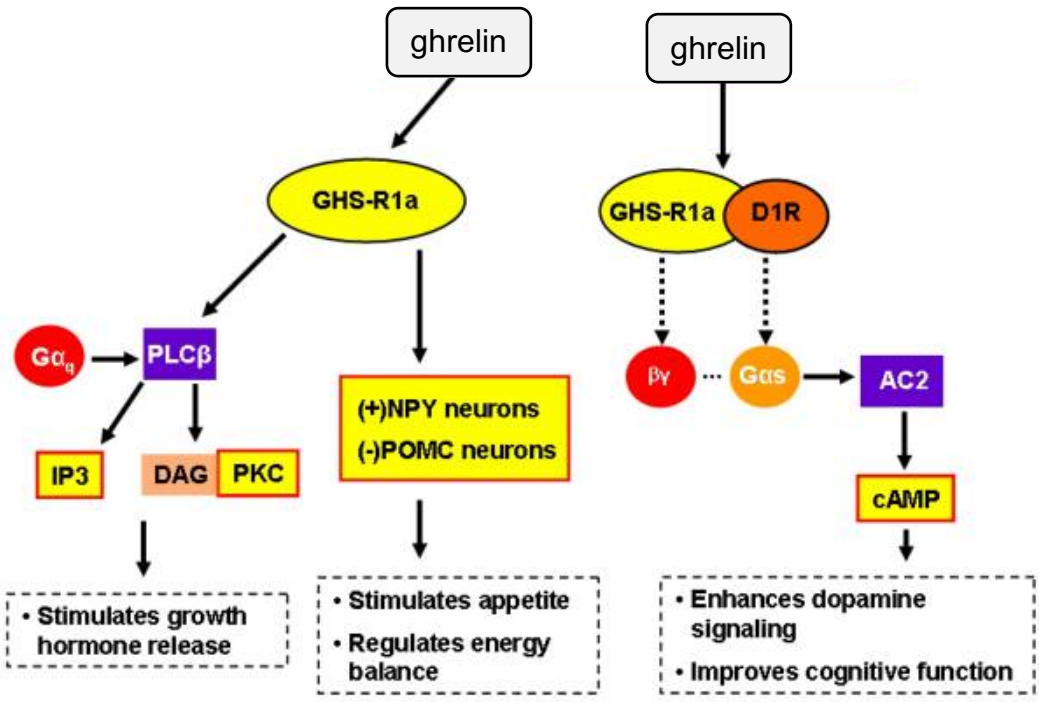

Figure 11. Ghrelin receptor signaling pathways and functions in the CNS. The PLC/PKC/IP3 pathway is the dominant pathway. In the hypothalamus, ghrelin activates Neuropeptide Y (NPY)-containing neurons and inhibits POMC neurons. Ghrelin also augments dopamine signaling, which involves heterodimerization with the D1R (dopamine receptor). AC2: adenylyl cyclase-2, D1R: dopamine receptor 1, DAG: diacylglycerol, IP3: Inositol(1,4,5)triphosphate, NPY: neuropeptide Y, POMC: pro-opiomelanocortin. Modified from Cong et al. (2010).

As mentioned before, the ghrelin receptor has high constitutive signaling activity through the phospholipase C (PLC) pathway (Holst et al., 2004). Up to 50% of the ghrelin receptor's activity is constitutive. By contrast, no constitutive activity has been detected for the motilin receptor. Interestingly, in the intestine, no ligand has been identified for the ghrelin receptor. This raises the possibility that ghrelin receptor activity in the gut may be constitutive only.

Motilin Receptor

The motilin receptor was discovered in 1999 in the human GI tract (Feighner et al., 1999). Before the discovery, this receptor was an orphan GPCR called GPR38, now called MLNR. As previously mentioned, Feighner et al., 1999 found there to be a 52% identity between GPR38 and GHS-R. Since there was a similarity between these two receptors, Feighner et al., 1999 tested several peptide and nonpeptide molecules in a stable cell line that involved intracellular signaling. They ultimately discovered the motilin receptor through a PLC assay that detected calcium release by aequorin, a bioluminescent calcium-sensitive reporter protein.

The motilin receptor is comprised of 412 amino acids and has seven TM domains (Feighner et al., 1999). This receptor is found on circular smooth muscle cells in the stomach and intestine (Feighner et al., 1999; Huang et al., 2005; Holst et al., 2004). The motilin receptor controls two signaling pathways, one for initial contraction and another for sustained contraction of smooth muscle (Figure 12) (Huang et al., 2005). Once the ligand binds to the

motilin receptor, it becomes internalized by the GRK2-dependent mechanism. Both pathways are regulated by the coupling of two subunits $G\alpha_q$ and $G\alpha_{13}$. The MLC kinase pathway is the initial contraction pathway and it is calcium dependent. The MLC phosphatase pathway is the sustained contraction pathway and is Ca^{2+} independent.

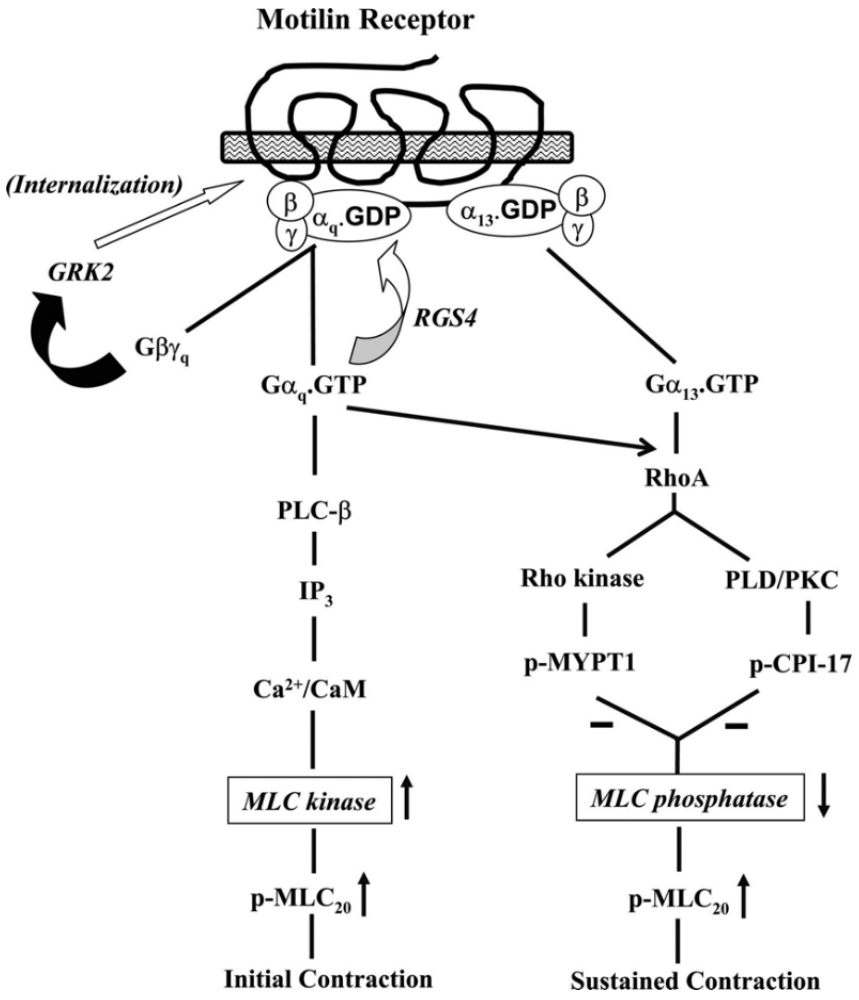

Figure 12. Signaling pathways of the motilin receptor. The receptor is activated when it binds the motilin hormone. Smooth muscle contractions are mediated by Ca^{2+} dependent and independent mechanisms. CaM: calmodulin, GRK2: G protein-coupled receptor kinase 2, IP_3: inositol triphosphate, MLC: myosin light chain, PKC: protein kinase C, PLD: phospholipase D, p-CPI-17: C-kinase potentiated protein phosphatase-1 inhibitor, p-MYPT1: myosin phosphatase target subunit 1, RGS4: regulator of G protein signaling 4. Huang et al. (2005).

Ligand binding

The ghrelin hormone is a 28 amino acid peptide that was first isolated in rat stomach tissue (Kojima et al., 1999). This hormone is released from endocrine cells within oxyntic glands in the stomach and small intestine of humans (Date et al., 2000; Holst et al., 2004). There are two different types of the ghrelin hormone, acylated and nonacylated. Kojima et al. (1999) found that the ghrelin hormone must become acylated at Ser3 to carry out its biological activity, in this case the release of GH.

Because most GPCRs use the PLC pathway, which induces an influx of calcium ions, Kojima et al. (1999) used a PLC assay to determine if both motilin and ghrelin activate GHS-R1a. They found an increase in Ca^{2+}-influx, when bound by ghrelin, but not by motilin. indicating GHS-R1a was activated when bound by ghrelin. Therefore, they concluded that only ghrelin can bind to GHS-R1a.

Ghrelin is acylated by the ghrelin O-acyltransferase (GOAT) (Yang et al., 2008). The GOAT attaches a fatty acid onto Ser3 residue. The GOAT-ghrelin system has been shown to be a nutrient sensor (Kirchner et al., 2009). It has also been shown when mice lack GOAT there is a decrease in body weight, thus supporting the idea that the GOAT-ghrelin system is a nutrient sensor. The GOAT-ghrelin system senses the presence of nutrients rather the absence of nutrients (Kirchner et al., 2009).

Previous experiments showed that when the ghrelin hormone was administered to rodents, it led to an increase in food intake and weight (Tschop et al., 2000). The ghrelin hormone is constantly circulating throughout the bloodstream increasing and decreasing in concentration depending on the fasting and postprandial states of the GI tract (Cummings et al., 2001; Gnanapavan et al., 2002). Ghrelin decreases after a meal and increases right before

a meal (Cummings et al., 2001). Cummings et al. (2001) saw that, in human subjects, the ghrelin hormone is involved in meal initiation. Before a meal, ghrelin levels increased by 78% and decreased within an hour after the meal.

The motilin hormone was first discovered in the upper intestinal mucosa of swine (Brown et al., 1971). It is a 22 amino acid peptide that binds to the motilin receptor. When the hormone is released from enterochromaffin cells from the upper small intestine, it binds to the motilin receptor found on enteric neurons in the human duodenum and colon. In return, this binding induces smooth muscle contractions in the gut wall (Brown et al., 1971; Dass and Hill et al., 2003). The contractions are dependent on motilin concentration (Dass and Hill et al., 2003). These contractions occur during the interdigestive state in the antrum, which is the lower part of the stomach, and in the duodenum, which is the first part of the small intestine.

An amino acid sequence analysis by Dass and Hill et al. (2003), showed that the closest family member to the motilin receptor was GHS-R1a. However, when human and rat ghrelin was administered to rat stomach and colon in concentrations between 0.01-10 μM, there was no muscle tension or nerve-evoked contractions (Dass and Hill et al., 2003). These data support the idea that the ghrelin hormone cannot bind to the motilin receptor.

Since the discovery of the ghrelin receptor and the motilin receptor, agonists and antagonists have continued to be developed, studied, and tested as potential treatments for gastrointestinal diseases and disorders. The roles of the ghrelin receptor are still being studied. Some studies have shown the receptor to be involved with food intake and gut motility; however, others suggest the receptor is also involved in behavior such as anxiety, impulsiveness, cognition, and pain (Howick et al., 2017). The ghrelin receptor has far more

diverse roles than other receptors in the GHS-R family, even though it is very similar in identity to the motilin receptor. The receptors are similar; however, the hormones are specific to each receptor. Overall, studies have identified the different locations, functions, and signaling pathways for the ghrelin receptor and the motilin receptor.

In zebrafish, the expression of the ghrelin receptor and motilin receptor have not been carefully studied during development and maturation, and their functions are still poorly understood. In previous experiments, researchers have detected the ghrelin receptor and motilin receptor in the adult zebrafish intestine by immunohistochemistry, however it was not known which cell types express either receptor (Olsson, 2008). In embryonic, larval, and juvenile zebrafish, neither receptor has been studied in terms of gene expression or function. However, studies by Kitazawa et al. (2017) identified that the motilin receptor and the motilin hormone are present in zebrafish, but they are not sure if it was involved in gut motility.

Objectives of This Study

The primary goal for this study is to evaluate the roles of *ghsr1a*, the ghrelin receptor gene, and *mlnr*, the motilin receptor gene, to better understand how these genes function in the development and maturation of normal gut motility. I will use the zebrafish model across multiple life stages including embryonic, larval, juvenile, and adult stages. To determine where these genes are expressed, *ghsr1a* and *mlnr* will be mapped using in situ hybridization or antibody staining across a stage series. Since the ghrelin receptor and the motilin receptor are from the GHS-R family, a protein sequence alignment will be performed for comparison. To further understand their roles in gut motility, functional assays will be performed using

larval zebrafish. This investigation may contribute to developing new potential therapies, medications, and treatments for populations affected by gastrointestinal diseases.

Materials and Methods

Zebrafish Husbandry

Wild type and *mlnr* mutant zebrafish (*Danio rerio*) were housed in the Appalachian State University animal facility and maintained following standard procedures (Westerfield, 2007). The *mlnr* line was purchased from the Zebrafish International Resource Center and was regenerated from frozen sperm. The zebrafish were maintained on a 14-hour daily light cycle from 9:00 am to 11:00 pm. All zebrafish were fed at 9:00 am with dry food and 3:00 pm with 48-hour old brine shrimp (*Artemia franciscana*). Several water quality tests were conducted daily, including conductivity, pH, temperature, and total dissolved solids (TDS) of the water supply. The conductivity was kept between 450-600 microsiemens and acidity between pH 6.8-7.2. The temperature fluctuated between 27ºC and 28.5ºC and the TDS was maintained at 0 ppm. All procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University.

To conduct experiments on larval zebrafish, adult zebrafish were bred, and eggs were collected to raise larvae. To breed zebrafish, several male and female zebrafish were netted and put into breeding tanks so that the fertilized eggs could be collected. Eggs and larvae were maintained on a daily regimen as described in Norton et al. (2019). Briefly, eggs were put into a glass culture dish filled with approximately 120 mL of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) with the addition of 0.01% methylene blue. Before the first 24 hours after the eggs were fertilized, they were bleached to limit fungal growth. Eggs were treated with a solution of 100 µL bleach and 175 mL facility water and rinsed thoroughly three times with fresh facility water. The bleached eggs were transferred back into glass bowls containing fresh E3 medium at a density of 50 eggs per

bowl. They were incubated at 28.5℃ until 5 dpf. At 3 dpf, the embryos were manually dechorionated. At 5 dpf, the larvae were transferred into nursery tanks that were maintained at 28.5ºC. From 5-9 dpf, the larvae were fed two times daily with dry food and one time with newly hatched 24-hour old San Francisco Bay strain of brine shrimp. The dry food was Golden Pearls (GP) Reef and Laval Diet (Brine Shrimp Direct). An equal amount of GP5-50 and GP50-100 was suspended with E3 medium and stored at 4℃. The brine shrimp were cultured in a shrimp hatchery filled with approximately 725 mL of 30 grams/Liter Instant Ocean salt dissolved in deionized reverse osmosis water.

Gut Transit Assay, Basic Protocol

The gut transit assay used approximately 50 wild-type fish per group. Larvae were maintained at a density of 50 larvae per tank in 250 mL of 0.5x E3 medium. The evening before the experiment, the fish were fasted overnight. To fast the fish, they were transferred to fresh tanks before the 3:00 pm feeding time and then fed as usual. At 5:00 pm, the tanks were cleaned thoroughly with a Pasteur pipet to remove loose debris. After cleaning, the fish were transferred to a new tank and any remaining debris was removed by siphoning the tank 2-3 times as described (Norton et al., 2019). Each tank was labeled as “fasting” on the lid and the side of the tank.

The following morning, larvae were fed brine shrimp at 9:00 am. After 15 minutes of feeding, larvae were transferred to a 100 mm glass Petri dish. This was accomplished by removing most of the water from the fish tank, using a siphon. Larvae were then carefully poured into the Petri dish. Unless otherwise stated, the Petri dish was maintained on a 28.5ºC heat block. Larvae were anesthetized with 10-20 drops of 0.4% tricaine and screened for the

presence of a shrimp in the intestinal bulb. During the screening process, the fish were positioned laterally using a fishing line probe. Positive larvae were transferred into a fresh tank using a wide bore fire-polished Pasteur pipet and tanks were returned to the nursery until larvae were imaged.

Detection of Decreased Gut Motility

Standard gut transit assay procedures were followed except that 15 minutes after larvae were fed, half of the tanks were transferred to a nursery maintained at room temperature (20.0℃) while the other half remained in nurseries maintained at 28.5ºC. To maintain larvae at room temperature during screening, the Petri dish was held on the countertop instead of on the heat block. Images were taken at 3 hours, 4 hours, 6 hours, or 7 hours post-feeding. At each timepoint, a new tank was imaged so that larvae were not imaged twice.

Detection of Increased Gut Motility

Standard gut transit assay procedures were followed except the shrimp feedings were staggered at 9:00 am, 9:30 am, and 10:00 am. During the 30 minutes between each shrimp feeding, the fed larvae were screened as described previously. Once the larvae were screened, half of the tanks were administered 2.5 mL of pre-warmed 200 mg/L $MgSO_4$ (Zhou et al., 2014). The remaining tanks were mock treated with 2.5 mL of pre-warmed 0.5x E3. The larvae were exposed to treatment until they were imaged at 1 hour, 2 hours, 3 hours, or 4 hours post-feeding. At each timepoint, a new tank was imaged so that larvae were not imaged twice.

Live Imaging

Ten minutes before the imaging time points, the larvae were transferred to a Petrie dish and anesthetized, as described above. The anesthetized larvae were transferred to a mold (World Precision Instruments) made from 3% agarose in E3 medium. The transfer was done using a wide bore fire-polished Pasteur pipet. While on the mold, larvae were submerged in warm E3 medium. The larvae were imaged at regular intervals to track gut transit. At each timepoint, a new tank was imaged so that larvae were not imaged twice.

Larvae were imaged using either an Olympus SZX12 stereomicroscope or a Leica M80 stereomicroscope with a Canon T5i camera connected. Larvae were anesthetized and transferred to an agarose mold as described above. The images were taken once the larvae were positioned on their side using a fishing line probe. To image both the control groups and the treated groups at regular intervals, the experiment was performed by two people working simultaneously.

Sequence Alignment

Protein sequences were obtained from the NCBI RefSeq protein database and aligned using BLASTp with the Needleman-Wunsch Global Align Sequences program from NCBI (Altschul et al., 2005). The accession numbers used were NP_940799 (growth hormone secretagogue receptor type 1 isoform 1a) and NP_001498 (motilin receptor).

RT-PCR primers

The *ada*, *apq4*, and *slc10a2* primers were designed using Primer-BLAST (Ye et al., 2012). The expression of *ada* is restricted to the intestinal bulb, the expression of *slc10a2* is

restricted to the small intestine, and the expression of *apq4* is restricted to the posterior intestine (Lickwar et al., 2017; Tingaud-Sequeira et al., 2010). The *ghsra* primers were from Eom et al. (2014) and the *mlnr* primers were from Liu et al. (2013). The primers for *ghsra* and *mlnr* were confirmed through Primer-BLAST. Because BLAST analysis suggested that the published *mlnr* primers could amplify *nrp2a*, a second pair of *mlnr* primers (*mlnr2*) was designed.

Table 1. PCR primers.

Name	Direction	Sequence	Expected size (bp)
ada	Forward Reverse	5'-ATTGGGCACGGATACCACA-3' 5'-GCATGCCGTAGGCCTCATA-3'	161
aqp4	Forward Reverse	5'-AGTCTGAGGAGGAATGACAAGC-3' 5'-GTTGCGATGGACAAGCCAAA-3'	267
ghsra	Forward Reverse	5'-CCTCAGTGCAGCAATCAACC-3' 5'-GCATGGCGAACTCTCTCCTT-3'	136
mlnr	Forward Reverse	5'-GCCGAAAGTTGTGGAAGAGT-3' 5'-CAGGTAGAAGAGCACCATCGAG-3'	227
mlnr2	Forward Reverse	5'-GATACGCCATCGAGTCAGGG-3' 5'-ATGAGTGAAGAGGAAGCGGC-3'	260
slc10a2	Forward Reverse	5'-ATCTGTGGTGGGAATCGTCC-3' 5'-GCGTTCTGCATGCCTGTTTC-3'	143

Intestinal Isolation and Dissection

Intestines were isolated from adult wild type fish. Dissection was performed as previously described (Eames Nalle et al., 2017). Briefly, the fish were euthanized using cryoanesthesia followed by decapitation. The specimen was oriented semi-laterally on a dissection dish by pinning through the fleshy part of the body dorsal to the anal fin. Throughout the dissection, the specimen was submerged in 0.5x E3 medium. A cut was made

with Vannas spring scissors along the ventral midline just past the pelvic girdle. At the caudal end of the incision, a perpendicular cut was made that extended dorsally into the axial muscles. The last cut was parallel to this, on the opposite side of the body (Eames Nalle et al., 2017). Forceps were used to pull the resulting tissue flaps apart to widen the opening to allow removal of the organs from the body cavity. To isolate the intestine, the attached organs were removed including the gonads, heart, spleen, gall bladder, liver, and swim bladder (Figure 13A). The intestine was then cut into segments: intestinal bulb (IB), the proximal region of the intestinal loop, and the distal small intestine and colon (Figure 13B-D).

Survival Analysis

The general health of the *mlnr* fish line was assessed using a survival curve generated by GraphPad Prism. The line was shipped from ZIRC and received as 128 fertilized eggs. Survival was tracked from the date that the eggs were shipped through to 20 dpf. The Kaplan-Meier method was used to generate the curve (Machin et al., 2006).

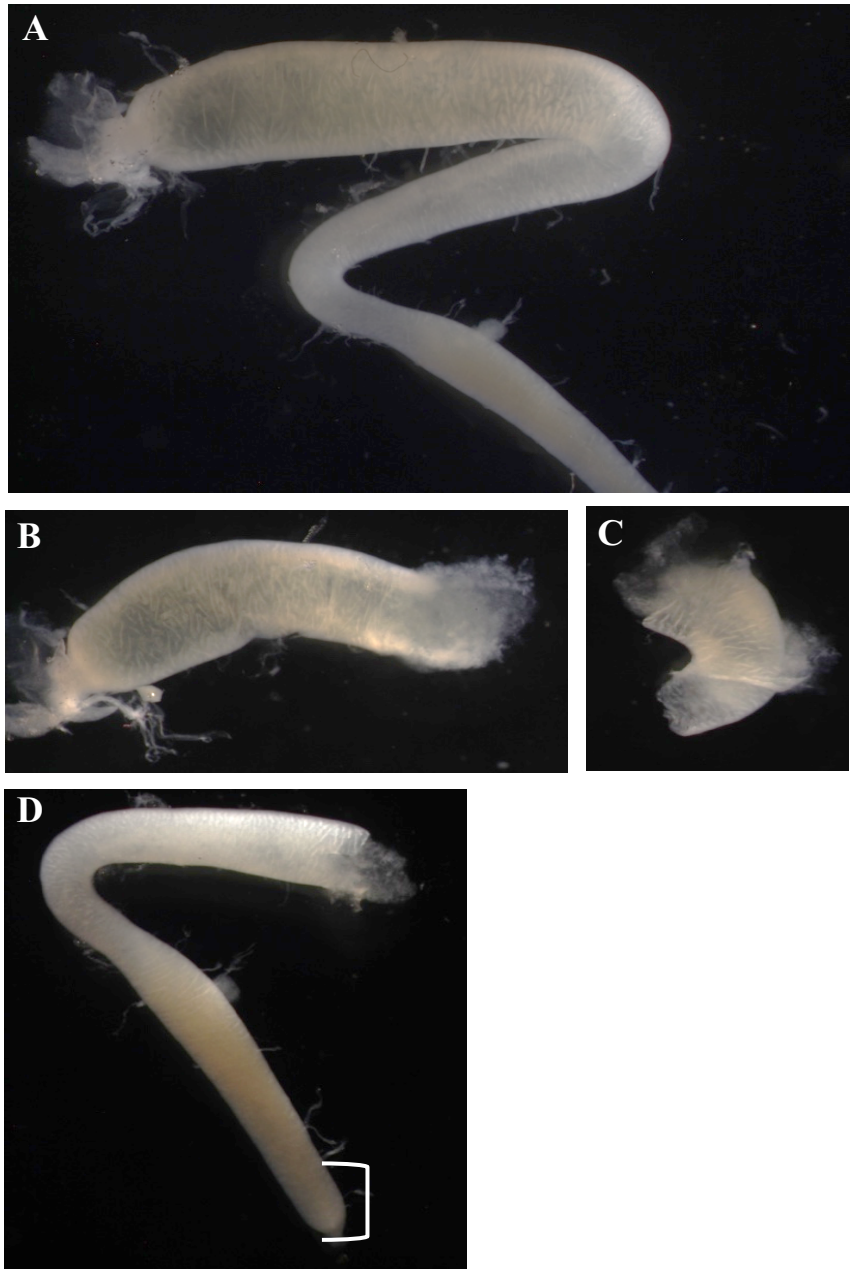

Figure 13. The isolated intestine and segments of an adult wild type zebrafish. A. Isolated intestine, B. Intestinal bulb, C. Proximal intestinal loop and D. Distal small intestine. The bracket indicates the colon.

Results

Intestinal Motility in Wild Type Zebrafish

To study GI motility, an assay was designed to allow observation of intestinal transit in live, intact zebrafish. Larval zebrafish have a transparent body wall and, therefore, it is possible to observe the intestinal wall and the contents of the intestine without using dissection. Between meals, when the intestine is empty or largely empty, folds in the intestinal wall are visible under a light microscope. These folds, called villar ridges, create more surface area for absorption of nutrients. When a meal is eaten, these folds flatten as the intestinal bulb becomes distended. As digestion proceeds, and the intestine empties, the villar ridges reappear. Thus, the presence or absence of villar ridges is an important aspect of tracking intestinal transit. Another aspect is following the ingested meal as it moves through the GI tract, from the intestinal bulb to the colon. The gut transit assay used here involved feeding a meal of brine shrimp to the larvae. Previous experiments (unpublished) found that larval zebrafish ate only one brine shrimp during a feeding session. Thus, the meal size is controlled for the gut transit assay.

When larval zebrafish eat brine shrimp, the bright orange shrimp is visible in the distended intestinal bulb. During digestion of the shrimp, orange pigmented material can be observed moving through the GI tract, as shown in Figure 14A. This figure shows representative images from a gut transit assay that extended over a period of five hours. The distended intestinal bulb is shown in panel A, imaged within an hour of feeding. Within 3 hours after feeding, the intestinal bulb was less distended, contained little orange material, and mostly undigested material remained, as shown in panel B. By hours four and five, only

gray material was visible in the intestinal bulb (panel C, D). This gut transit assay shows that it takes four hours for all intestinal contents to exit the intestinal bulb.

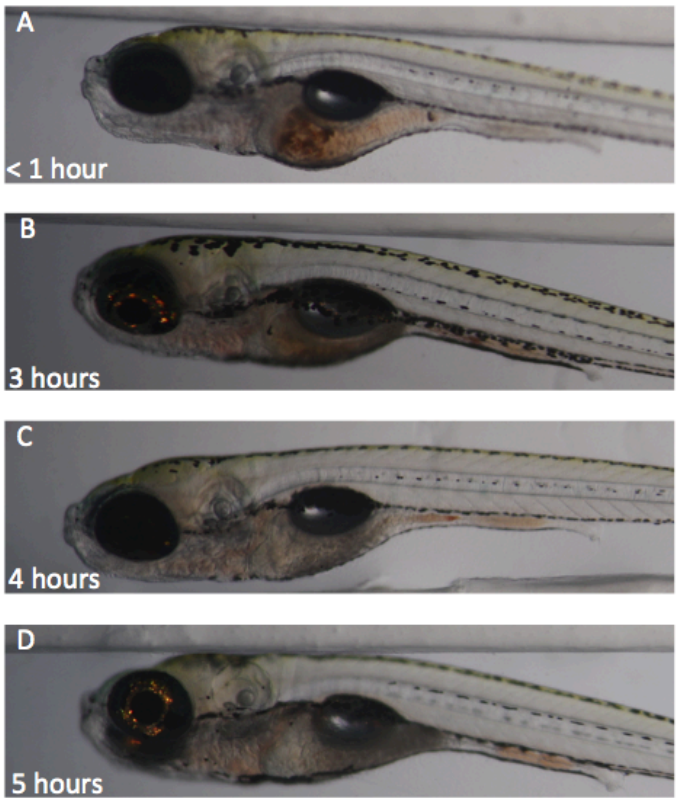

Figure 14. A gut transit assay over a period of five hours. A. Intestinal Bulb (IB) is distended after eating a brine shrimp. B. Presence of waste in IB and posterior intestine. C-D. Some gray undigested material in IB and some orange material in posterior intestine. The heads are to the left and the tails are to the right. All specimens are live, wild-type larvae at 9 dpf. n = 48 – 73 per timepoint.

Detection of Changes in Gut Motility

Pilot Study for Room Temperature Feeding

To test whether the gut transit assay could distinguish delayed transit versus normal transit, my goal was to expose the larvae to cool water temperature during digestion. As part of designing the assay, I first tested how rapidly the tank water would drop to room

temperature when tanks were transferred from the normal 28.5ºC of the larval nursery to a nursery set to 20.0ºC. Tank temperature changes were monitored by placing a thermometer into each tank and recording the values at regular intervals. As shown in Figure 15, I found that the tank water temperature dropped to 24.5°C, on average, within 10 minutes. It took roughly 50 minutes for the tanks to drop down to room temperature at 20.2°C. Since the temperature change was well-within the 3-hour timeframe of the gut transit assay, I concluded that I could move the tanks from the warm 28.5°C nursery to the room temperature nursery to apply a temperature treatment.

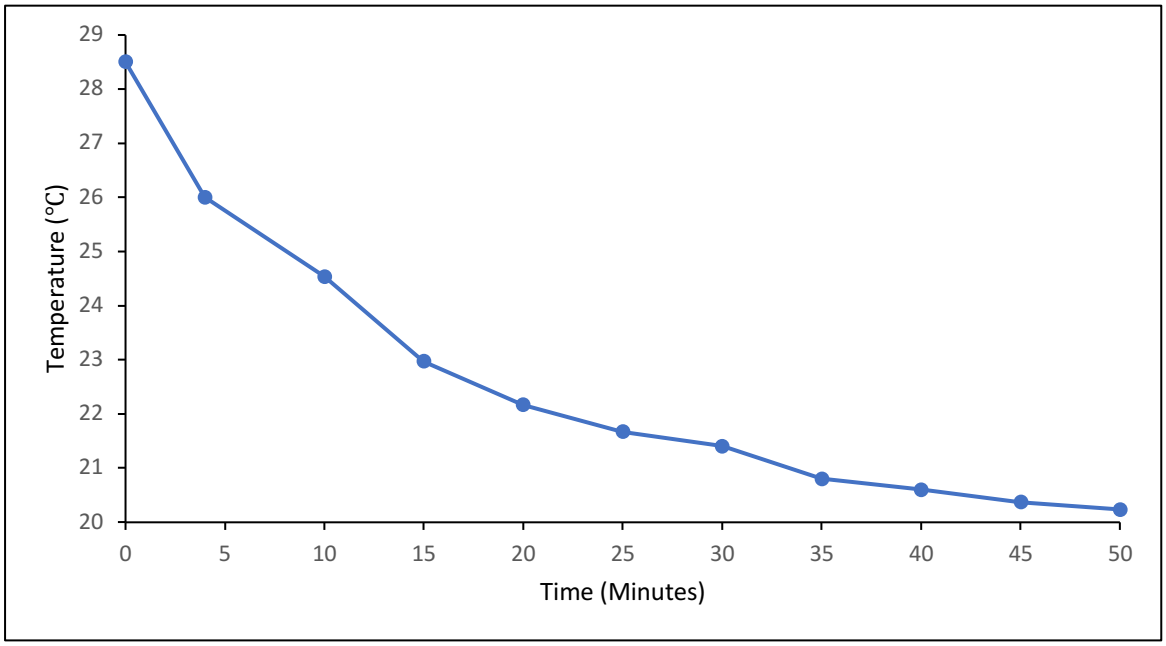

Figure 15. Average temperature change over a period of 50 minutes. The tank water temperature was monitored as it dropped from the 28.5ºC starting value. The experiment was performed in triplicate. The datapoints are averages.

Cooling Decreases Gut Motility

Next, I tested whether the gut transit assay could detect a delay in intestinal transit by comparing intestinal transit times for control larvae maintained at their normal temperature (28.5°C) versus cool temperature-treated larvae that were transferred to the room temperature nursery (20.0°C) during digestion. After the larvae were fed shrimp, half of the tanks were placed in the room temperature nursery.

For the control group, gut transit proceeded as expected and was completed within 4 hours after feeding (Figure 16A, B). For the cool temperature-treated group, gut transit was delayed (Figure 16C-F). At hour 3 after feeding, the treated group still had a distended intestinal bulb filled with orange material, indicating that it was filled with food. By contrast, the control group had a flattened intestinal bulb with little to no food remaining. At hour 4 after feeding, the control group showed that the food had been digested. This was indicated by the reappearance of villar ridges in the intestinal bulb and a lack of orange material. Some gray debris remained in the intestinal bulb, as expected. The debris was likely the indigestible fiber from the shrimp exoskeleton. For the treated group at hour 4, food was still present in the intestinal bulb. By hours 6 and 7, intestinal transit in the treated group was similar to hours 3 and 4 of the control group. Overall, there was roughly a 3-hour delay in intestinal transit for the cool temperature-treated group. Therefore, I concluded that the gut transit assay can detect a decrease in gut motility.

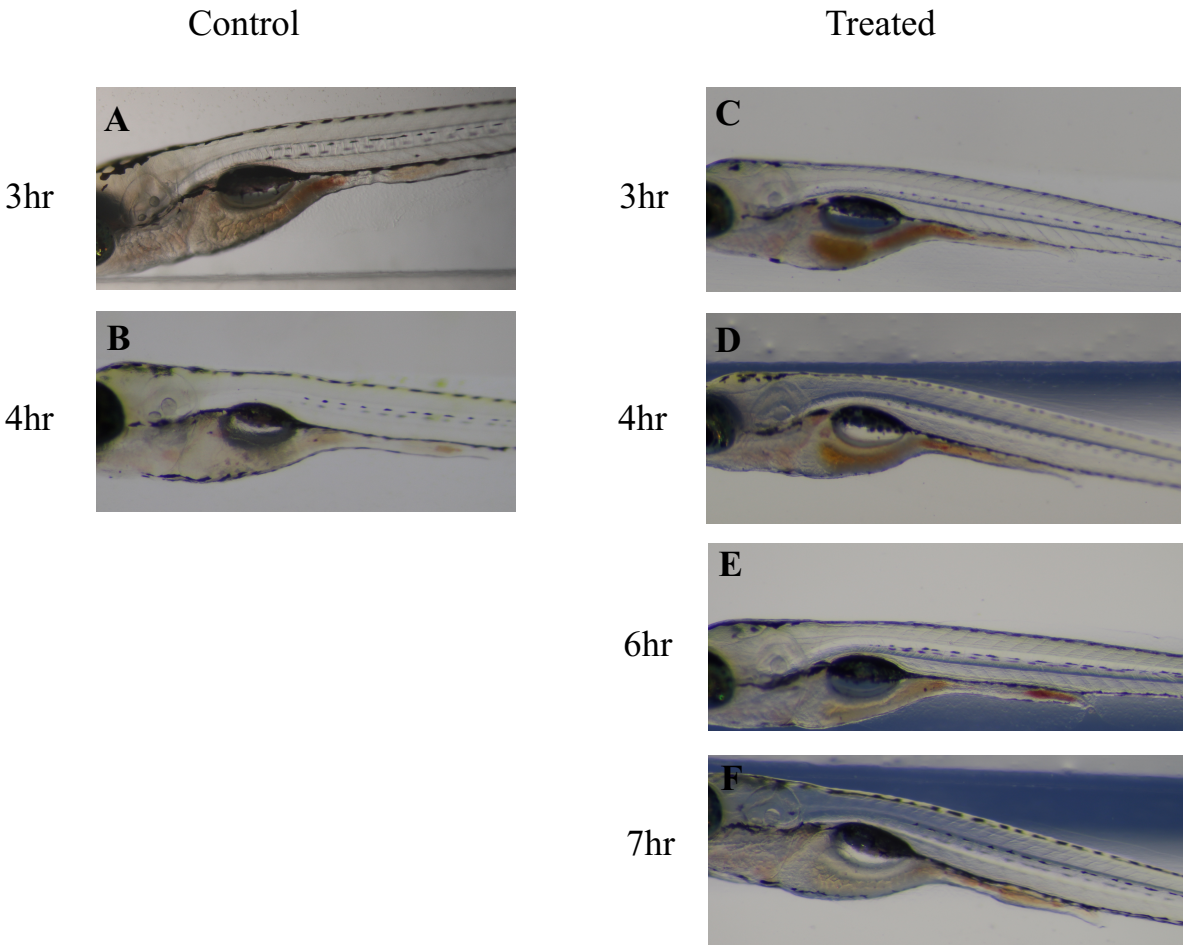

Figure 16. The effects of cooling on gut motility. A-B. Wild type larvae maintained at 28.5ºC throughout feeding and digestion. C-F. Wild type larvae transferred to a room temperature nursery 15 minutes after feeding. The heads are to the left and the tails are to the right. All specimens are 9 dpf and imaged live. The experiment was performed twice. Sample sizes were A: *n* = 17, B: *n* = 15, C: *n* = 7, D: *n* = 8, E: *n* = 13, F: *n* = 15.

$MgSO_4$ Treatment Increases Gut Motility

To test whether the gut transit assay could distinguish increased transit versus normal transit, my goal was to expose larvae to a treatment that was expected to increase gut motility, and thus increase gut transit. The experiment compared mock-treated larvae (Figure 17A-D) with larvae exposed to 200 mg/L $MgSO_4$ (Figure 17E-H) during the digestion period. At hour 1, both groups had distended intestinal bulbs. At hour 2, the mock-treated

group had food present in the intestinal bulb while the $MgSO_4$ treated group only had debris in the intestinal bulb. At hour 3, the mock-treated group had little to no food present in the intestinal bulb, whereas the $MgSO_4$ had an empty intestinal bulb. At hour 4, the mock-treated and the $MgSO_4$ treated groups had empty intestinal bulbs. At hour 4, both the mock-treated looked similar to hour 3 larvae treated with $MgSO_4$. Based on the live images, there was a 1 hour increase in intestinal transit with the addition of $MgSO_4$. Therefore, I concluded that the gut transit assay can detect an increase in gut motility.

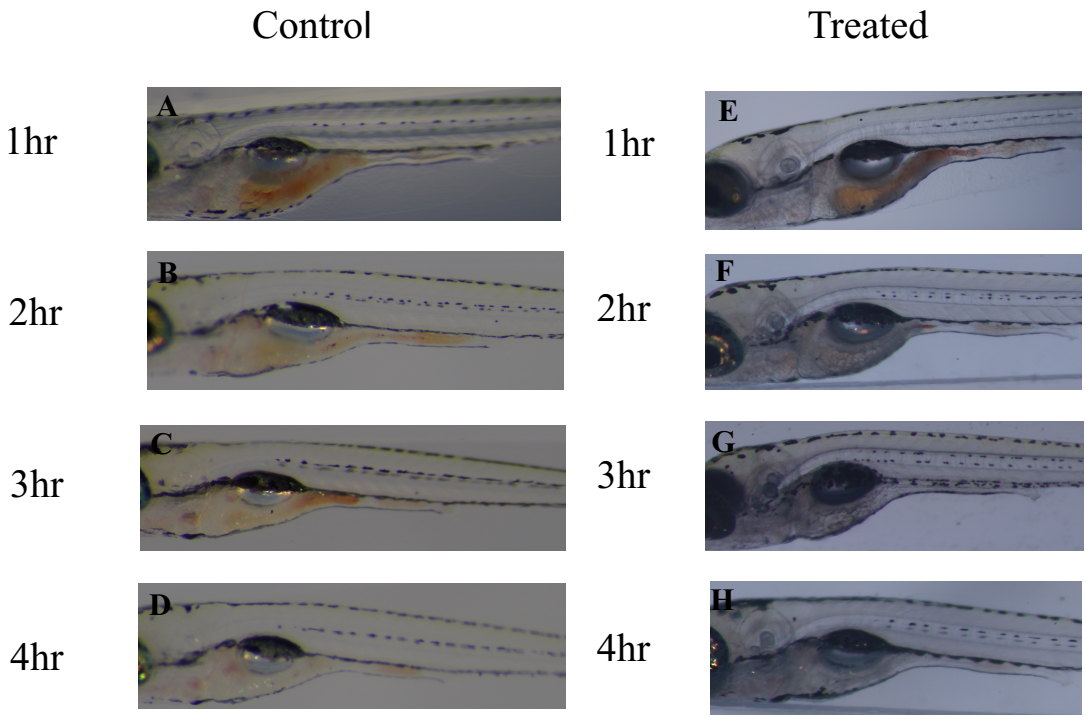

Figure 17. Intestinal transit assay with the addition of $MgSO_4$ over a period of four hours. A-D. Larvae mock treated with E3 medium. E-H. Larvae treated with 200 mg/L $MgSO_4$. The heads are to the left and the tails are to the right. All specimens are live, wild-type larvae at 9 dpf. The experiment was performed five times. Sample sizes were A: $n = 22$, B: $n = 5$, C: $n = 4$, D: $n = 6$, E: $n = 14$, F: $n = 7$, G: $n = 7$, H: $n = 4$.

Comparison of GHS-R Intestinal Receptors

Early studies found that the human GHSR and MLNR receptors shared 52% identity at the amino acid level (Feighner et al., 1999; McKee et al., 1997). However, when I repeated this study using more recent sequences from the NCBI RefSeq database, I found that the sequences were only 44% identical, as shown in Figure 18. This is consistent with a recent review by De Smet et al. (2009) who also reported a 44% identity. Not only are they similar in sequences, but also, they are structurally similar as well. Overall, these two receptors are structurally similar and they both have important roles in gut motility.

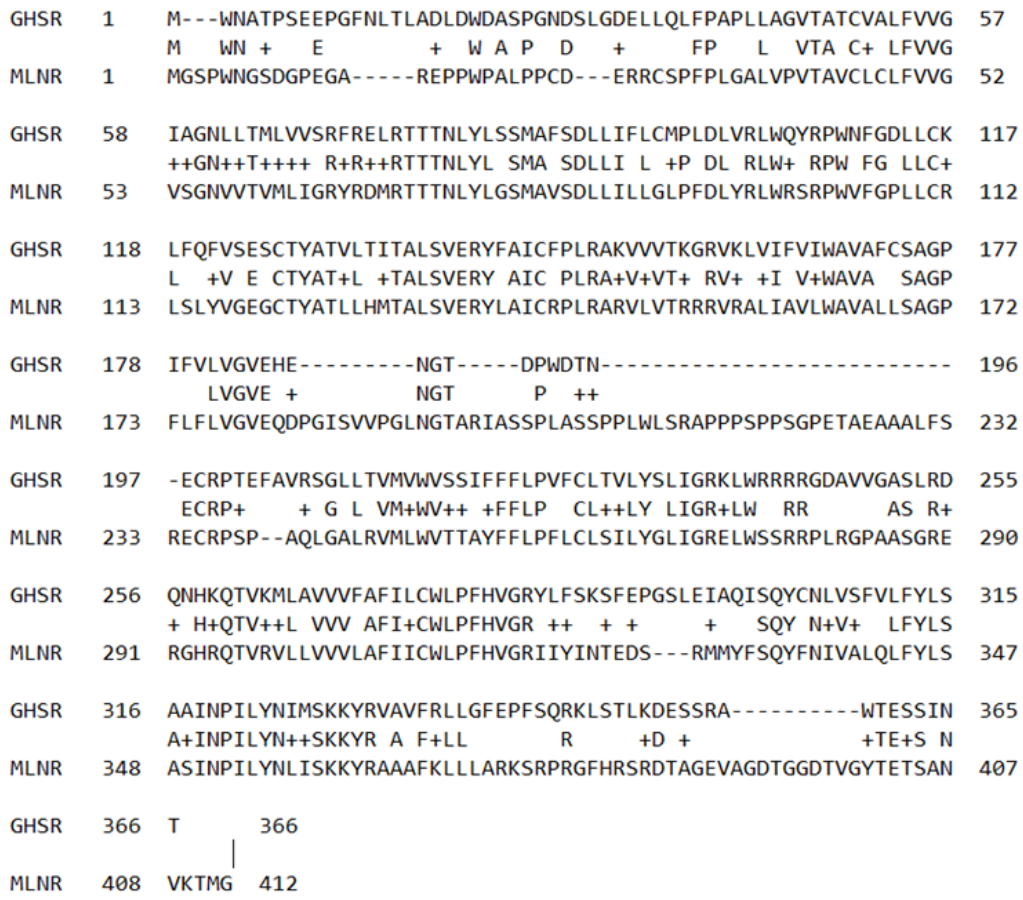

Figure 18. Amino acid sequence alignment for human ghrelin receptor and motilin receptor. Global alignment analysis indicates the sequences are 44% identical. Accession numbers NP_940799 (366 aa, growth hormone secretagogue receptor type 1 isoform 1a), NP_001498 (412 aa, motilin receptor). Program: Needleman-Wunsch Global Align Protein Sequences, from NCBI (Altschul et al., 2005).

Motilin Receptor Functional Studies

The *mlnr* mutant zebrafish line has never been studied. For the *mlnr* mutant line, I predict that larvae will have an impaired migrating motor complex and they will therefore have trouble sweeping undigested debris from the gut between meals. This mutant line can be followed by non-invasive imaging until the larvae enter metamorphosis. At this point, pigmentation of the body wall will prevent imaging the intestinal contents in live animals. If I

find that the intestine is progressively impacted by the *mlnr* mutation, then I may decide to euthanize and dissect older specimens in order to observe the intestine.

In preliminary work, the *mlnr* mutant line was studied by tracking the survival rate of a cohort of 128 embryos through to 20 dpf. The embryos were derived from wild type eggs fertilized in vitro with sperm that carried the mutation. All embryos were tracked, without determining genotypes. On 20 dpf, there was an 87.5% survival rate for the *mlnr* mutant zebrafish line (Figure 19). From 14 dpf to 20 dpf there were no recorded deaths. I concluded that the defect is probably not lethal, at least for young fish, because the survival rate was high.

The motilin receptor will also be studied by using the small molecule antagonist ANQ 11125 (Tocris). Preliminary work suggests that the motilin receptor would begin to be active by 3 hours after feeding. The antagonist is water soluble and will be directly added to the tank water. Therefore, the antagonist will be added to the water 2 to 3 hours after feeding. Larvae will be imaged beginning at 5 hours post feeding. I predict that the undigested remains of the meal will be retained in the intestinal tract for a prolonged period compared to mock-treated controls.

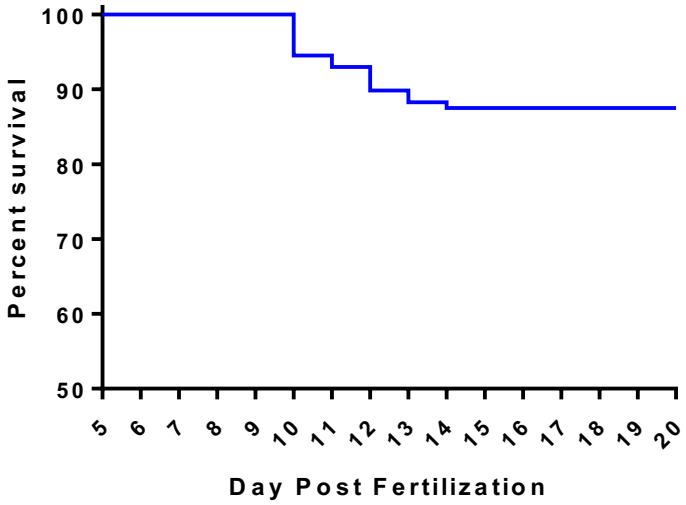

Figure 19. Survival rate of the *mlnr* zebrafish line. Sixteen deaths were recorded from a starting population of 128. Survival was plotted using the Kaplan-Meier method.

Gene Expression Mapping

To complement studies of gut function, my next objective was to map the expression of candidate genes in a temporospatial manner. My focus was on *gshr1a*, the ghrelin receptor, and *mlnr*, the motilin receptor, because of their roles in coordinating gut smooth muscle contractions. In collaboration with others, I searched databases, including PubMed and the Zebrafish Information Network, to survey what was already known for expression patterns of *ghsra* and *mlnr*. As shown in Table 2, expression of these receptors in zebrafish has been investigated only in adults (Eom et al., 2014; Kitazawa et al., 2017). Therefore, my goal was to map expression using RT-PCR over different developmental stages. This would allow me to build an expression map along the anterior-posterior axis and give me a gene

expression profile of the major anatomical segments: intestinal bulb, small intestine, colon, and the poorly-described proximal loop region.

Table 2. Intestinal gene expression patterns.

Gene	Embryonic	Larval	Juvenile	Adult
Ghrelin receptor (*ghsra*)	Unknown	Unknown	Unknown	RT-PCR only
Motilin receptor (*mlnr*)	Unknown	Unknown	Unknown	RT-PCR only

Based on published studies, I have identified three genes that are predicted to be expressed in specific gut regions in a non-overlapping manner, as schematized in Figure 20. Studies by Lickwar et al. (2017) suggested that *ada* is expressed only in the intestinal bulb and that *slc10a2* is expressed only in the small intestine of zebrafish. Other studies by Tingaud-Sequeira et al. (2010) suggest that *aqp4* is expressed only in the colon. Therefore, these genes are expected to serve as positive controls for RT-PCR experiments.

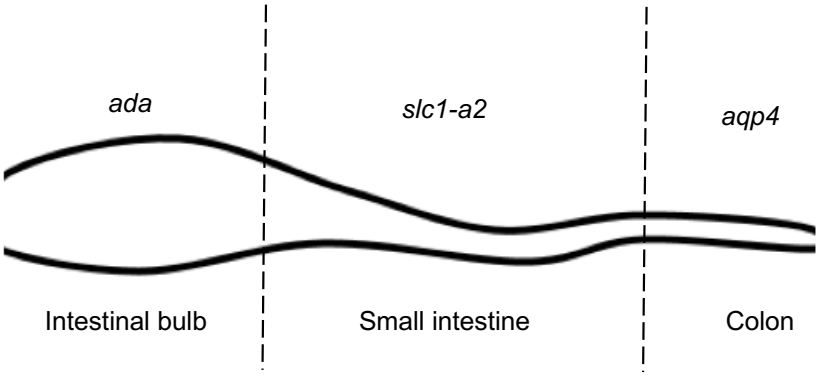

Figure 20. Predicted gene expression in the zebrafish intestine.
The intestine is shown without the intestinal loop, for clarity.

Since the proximal loop of the small intestine has not been clearly defined, anatomically or molecularly, the location of these receptors will help identify which segment the intestinal loop belongs to, if the hypothesized location of the receptors is correct. After mapping the anterior-posterior expression of these five genes in the adult intestine by RT-PCR, the next step will be to perform in situ hybridization on embryos, and antibody staining on sectioned tissues from older specimens. These approaches will enable localizing gene expression or proteins to specific tissues or cell types.

Discussion

The long-term goal of this study is to understand how the ghrelin receptor and motilin receptor regulate normal gut motility. This work contributed to the development of a novel gut motility assay and it helped to establish that the assay can be used to observe gut movements and emptying in live zebrafish, thus allowing for the detection of decreases and increases in gut motility. This assay will be useful for functional studies of intestinal receptors including the ghrelin receptor and motilin receptor.

Intestinal Motility in Wild Type Zebrafish

In live specimens, the larvae can be used to track intestinal transit using a gut transit assay. The assay works because the body wall is transparent and the ingested meal is bright orange, allowing observation of the intestinal contents. As the larvae mature, they develop skin pigment and the assay no longer works.

The first live gut transit assay used a fluorescent polystyrene tracer in larval zebrafish (Field et al., 2009). The non-digestible fluorescent polystyrene tracer replaced the live prey from their diet, allowing visualization of intestinal transit in larvae. The experiments were performed at 7 dpf, and the results showed that it took between 6-24 hours for the fluorescent polystyrene tracer to exit the intestinal tract. Brady et al. (2017) repeated the study and similarly found that many zebrafish took 24 hours for the tracer to completely empty the intestinal tract. However, zebrafish are known to have relatively fast digestion, with larvae requiring at least three feedings per day (Westerfield 2007). Based on Brady et al. (2017) and Field et al. (2009), the fluorescent polystyrene tracer would not be suitable when observing normal gut transit rates.

This is validated in the new assay developed here, where it took 4 hours for the larval zebrafish to empty intestinal contents out of the intestinal bulb, essentially allowing the experiments to be performed in a time-efficient manner. The new assay uses a natural diet, brine shrimp, instead of the plastic fluorescent polystyrene tracer. In the study from Brady et al. (2017), the larvae had shown spitting behavior. Spitting behavior shows that the fish detect inedible food, whereas in the new gut transit assay brine shrimp are part of their natural diet and are palatable. Additionally, it is known from previous experiments (unpublished study), that larval zebrafish will eat only one brine shrimp during feeding, allowing a controllable meal size. This helps to ensure that when tracking intestinal transit times, the experiments are consistent. Overall, the newly-developed assay using brine shrimp allowed digestion to occur naturally. The intestinal transit rate was consistent throughout each time point of the assay.

Cooling Decreases Gut Motility

When the fish were transferred to room temperature during digestion, the gut transit assay detected a 3-hour delay in intestinal transit. A temperature effect on digestion has not been studied in zebrafish. However, it is known that in colder temperatures, metabolism will slow in cold-blooded species such as zebrafish. Since zebrafish are unable to regulate their body temperature, lowering the water temperature of the tank led to a slowed metabolism. This cooling experiment showed that the assay can detect decreases in gut motility, therefore allowing future studies of the functions of intestinal receptors, especially with respect to using receptor antagonists or mutant fish lines.

$MgSO_4$ Treatment Increases Gut Motility

When $MgSO_4$ was administered, the assay was able to detect an increase in gut motility. This is similar to Zhou et al. (2014) who treated zebrafish with concentrations ranging from 200-2,000 mg/L $MgSO_4$ and found that there was an increase in gut motility. In this study, I found that the addition of 200 mg/L $MgSO_4$ resulted in intestinal transit that was 1 hour faster than in vehicle-treated controls. $MgSO_4$ is an osmotic laxative that can increase osmotic pressure in the intestinal tract (Izzo et al., 1996). Ikarashi et al. (2011) found that $MgSO_4$ laxative effect is not solely dependent on osmotic pressure alone but also on the water channel aquaporin-3 (AQP3). In rats, $MgSO_4$ increased water influx through AQP3, into the intestinal tract (Ikarashi et al., 2011). The increase of osmotic pressure in the intestinal tract and AQP3 caused a rapid transfer of water from the vascular side to the luminal side of the intestine, creating the laxative effect (Ikarashi et al., 2011). The effect of $MgSO_4$ treatment in zebrafish may be similar to that seen in rats. This experiment showing increased gut motility suggests that the gut transit assay will allow future studies of the functions of intestinal receptors, especially with respect to using receptor agonists

Comparison of GHS-R Intestinal Receptors

Even though the ghrelin receptor and the motilin receptor are similar in sequence and structure, I hypothesized that they will have different functions in zebrafish. As mentioned previously, McKee et al. (1997) found the amino acid sequences were 52% identical. However, when I ran the sequence alignment through, I found that the sequences were only 44% identical, consistent with a report by De Smet et al. (2009). It is unclear why the more recent analyses differ from the original report. During the recent analysis, I used protein

sequences that were obtained from the NCBI RefSeq protein database and aligned them using BLASTp with the Needleman-Wunsch Global Align Sequences program from NCBI (Altschul et al., 2005). However, the original studies used the Wisconsin Package Genetics Computer Group (GCG) software (Womble, 2000). Since different software programs were used to compare sequence similarity between the ghrelin receptor and motilin receptor, this could be the cause of the discrepancy between the sequence identity percentages.

Motilin Receptor Functional Studies

The *mlnr* mutant line has never been studied. Therefore, I did not know whether the *mlnr* mutation would be homozygous lethal or not. To begin to test this, I tracked the daily survival rate of a cohort of 128 embryos as they developed through to 20 dpf and found a high survival rate of 87.5%. It is therefore possible that the *mlnr* mutation is not lethal, at least for young fish.

Future Directions

Receptor Functional Studies

The embryos were derived from wild type eggs fertilized with sperm from a male carrying the *mlnr* mutation. However, it is not known whether the male parent was homozygous versus heterozygous for the mutation. Depending on the zygosity of the male parent, the resulting embryos could have been 50% heterozygous or 25% heterozygous. Genotyping the fish will be an important next step in this project.

The motilin receptor small molecule antagonist ANQ 11125 (Tocris) has been studied in porcine and rabbit species (Depoortere et al., 1995; Peeters and Depoortere, 1994). Peeters

and Depoortere (1994) discovered this motilin antagonist, and when administered, it blocked motilin-induced contractions in the smooth muscle tissue of rabbit at low concentrations. This antagonist is specific only for the motilin receptor and outcompetes motilin for binding. This small molecule antagonizes a class of motilin receptor agonist called motilides (Peeters and Depoortere, 1994). When administered to zebrafish, the sweeping motions that occur between meals should be blocked, resulting in a delay in intestinal transit. If the debris remains in the intestine for a long period, the larvae could become susceptible to small intestinal bacterial overgrowth (SIBO). SIBO is caused by the backup of waste that leads to overgrowth of bacteria in the small intestine. SIBO has not been studied in zebrafish with the direct involvement of motilin (Deloose and Tack, 2016). Similar experiments will be performed on the ghrelin receptor in the future.

To study the function of the ghrelin receptor, antagonists, agonists, and a mutant line could be used. A *ghsra* mutant zebrafish line would be beneficial for testing the function of the ghrelin receptor in the intestinal tract. Plans are underway to obtain the *ghsra*^{sa15867} mutant line from ZIRC. For the *ghsra* mutant line, I predict that there will be a delay in intestinal transit in the zebrafish because they will have trouble digesting food. I further hypothesize that homozygous mutants will have more severe defects than heterozygous mutants. There could be a gene dosage affect. In order to detect this, the zebrafish will have to be raised along with a group of wild-type zebrafish and compared by the gut transit assay. All of this can be detected by using non-invasive imaging until metamorphosis. At this point, pigmentation of the body wall will prevent imaging the intestinal contents in live animals. If I find that the intestine is progressively impacted in larvae because of the delay in digestion by the *ghsra* mutation, then I may decide to euthanize and dissect older specimens in order to

observe the intestinal contents. Since these zebrafish will have a gut motility disorder, they might not eat as much since there is a delay in digestion, ultimately leading to smaller adult zebrafish when compared to wild-type zebrafish. In order to detect this, the zebrafish will have to be raised along with a group of wild-type zebrafish and compared once adulthood is reached.

A small molecule antagonist can also be used to test the function of the ghrelin receptor. The ghrelin receptor will be antagonized with [D-Lys^3]-GHRP-6 (Tocris). To study the ghrelin receptor, the antagonist will be added to the water just prior to feeding at 9 dpf. A gut transit assay will then be performed. I hypothesize there will be a delay in intestinal transit. The antagonist experiment will complement experiments with the *ghsra* mutant line. In reciprocal experiments, I will treat larvae with MK 0677 (Tocris), a ghrelin receptor agonist. I hypothesize that gut motility will be increased, and therefore, I expect to observe a faster gut transit. The ghrelin receptor agonist experiment will also complement the studies using the *ghsra* mutant line.

Receptor Expression Analysis

It has been shown by RT-PCR that *ghsr1a* and *mlnr* are expressed in the adult zebrafish intestine (Eom et al., 2014; Kitazawa et al., 2017). However, it is not known what cell type expresses either receptor. In rodents and humans, the ghrelin receptor is located on neuroendocrine cells of the GI tract (Dass and Munonyara et al., 2003; Holst et al., 2004). In rabbits and humans, the motilin receptor is found on circular smooth muscle cells in the stomach and intestine (Feighner et al., 1999; Huang et al., 2005; Holst et al., 2004).

Because the intestine is immature at hatching and continues to develop until adulthood is reached, the receptors should be studied across all developmental stages including embryonic, larval, juvenile, and adult stages. I expect during the embryonic and early developmental stages of zebrafish that the motilin receptor will be present in either the mid- or distal-intestine and the ghrelin receptor will be present in the intestinal bulb that will be found by using in situ hybridization. For the later development stages of zebrafish, I expect that the motilin receptor will be present in either the mid- or distal-intestine and the ghrelin receptor will be present in the intestinal bulb that will be found by immunohistochemistry.

It is unknown which cells in the zebrafish intestinal tract express the ghrelin receptor and motilin receptor. The location of the receptors in adult zebrafish will be studied through dissection of the intestine into three segments: intestinal bulb, mid-intestine, and distal intestine. RT-PCR will identify which segment the receptors are in. The receptor cell expression will be found through antibody staining. To identify the receptor-expressing cells, antibody staining will label the cells where they are found. Double labeling will be used, and this refers to the use of two antibodies being used simultaneously. Secondary antibodies will be labeled with FITC or rhodamine. DAPI will be used to label the nuclei. I expect the vagal afferent neurons to express the ghrelin receptor. The vagal afferent neurons have been known to produce peptides that are involved with feeding in rats (Date et al., 2002). As previously mentioned, the ghrelin receptor is on neuroendocrine cells in the GI tract. Neuroendocrine cells are a part of the abdominal vagal afferent neurons and are classified as chemosensers (Latorre et al., 2015). The motilin receptor is found on smooth muscle cells in the GI tract;

therefore, I expect the cholinergic and serotoninergic neurons to express the motilin receptor. These neurons control the enteric smooth muscle in zebrafish (Uyttebroek et al., 2010).

Overall, these approaches should elicit changes in the MMC and to observe the effects on gut motility. However, graded effects of MMC disruption may be difficult to detect using a gut transit assay. The real value of the antagonist and agonist will be the ability to add the small molecules directly to the fish water to disrupt gut motility at specific time points (*e.g.* after feeding) and at specific ages. In conclusion, I would expect when genes in the intestinal tract become disrupted, the enteric nervous system will not function properly, leading to many different gastrointestinal diseases that involve any type of motility.

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Vita

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