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Morphogenesis and motility of the *Astyanax mexicanus* gastrointestinal tract

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Abstract

Through the course of evolution, the gastrointestinal (GI) tract has been modified to maximize nutrient absorption, forming specialized segments that are morphologically and functionally distinct. Here we show that the GI tract of the Mexican tetra, *Astyanax mexicanus*, has distinct regions, exhibiting differences in morphology, motility, and absorption. We found that *A. mexicanus* populations adapted for life in subterranean caves exhibit differences in the GI segments compared to those adapted to surface rivers. Cave-adapted fish exhibit bi-directional churning motility in the stomach region that is largely absent in river-adapted fish. We investigated how this motility pattern influences intestinal transit of powdered food and live prey. We found that powdered food is more readily emptied from the cavefish GI tract. In contrast, the transit of live rotifers from the stomach region to the midgut occurs more slowly in cavefish compared to surface fish, consistent with the presence of churning motility. Differences in intestinal motility and transit likely reflect adaptation to unique food sources available to post-larval *A. mexicanus* in the cave and river environments. We found that cavefish grow more quickly than surface fish when fed *ad libitum*, suggesting that altered GI function may aid in nutrient consumption or absorption. We did not observe differences in enteric neuron density or smooth muscle organization between cavefish and surface fish. Altered intestinal motility in cavefish could instead be due to changes in the activity or patterning of the enteric nervous system. Exploring this avenue will lead to a better understanding of how the GI tract evolves to maximize energy assimilation from novel food sources.

Keywords

Astyanax mexicanus; Gastrointestinal tract; Gut morphogenesis; Enteric nervous system; Intestinal motility; Evolution

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.06.004.

Introduction

The GI tract (gut) digests food and absorbs nutrients, providing the fuel for all biological processes. The gut has undergone extensive evolutionary adaptation across taxa, to allow animals to thrive on diets with different nutrient compositions (Stevens and Humes, 1995). As critical as this is for survival, compared to evolution of morphology and behavior, far less is known about digestive and metabolic evolution.

The GI tract is a multilayered tube; epithelial cells form the lumen and are surrounded by layers of connective tissue (mucosa) and smooth muscle. The mucosa directly interfacing the epithelia contains glands and blood vessels essential for digestion and transport of nutrients, respectively. The enteric nervous system forms extensive networks within the gut wall that sense nutrients, control enzyme and hormone secretions, and coordinate intestinal motility (e.g. peristalsis) (Furness, 2012).

Each portion of the gut, from anterior to posterior, has a specialized function and morphology reflected in the composition of epithelial cell types, thickness and organization of the muscle and mucosal layers, and architecture of the enteric nervous system (Thompson et al., 2018). The total length of the gut, and proportionate length of specialized segments varies across species and is related to diet. For example, carnivores tend to have a large stomach and overall shorter gut to prioritize protein digestion and rapid absorption, while herbivores have a longer small intestine to provide greater area for digestion and absorption of tough plant material (Stevens and Humes, 1995). The intestinal epithelium is the site of nutrient absorption and its surface area is maximized in the confines of the lumen by folding into various patterns. In humans, mice, and chickens, finger-like villi project into the intestinal lumen. However, in other species, ridges, zig-zags, and honeycomb patterns are observed (Walton et al., 2018).

Contractions of the gut's smooth muscle layers create intestinal motility patterns including wave-like movements, or peristalsis, that push the contents of the gut forward. This process, in the context of the length of the gut, determines the time it takes for food to be digested and evacuated (intestinal transit time). While intestinal transit time has been measured in a number of animals (Stevens and Humes, 1995), in general there is a dearth of information on how peristaltic contractions differ between species, and even less is known about how these differences are mediated. Studies using the zebrafish, *Danio rerio*, have taken advantage of the transparent larvae to visualize peristalsis and make links to intestinal transit and enteric nervous system development (Heanue et al., 2016b). However, in the absence of extensive comparative data, how intestinal motility patterns and the enteric nervous system are specialized for different diets, and the functional impact on nutrient absorption remains unclear.

From studying mouse and chick development, we understand that morphogenesis of the intestinal epithelium is driven by molecular and physical interaction of the early epithelium with the underlying mesenchyme and muscle layers (Shyer et al., 2013; Walton et al., 2016), and that specification of the gut into regions is set up during embryogenesis by patterns of gene expression (Thompson et al., 2018). While some studies have focused on comparing

gene expression in the GI segments of mammals, birds, and amphibians (Smith et al., 2000), we lack an understanding of the developmental mechanisms that generate species-specific gut morphology and motility. These studies have been hampered by the inability to make functional comparisons between distantly related species, or to access the embryos of closely related species in the process of diversifying.

The Mexican tetra, *Astyanax mexicanus*, is a teleost fish with river- and cave-adapted populations that have evolved to thrive on diets of different composition and availability (Espinasa et al., 2017; Mitchell et al., 1977). The river (surface) fish likely consume insects and plants in abundance, while adult cavefish live in perpetual darkness and can survive much of the year without food, dependent on detritus from seasonal flooding and/or nutrients brought in from bat droppings (Espinasa et al., 2017; Mitchell et al., 1977). There are 30 known geographically-isolated cave populations that are derived from several independent invasions of surface fish (Fumey et al., 2018; Gross, 2012). Surface and cave populations are interfertile, and methods for breeding the fish in the lab produce thousands of embryos with each spawn (Elipot et al., 2014; Hinaux et al., 2011). *A. mexicanus* has proven to be a powerful model to investigate the developmental and genetic basis of the evolution of morphology (McGaugh et al., 2014; Powers et al., 2017; Protas et al., 2006), behavior (Hinaux et al., 2016; Jaggard et al., 2017; Kowalko et al., 2013; Yoshizawa et al., 2015), and metabolism (Aspiras et al., 2015; Moran et al., 2014; Riddle et al., 2018).

Here we describe the morphological and functional development of the *A. mexicanus* GI tract. We found that post-larval cavefish have evolved intestinal motility that favors more stomach-like churning activity. This results in slower transit of live food from the stomach to midgut which could provide superior digestion of prey found in the caves. We found that cavefish grow more quickly than surface when fed *ad libitum*, possibly due to increased nutrient consumption or absorption. We examined the number of enteric neurons and organization of smooth muscle in the GI tract and found no difference in enteric neuron density or smooth muscle appearance between the populations. Our study provides foundational work for investigating the genetic and developmental aspects of gut evolution, morphology, and function using *A. mexicanus* as a model.

Methods

Fish husbandry

Fish were bred in standalone tanks and spawning was induced by gradual increase in temperature over 2-3 days. Time of spawning was determined using available developmental staging table (Hinaux et al., 2011) and occurred between 9 p.m. and 6 a.m. Fertilized embryos were grown in 3ppt salt water in 1 L tanks at a density of 20 fish per tank. At 5 dpf, a mixture of live marine rotifers (Reed Mariculture) and Rgcomplete (APBreed™) were added to the tank at visible densities. Rotifers were cultured according to the Reed Mariculture protocol (Reed Mariculture, 2018). Fish were maintained on a 14:10 light: dark cycle at room temperature (25 °C). All experiments were carried out during the second half of the light cycle.

Immunohistochemistry and microscopy

Immunostaining was carried out as described (Heanue et al., 2016a). The following antibodies were used; 1:250 HuC/HuD Neuronal Protein Mouse Monoclonal Antibody (Invitrogen, A-21271), 1:500 Alexa Fluor® 647 Phalloidin (Thermo Fisher), 1:500 goat anti-mouse Cy3. Samples were mounted in prolong gold with DAPI and imaged with Zeiss LSM 780 confocal microscope with ZEN software. To quantify enteric neuron number, we took a z-series image of the gut in wholemount using a Keyence BZ-9000 fluorescence microscope. We used the z-series to generate a maximum intensity projection that was used with the Keyence BZ hybrid cell count software.

Intestinal motility measurement

Fish were fasted for 24–48 h to clear food from the gut. Fish were submerged in 140 ppm tricane solution and placed on their right side into the grooves of an agarose mold (Adaptive Science Tools, i34). Images were taken at 1 frame per second for 16 min using a Leica M165SC stereomicroscope and software. For the purpose of video analysis, a custom plugin was developed for the freely available ImageJ (Rueden et al., 2017) image-processing platform and is provided with this paper (Supplemental data 1). The plugin allows the construction of spatiotemporal maps of movement along the gut wall throughout the recording duration as reported by previous methods (Heanue et al., 2016a; Holmberg et al., 2007), and automatically calculates contraction parameters: contraction intervals, contraction frequency (waves^{-1}), travel distance (mm), and the average velocity of a contraction (mm s^{-1}) based on user input.

Fluorescent microsphere intestinal transit assay

To assess intestinal transit, we used a method developed in *Danio rerio* (Field et al., 2009). We fasted 11dpf fish for 24 h and then fed them 2 mg of tracer (food mixed with 2.0 μm polystyrene microspheres, Invitrogen) in 10 cm dishes with 20–30 fish per dish. Fish with food exclusively in the stomach region after 20 min were removed from food and tracer location was recorded over time by anesthetizing the fish briefly in 140 ppm tricane solution.

Rotifer intestinal transit assay

Rotifers were cultured in 1.5 L RO water with 4tsp Instant ocean aquarium sea salt and 1/8tsp astaxanthin powder (Kens Fish, Tauton MA). *A. mexicanus* were placed into individual wells of a 12-well plate at 9dpf and withheld from food for 48 h. Approximately 200 rotifers were then added to each well. After 5 min, the fish were anesthetized in 140 ppm tricane and viewed with a stereomicroscope to determine the number of rotifers consumed. The fish were then transferred to wells without food and the location of ingested food was determined at multiple time points by anesthetizing the fish briefly in 140 ppm tricane solution.

Gavage

Fish were anesthetized in 140 ppm tricane solution and a pulled microcapillary needle was used to deliver $<1 \mu\text{L}$ of Fluorescein isothiocyanate-dextran (Sigma-Aldrich, 46945) into the GI tract via the mouth (adapted from (Cocchiari and Rawls, 2013)).

Results

Development and morphology of the intestinal lumen

The epithelial lining of the vertebrate gut develops from a narrow sheet of endoderm. In zebrafish, the endoderm derives from a layer of flattened cells overlying the embryo yolk (Wallace and Pack, 2003). After a day, the cells meet at the embryo midline and form a bilayer. A few hours later they develop apicobasal polarity and separate at the apical surface to form the intestinal lumen (Ng et al., 2005).

This process appears similar in *A. mexicanus* (Fig. 1). At 18hpf, the urogenital opening is visible at the posterior end of the yolk extension (Fig. 1a, yellow arrow), and moves further away from the yolk as the embryo elongates (Fig. 1b, Supplemental movie 1). At the approximate time of hatching (36hpf), the primitive gut is visible as a ribbon of tissue ending in the future site of the anal pore (Fig. 1c) that continues to elongate as the larvae grow (Fig. 1d). At 3.5dpf there is a visible bilayer of cells that has established apicobasal polarity (Fig. 1h) and starts to open along the apical border at multiple points along the GI tract (Fig. 1e-e'). At 4.5dpf, the lumen continues to expand with the widest regions appearing anteriorly, just above the yolk (Fig. 1f); the more posterior regions start to show folding patterns (Fig. 1i). By 5.5dpf, the region of the GI tract ventral to the developing swim bladder is surrounded by clearly distinguishable muscle layers, and narrow epithelial ridges extended into the lumen (Fig. 1j). Just posterior to the yolk, the lumen is wider with a flat epithelial surface (Fig. 1k). In the midgut, the lumen has a more irregular surface as the epithelial cells begin to change shape (Fig. 1l). At this stage, the gut starts to show localized contractions (Supplemental movie 2) and the lumen fills with green bile (Supplemental Fig. 1). By 6.5dpf the larvae are actively feeding and the digestive system is fully functional.

Elaboration of the digestive system

The digestive organs are easily visualized at later stages of development (Fig. 2a-a'). The liver is visible at the ventral side of the fish and is connected to the gallbladder. The gallbladder empties bile into the lumen of the gut via the common bile duct. The pancreas is more difficult to visualize with light microscopy, but sits just beneath and slightly right of the swim bladder. A single insulin- and glucagon-producing islet is present at this stage at its anterior end (Fig. 2a', blue with orange circle, (Riddle et al., 2018)).

Distinct regions of the GI tract are apparent at this stage (Fig. 2b). Most anteriorly, the narrow esophagus connects the mouth with the stomach. The stomach lumen is wider than the other portions of the gut and the morphology of the epithelium appears as a series of mounds (Fig. 2b). Posterior to the swim bladder, the midgut epithelium is also characterized by a series of mounds (Fig. 2c-c') that appear to be elaborated from earlier stages where columnar cells are surrounded by cuboidal cells (Fig. 2d). The hindgut has a more irregular epithelium with wider and flatter folds (Fig. 2e, f, i). Posterior to the hindgut, the epithelium of the rectum is organized into longitudinal ridges that coalesce at the anal pore (Fig. 2b, b').

The mid- and hindgut are connected by a sphincter; a ring of muscle that surrounds the tube and forms a valve that can be opened and closed (Fig. 2e, f, Supplemental movie 3). The hindgut is functionally distinct from the other gut regions as evidenced by comparing

absorption. Fluorescently labeled dextran of high molecular weight is exclusively absorbed into the epithelium of the hindgut and excluded from the stomach, midgut, and rectum (Fig. 2g-i).

Growth and intestinal motility in surface fish and Pachón cavefish

We compared post-larval surface fish to cave-adapted fish from the Pachón cave. The Pachón cave is perched and less impacted by seasonal flooding than other caves. While the Pachón cave, thus, lacks periodic influx of nutrients from the outside, it contains resident aquatic crustaceans in addition to a bat population as potential sources of food (Espinasa et al., 2017). We monitored growth in post-larval surface and Pachón populations and found that Pachón cavefish are slightly bigger than surface fish two days after feeding begins, 7.5dpf (Fig. 3, average 4.79 Pachón versus 4.69 mm surface, $p = .17$, $n = 8,8$). The size difference is exaggerated as the fish develop to 11.5dpf (average 5.32 Pachón versus 4.98 mm surface, $p = .01$, $n = 8,8$) and 13.5dpf (average 5.73 Pachón versus 5.30 mm surface, $p = .10$, $n = 9,8$). We found that the overall external anatomy of the digestive system of the Pachón cavefish and surface fish appears similar at 8.5-12.5dpf (Fig. 3a,b, Fig. 4a,b). We hypothesized that intestinal physiology may contribute to the observed differences in growth.

We found that surface fish and Pachón cavefish exhibit differences in GI movement (motility). We took time-lapse recordings of the GI tract in lightly anesthetized fish at 8.5dpf and 12.5dpf and generated spatiotemporal maps to quantify the frequency of contractions, and direction and distance of waves created by the contractions (Fig. 4, Supplemental movie 4,5). We found that at both stages, contractions in the surface fish gut primarily generate peristaltic waves in the midgut that travel from anterior to posterior (antereograde, Fig. 4c,e). During a 16-min recording of 12.5dpf fish, 4 out of 6 Pachón cavefish exhibited peristaltic waves compared to 6 out of 6 surface fish. In fish that exhibited waves, the average wave number was lower in Pachón (average of 5.5 waves versus 9 waves). The peristaltic waves in Pachón also traveled a significantly shorter relative distance (Fig. 4e), mean of 28% versus 40% of gut in Pachón versus surface at 8.5dpf ($n = 6, 8$, $p = .03$, two-tailed t -test) and mean of 26% in Pachón versus surface at 12.5dpf ($n = 4, 6$, $p = .008$, two-tailed t test).

We observed additional contractions in the stomach region that produced waves traveling in both directions (antereograde ↔ retrograde), creating a churning motility (Fig. 4, Supplemental movie 5). The retrograde contractions are mostly absent in surface fish; during a 16-min recording, 5 out of 14 surface fish showed retrograde contractions compared to 15 out of 16 Pachón cavefish (combined 8.5dpf and 12.5dpf, $p = .04$ fishers exact test). In surface fish, the bi-directional waves travel a short distance (Fig. 4c). In comparison, the retrograde waves in the Pachón gut are more pronounced and the churning motility spans a larger portion of the gut (Fig. 4d), mean of 35% versus 20% of the gut in Pachón versus surface at 8.5dpf ($n = 9, 6$, $p = .002$, two-tailed t -test) and 39% versus 21% of the gut in Pachón versus surface at 12.5dpf ($n = 6, 4$, $p = .004$, two-tailed t -test). Both populations exhibited contractions near the anal pore at similar frequencies (average of 1 every 15 s in surface ($n = 6$) and 1 every 13 s in Pachón ($n = 6$) at 12.5dpf, Supplemental movie 4,5).

These contractions produce retrograde waves in the rectum that travel similarly short distances (mean of 26% of gut in surface and 31% of gut in Pachón at 12.5dpf).

Gastrointestinal transit time

To determine how differences in motility influence the movement of food through the gut, we measured digestive transit time using two different foods: powdered food mixed with fluorescent particles, and live prey (Figs. 5 and 6). To quantify differences in transit we designated 4 zones in the gut (Fig. 5a) and recorded the position of the food (zone number) at multiple time points after feeding (Fig. 5b). Zone 1 is directly under the swim bladder and represents the stomach, zone 2 is the transition from the stomach to the midgut, zone 3 is the midgut, and zone 4 includes the hindgut and rectum. We found that powdered food mixed with fluorescent microspheres passes through the Pachón gut more quickly (Fig. 5b). Five hours after eating, most surface fish have food in the midgut, while most Pachón have already passed the food (Fig. 5b, $p = .0005$, Kruskal-Wallis chi-squared). By 24 h, the food was eliminated from most of the fish from both populations.

Post-larval surface fish and Pachón cavefish both consume live food in the wild (Espinasa et al., 2017; Mitchell et al., 1977). We therefore tested the transit time of live rotifers. We fed rotifers a diet containing astaxanthin powder; a bright-red indigestible pigment produced from microalgae. This allowed us to easily visualize the rotifers outside and inside the gut (Fig. 6a,c). We withheld the fish from food for 48 h and then provided them with rotifers at a final density of approximately 200/mL. We found that after five minutes, most fish had a full stomach (Fig. 6c). We did not observe a significant difference in the total number of rotifers consumed between populations (Fig. 6b, surface mean 12.9 ($n = 47$), Pachón mean 12.3 ($n = 25$)).

We visualized rotifer transit over time and found that within 1.5 h, rotifers were no longer individually distinguishable (Fig. 6d). As the digested material progresses through the gut, it becomes darker and more condensed (Fig. 6d-f). The red astaxanthin powder was however clearly visible in the feces (Fig. 6h). In most fish, rotifers were digested and eliminated by 24 h (Fig. 6g). To compare transit between Pachón cavefish and surface fish, we recorded the position of digested food at multiple time points. We found that at the earliest time point (1.5 h), the digested material is significantly less advanced in Pachón (Fig. 6i, $p = .01$, Kruskal-Wallis chi-squared). At this time point, most Pachón have food in the stomach region (zone 1,2) while most surface fish have food in the midgut (zone 3). We did not find a significant difference at 4-5h, or 19-24 h ($p = .58, .08$, Kruskal-Wallis chi-squared).

Enteric neuron number and smooth muscle organization

We hypothesized that altered intestinal motility in cavefish may be linked to differences in the enteric nervous system or intestinal smooth muscle. We used a pan-neuronal antibody (HuD) to visualize the enteric neurons in whole larvae (Fig. 7a,d), and took a z-series of images through the gut to generate a 2-dimensional z-stack (Fig. 7b,e). We used the z-stack to compare the total number of enteric neurons in each segment of the gut between Pachón cavefish and surface fish at 10.5-11.5dpf (Fig. 7c,f). The average number of neurons in each region of the gut is greater in Pachón (Fig. 7g), adding up to a greater total number of

neurons (average 675 versus 610, $p = .13$). The number of neurons in the rectum is significantly greater in Pachón (Fig. 7g, average 80 versus 61, $p = .001$). However, each area of the gut is also greater in Pachón (Fig. 7h, average total area 27.8 versus 23.6 ($\mu\text{m}^2/10,000$), $p = .05$). The density of enteric neurons is therefore not significantly different in any region of the gut (Fig. 7i, average density of entire gut 101.1 vs. 108.1, $p = .44$). We found that Pachón are longer than surface fish at 11.5dpf (Fig. 3, mean 5.3 vs. 5.0 mm, $p = .01$) and there is a significant correlation between gut area, fish length, and neuron number (pearson's test, $p < .05$ for each comparison). In summary, Pachón grow more quickly than surface fish and are significantly larger at 11.5dpf with a proportionately larger gut and greater number of enteric neurons.

We analyzed the morphology of intestinal smooth muscle using wholemount immunostaining (Fig. 8). The muscle along the entire GI tract is composed of outer longitudinal and inner circumferential layers. We did not observe a notable difference in the organization of smooth muscle in the Pachón posterior stomach, compared to anterior and posterior midgut; the regions that show different contractile patterns (Fig. 8b). We also did not observe an obvious difference in any region when comparing the smooth muscle of Pachón cavefish to surface fish (Fig. 8a,b). In summary, we found that the early morphogenesis of the gut occurs similarly in surface and cave forms but that cavefish grow more quickly than surface fish at post-larval stages. Pachón cavefish exhibit primarily churning motility in the gut and have slower transit of live food. Our analysis did not reveal differences in the density of enteric neurons or organization of smooth muscle layers between surface fish and Pachón cavefish.

Discussion

We found that the *A. mexicanus* digestive tract develops similarly in surface and cave forms based on analysis of the transparent whole larvae as well as histological sections. Our findings are in line with previous studies showing that surface and Pachón embryogenesis and early post-larval development is synchronous (Hinaux et al., 2011). We found that the gut lumen starts from a bilayer of cuboidal cells that take on apicobasal polarity and begin to unzip at the apical borders at 3.5dpf. In both populations, the lumen is visible at 4.5dpf and the gut is functional at 5.5dpf. Numerous studies have focused on lumen morphogenesis in the Zebrafish (*Danio rerio*) (Alvers et al., 2014; Bagnat et al., 2007; Ng et al., 2005). The zebrafish intestinal lumen is formed at 3dpf and the digestive system is fully functional at 5dpf when feeding starts (Ng et al., 2005). Although *Astyanax* develops more slowly than *Danio* and feeding starts between 5.5dpf-6.5dpf (Hinaux et al., 2011), the process of lumen morphogenesis appears similar.

In contrast, later morphogenesis and differentiation results in a more complex GI tract in *A. mexicanus*. The post-larval zebrafish gut consists of an esophagus, intestinal bulb, intestine, and anal pore (Wallace et al., 2005). The “intestinal bulb” region in cavefish becomes the stomach in adult forms while the zebrafish remain stomachless as do all fish of the family Cyprinidae. *A. mexicanus* also has a morphologically and functionally distinct hindgut that is connected to the midgut via a sphincter. The midgut sphincter is similar to the ileocecal junction that separates the human small and large intestine (Pollard et al., 2012). The

terminal end of the *A. mexicanus* GI tract has longitudinal epithelial ridges and appears more muscular than other portions of the gut, also similar to the human rectum. Studying the *A. mexicanus* GI tract provides an opportunity, not available in zebrafish, to uncover principles of stomach and sphincter formation and function in a model where they can be easily visualized *in vivo*. Indeed, cavefish have reduced or absent pigment allowing easier visualization of all internal organ development.

Efficient digestion, absorption, and elimination serve to promote fish growth, which is under strong selection in the river and the cave. We found that Pachón grow more quickly than surface fish during postlarval development when fed *ad libitum* and housed at identical densities. In addition, Pachón accumulate fat earlier than surface fish during post-larval development (Xiong et al., 2018), consistent with accelerated nutrient accumulation and growth. These observations suggest that Pachón either absorb more nutrients from the food they eat, or are able to eat more frequently. To investigate these possibilities, we first measured gastrointestinal motility. We found that in cavefish, gut movement is characterized primarily by churning motility and in comparison, the surface fish gut exhibits primarily peristaltic waves. This difference in motility persists across post-larval stages, from 8.5dpf, when the fish are similar in size, to 12.5dpf when Pachón are greater in size, suggesting that the observed differences are not attributed to a delay in development. The intestinal motility patterns in surface fish are similar to those observed in zebrafish with the exception that *A. mexicanus* exhibit more pronounced retrograde contractions in the rectum (Holmberg et al., 2007).

To determine how motility influences the movement of food in the gut, we measured gastrointestinal transit time in post-larval fish. Interestingly, we observed that powdered food traveled more quickly through the gut of Pachón compared to surface fish. In the cave environment, adult fish consume mostly detritus, while post-larval fish consume crustaceans (Espinasa et al., 2017). It may be beneficial for post-larval fish to quickly eliminate dead material and prioritize consumption of prey with higher nutrient content. To investigate this possibility, we visualized the transit of live rotifers. We found that surface fish and Pachón cavefish consumed the same amount of rotifers over five minutes, in line with the findings of other studies focused on comparing ability to capture prey (Espinasa et al., 2014). The transit of rotifers into the midgut occurs more slowly in Pachón, consistent with the stomach churning motility patterns we observed. Churning motility in Pachón may have evolved to aid in digestion of hard-bodied crustaceans found in the cave.

Our results suggest that differences in motility and transit of food could contribute to energy assimilation and increased growth in Pachón. However, this does not exclude the possibility that the cavefish have other modifications to the GI tract such as differences in epithelial cell types and glands that alter enzyme sections or influence absorptive capacity. We found that the hindgut is the only site of absorption of large molecules in both surface fish and cavefish, but it is unclear which regions absorb glucose, fat, and protein. Analysis of cell type specification and gavage of other fluorescently-labeled nutrients may reveal important differences in differentiation and absorption that are not obvious by morphological or motility analysis alone.

To investigate a morphological basis for the differences in motility, we examined the enteric nervous system and intestinal smooth muscle. We did not observe a notable difference in the organization of smooth muscle, however we found that Pachón have a greater number of enteric neurons in each region of the gut at 11.5dpf. We found that the size of the gut was however also larger in Pachón at this stage and did not observe a difference in enteric neuron density. Our results suggest that enteric neuron density is not the driver for differences in motility. The differentiated enteric nervous system is a complex network of neuronal subtypes that interconnect, sense luminal contents and innervate effector tissue (Hao et al., 2016; Lasrado et al., 2017). The differences we observed in intestinal motility could be linked to altered enteric neuron connectivity, subtype specification, activity, or differences in the physical properties of the gut. The function of the enteric nervous system and its communication with the central nervous system has broad impacts on metabolism and activity that are only beginning to be appreciated (Mayer, 2011; Rao and Gershon, 2016). It is well known that cavefish have differences in appetite, activity, and metabolism. Investigating how the enteric nervous system has evolved in these fish therefore represents a promising direction for future research.

Enteric neurons and pigment cells in fish are derived from vagal neural crest cells (Shepherd and Eisen, 2011). In Zebrafish, vagal neural crest cells migrate in two chains alongside the gut starting at 32hpf and completely colonize the gut with enteric neurons by 72hpf. In *A. mexicanus*, neurons populate the gut by 5.5dpf when uncoordinated contractions start to appear. It is currently unclear if the migration of enteric neuron precursors occurs similarly in *A. mexicanus* compared to zebrafish. It is also not clear if this process differs between surface and cave forms, but interestingly there are known differences in pigment cell number and differentiation (Jeffery et al., 2016). Investigating neural crest specification in *A. mexicanus* may reveal important developmental events that give rise to cave-specific morphology and physiology.

Conclusions

Here we provide the first description of digestive system development and morphology in the cavefish *Astyanax mexicanus* and identify important differences between post-larval surface and cave forms. Future studies will focus on identifying the genetic and developmental basis of altered intestinal motility, taking advantage of the ability to create surface/cave hybrids for genetic mapping (Casane and Retaux, 2016). The availability of independently-evolved cave populations will allow us to also ask whether alterations to the GI tract by the same or different mechanisms is a common feature of cave-adapted *A. mexicanus*. Such studies may identify factors that influence regional differentiation of the gut, or enteric neuron subtype specification, connectivity, and function. It is unclear how intestinal anatomy and peristalsis in the adult forms influence the unique cavefish metabolism that promotes fat accumulation. This is an area ripe for discovery and could provide insight into the diseases that affect human GI function and metabolism, in addition to broadening our understanding of how animals thrive in environments with different sources of food.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Development of the *A. mexicanus* GI tract.

a, b, surface fish embryo 18 and 21 h post fertilization (hpf), yellow arrow points to the urogenital opening (end of gut), and future anal pore in images that follow (y: yolk). c, hatched larva at 1.5dpf. d, 2.5dpf; single ribbon of endoderm with no lumen. e, 3.5dpf; yellow box (e') highlights lumen formation. f, 4.5dpf; asterisks highlights expanding lumen. g, 5.5dpf; open arrows indicate approximate position (anterior to posterior) of cross sections shown in j, k, l. h, 3.5dpf cross section of the gut posterior to the yolk shows bilayer of cells with apicobasal polarity (max intensity projection of 5 μ m z-stack). i, 4.5dpf; cross section

of gut posterior to the yolk shows early lumen morphogenesis. j, ridged lumen morphology in the foregut region. k, wide and flat lumen in the region just posterior to the yolk. l, folded lumen in the more posterior gut. (scale bars: a-g 500 μm , h-l 20 μm).

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Fig. 2. Anatomy of the *A. mexicanus* digestive system.

a, Right lateral view of 12.5dpf *A. mexicanus* surface fish. b, overlay outlines swim bladder (sb, white), pancreas (p, orange circle indicates approximate location of single pancreatic islet), liver (red), gallbladder (gb, green), and GI tract lumen (yellow). b', Left lateral view of 12.5dpf surface fish gut. b' Overlay outlines esophagus (es; blue), stomach (green), midgut (yellow), hindgut (red), and rectum (re: orange). Arrow indicates the location of the sphincter valve connecting the mid and hindgut. (N, one of five previously undescribed ventral fin neuromasts). c-c'', transverse view of midgut epithelium at 11.5dpf shows mound morphology. d, cross section of midgut epithelium at 9.5dpf shows columnar cells adjacent to cuboidal cells (scale bar 50 μ m, asterisk in lumen). e, transverse view of hindgut sphincter, yellow arrow shows thick band of circumferential muscle. f, transverse view of hindgut sphincter showing valve opening (anterior is up, asterisks placed in lumen, scale bar 50 μ m). g, Surface fish gut (11.5dpf, outlined in yellow) directly after gavage of fluorescently-labeled dextran (dextran-FL) that is visible in the stomach (color image). h, Surface fish gut (12.5dpf) 24 h after gavage of dextran-FL shows signal exclusively in hindgut (greyscale image, sb; swim bladder, scale bar .5 mm). i, Confocal image of Pachón (11.5dpf) hindgut 24 h after gavage of dextran-FL (anterior is left, asterisks placed at the hindgut valve in overlay). Brightness and contrast adjusted to show detail in print version.

a Surface

b Pachón

c

Fig. 3. Surface and Pachón *A. mexicanus* exhibit differences in post-larval growth.

a, b image of similarly sized 12.5dpf surface fish and Pachón cavefish. c, length of post-larval fish of the indicated age. S (surface), P (Pachón), p-values from two-tailed t-test. For box plots: points represent individual fish, horizontal bars represent median, 25th, 50th, and 75th percentiles, and vertical bars represent 1.5 interquartile ranges.

Fig. 4. Surface and Pachón *A. mexicanus* display differences in intestinal motility.

a, b image of gut region in 8.5dpf surface and Pachón. Yellow line marks the gut lumen and region used to generate spatiotemporal maps (stms, sb; swim bladder). Stms of surface (c) and Pachón (d) gut motility (500 s, scale bar: 200 μ m, y-axis lines label gut regions as shown in Fig. 2b'). The surface fish gut (c) exhibits predominantly anterograde (oral to anal) waves (longer dotted arrows), and short retrograde $\leftrightarrow$ anterograde waves more anteriorly (shorter dotted arrows). The Pachón gut (d) exhibits waves that are predominantly retrograde (longer dotted arrow). The Pachón anterograde waves travel a shorter distance than in surface fish (shorter dotted arrow). Both populations exhibit retrograde contractions at anal pore at similar frequency (filled arrow). e, Anterograde wave distance as a percentage of total gut

length in 8.5dpf and 12.5dpf surface (S, n = 8,6) and Pachón (P, n = 6,4). f, Percentage of gut displaying churning motility in 8.5dpf and 12.5dpf surface (S, n = 6,4) and Pachón (P, n = 9,6). * $p < .05$, * * $p < .005$, students two-tailed t-test. For box plots: points represent individual fish, horizontal bars represent median, 25th, 50th, and 75th percentiles, and vertical bars represent 1.5 interquartile ranges.

Fig. 5. Pachón cavefish have more rapid intestinal transit of powdered food.

a, Image of 12.5dpf surface fish GI tract containing powdered food mixed with 2.0- μ m polystyrene microspheres. Zones are separated by dotted lines and arrow points to particle in zone 2. Zone 1 is directly under the swim bladder in the location of the stomach, zone 2 is the transition from the stomach into the midgut, zone 3 is the midgut, and zone 4 includes the hindgut and rectum. Fish with food in zone 1 were sorted into individual wells and the position of the food was determined at 2-3 h, 5 h, 22-24 h, and 48 h after sorting. b, Percentage of surface (n = 20) and Pachón (n = 37) with fluorescent particles in the

indicated position at the indicated time. Position of Pachón digestive material is significantly different from surface (more progressed) at 2-3 h and 5 h (p-value from Kruskal-Wallis chi-squared).

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Fig. 6. Pachón cavefish have slower intestinal transit of live food.

a, Image of rotifers with astaxanthin (red) in the GI tract (Scale bar .5 mm). b, number of rotifers consumed by surface fish (n = 47, mean = 12.9) and Pachón cavefish (n = 25, mean = 12.3) after 5 min. Median, 25th, 50th, and 75th percentiles are represented by horizontal bars and vertical bars represent 1.5 interquartile ranges. ns, $p > .05$ two-tailed students t-test. c, GI tract of 12.5dpf surface fish after 5 min of feeding, arrows point to individual rotifers. d, GI tract 1.5 h after eating; individual rotifers are no longer distinguishable and material is present in zone 1 and 2. e, GI tract with material in zone 2 and 3. f, GI tract with material in

zone 3 and 4. g, GI tract of surface fish shown in c after 24 h (scale bars: 0.5 mm). h, Fecal material. i, Percentage of surface fish and Pachón cavefish with digestive material progressed to the indicated zone (x-axis) at 1.5 h (n = 21,19), 4-5 h (n = 29, 28), and 19-24 h (n = 47,28). For digestive material that was in more than one zone, the most posterior zone was recorded. Position of Pachón digestive material is significantly different from surface (less progressed) at 1.5 h (Kruskal-Wallis chi-squared, p-value = 0.01).

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Fig. 7. Pachón cavefish have a greater number of enteric neurons but no difference in enteric neuron density compared to surface fish.

Surface fish (a) and Pachón cavefish (d) stained with pan-neuronal Hu antibody (10.5dpf, arrows show position of midgut sphincter, sb; swimbladder). Grey-scale z-projection of region of interest containing the entire GI tract (top) and image from hybrid cell count analysis from surface fish (b) and Pachón cavefish (e). Quantification of enteric neuron number in the indicated regions for surface fish (c, n = 8) and Pachón cavefish (f, n = 8). Average number of neurons (g), gut area (h), and neuron density (i) in 10.5-11.5dpf surface and Pachón (n = 8,8). p-values from two-tailed t-test *p < .05. Scale bar 500 μM . Brightness and contrast adjusted to show details in print version.

Fig. 8. Circumferential and longitudinal intestinal smooth muscle layers in surface fish and Pachón cavefish.

a, b Wholemount images along the length of the digestive tract of 11.5dpf fish stained with phalloidin:647 (anterior is left, ventral is up, black spots are pigment cells in surface fish, scale bar 50 μ M, brightness and contrast adjusted across entire image to show detail).