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The effects of protein and fiber content on gut structure and function in zebrafish (*Danio rerio*)

Samantha C. Leigh[1] · Bao-Quang Nguyen-Phuc[1] · Donovan P. German[1]

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Abstract Chemical reactor theory (CRT) suggests that the digestive tract functions as a chemical reactor for processing food. Presumably, gut structure and function should match diet to ensure adequate nutrient and energy uptake to maintain performance. Within CRT, dietary biochemical composition is the most important factor affecting gut structure and function in vertebrates. We fed *Danio rerio* (zebrafish) diets ranging from high- to moderate- to low-quality (i.e., ranging from high-protein, low-fiber to low-protein, high-fiber), and observed how gut length and surface area, as well as the activity levels of digestive enzymes (amylase, maltase, trypsin, aminopeptidase, and lipase) shifted in response to these dietary changes. Fish on the low-quality diet had the longest guts with the largest intestinal epithelial surface area and enterocyte cellular volumes. Fish on the moderate-quality diet had intermediate values of most of these parameters, and fish on the high-quality diet, the lowest. These data largely support CRT. Digestive enzyme activity levels were generally elevated in fish fed the moderate- and low-quality diets, but were highest in the fish fed the moderate-quality diet, suggesting that a diet with protein levels closest to that of the natural diet of *D. rerio* (they are omnivorous in nature) may elicit the best gut performance. However, fish fed the carnivore diet reached the largest terminal body size. Our results support CRT in terms of gut structure; however, our enzyme results do not necessarily agree with CRT and largely depend on which enzyme is discussed. In particular, the evidence for lipase activities being elevated in the fish fed the low-protein, high-fiber diet perhaps reflects a lipid-scavenging mechanism in fish consuming high-fiber foods rather than CRT.

Keywords Chemical reactor theory · Phenotypic flexibility · Digestive enzyme activity

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✉ Samantha C. Leigh
scleigh19@gmail.com

[1] Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697, USA

Introduction

Unlike carnivorous foods, herbivorous diets are relatively low in protein, and are encased in fibrous cell walls that make these foods more difficult to digest (Horn 1989; Choat and Clements 1998; Karasov and Martinez del Rio 2007). Thus, herbivorous vertebrates exhibit a range of feeding strategies and specialized digestive systems that allow them to subsist on these lower quality foods, including higher intake, longer and more voluminous digestive systems, elevated activity levels of carbohydrate-degrading enzymes, and more diverse enteric microbial communities that may aid in the digestive process (Horn 1989; Karasov and Martinez del Rio 2007; Clements et al. 2014; German et al. 2015; Sullam et al. 2015). When a shift to a high-fiber, low-protein diet occurs, over what time scales do changes in digestive strategies occur (Karasov and Douglas 2013)? In other words, what phenotypic changes to gut structure and function occur when an animal initially transitions to a high-fiber, low-protein diet?

We can begin to address this question by thinking of the digestive tract in terms of chemical reactor theory (CRT), which suggests that the gut serves as a chemical reactor for

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processing substrates (Penry and Jumars 1987). We can set up the following proportion to understand how digestive parameters interact and change to maintain optimal food processing:

$$\text{Digestive Efficiency} \propto \frac{\text{Enzyme Activities}}{\text{Substrate Concentration}} \propto \text{Time} \propto \frac{\text{Gut Size}}{\text{Digesta Transit Rate}}.$$

The "enzyme activities/substrate concentrations" part of this proportion suggests that an animal would need to have elevated enzyme activities for a high-concentration substrate if the animal is to achieve high digestibility for that substrate. Consistent with this, the "gut size/digesta transit rate" part of the equation suggests that as the rate of digesta transit increases, the size of the gut must also increase to maintain the same level of overall digestive efficiency. There is broad support for CRT in terms of gut size and function, including studies performed in ontogenetic (e.g., German et al. 2004; Moran et al. 2005; German and Horn 2006; Kim et al. 2014) and phylogenetic (e.g., Penry and Jumars 1987; Horn and Messer 1992; Batzli et al. 1994; Schondube et al. 2001; Horn et al. 2006; German et al. 2010a) contexts. Furthermore, several studies have examined plasticity of digestive enzyme activity levels in response to diet switching, with many finding support for CRT (Table 1, e.g., Reimer 1982; Sabat et al. 1998, 1999; Levey et al. 1999; Caviedes-Vidal et al. 2000; German et al. 2004; Hakim et al. 2006, 2007; Kohl et al. 2016). For instance, Reimer (1982) fed the Amazonian characin *Brycon melanopterus* diets varying in carbohydrate, lipid, and protein content, and the activity levels of amylase, lipase, and trypsin were elevated in fish fed the diets rich in carbohydrate, lipid, and protein, respectively, thus supporting CRT. However, Sabat et al. (1998, 1999) observed differing trends in South American birds and rodents. Granivorous birds, *Zonotrichia capensis* and *Diuca diuca*, were fed two different diets: a carbohydrate-free/high-protein and carbohydrate-containing/low-protein diet. Aminopeptidase activity was significantly elevated in the birds consuming the carbohydrate-free/high-protein diet, which aligns with CRT. However, maltase and sucrase activities were also elevated in the birds consuming the carbohydrate-free/high-protein diet, which does not align with CRT. The authors attributed these elevated activities to a non-specific increase in response to the elevated protein in the diet (Sabat et al. 1998). Enzymes are proteins, so a higher-protein diet likely equated to greater amino acid availability and hence, greater enzyme synthesis across the board. This increase in enzyme production in response to elevated amino acid availability is similar to what is seen in microbial systems (e.g., Allison and Vitousek 2005; Allison et al. 2014). Sabat et al. (1999) fed two rodent species [*Phyllotis darwini* (omnivore) and *Octodon degus* (herbivore)] different diets and found similar results to the birds in the omnivore, but not the herbivore, suggesting that species with different natural diets and evolutionary histories can show different responses to diet switching. This was corroborated by German et al. (2004), who showed that more generalist prickleback fishes showed large changes in enzymatic activities in response to a dietary change, whereas specialists did not. In other words, for the enzymatic piece of the CRT equation, the results are mixed, depending on the natural diet and evolutionary history of the organism under study. Moreover, carbohydrase (e.g., amylase) activities tend to match with ingested substrate quantity, but the same cannot always be said for proteases and lipases and their respective substrates, as was reported for prickleback fishes, birds, and other vertebrates (Sabat et al. 1998, 1999; German et al. 2004, 2010a, 2016; Kohl et al. 2011).

Just as flexibility of gut function (digestive enzyme activity) has been observed in response to variations of diet composition, flexibility of gut structure (gut length) has also been observed in numerous vertebrates exposed to diets varying in protein, lipid, and carbohydrate content (Table 1, Linder et al. 1995; Sabat et al. 1998; Caviedes-Vidal et al. 2000; Garcia-Carreno et al. 2002; Elliott and Bellwood 2003; German and Horn 2006; Horn et al. 2006; Olsson et al. 2007; Davis et al. 2013; Zandoná et al. 2015; Kohl et al. 2016; Król et al. 2016; Martin et al. 2016; Calduch-Giner et al. 2016). Caviedes-Vidal et al. (2000) noted differences in overall small intestine length in house sparrows (*Passer domesticus*) on differing diets. Similarly, German and Horn (2006) found decreased gut length and mass in a species of prickleback fish (*Xiphister atropurpureus*) consuming a high-protein diet in comparison to those consuming their natural omnivorous diet; however, specialist herbivores did not show similar flexibility in gut size.

In this study, we used zebrafish (*Danio rerio*) as a vertebrate model (e.g., Ulloa et al. 2011; Liu and Leach 2011; Jing and Zon 2011; Watts et al. 2012; Sadler et al. 2013; Cheng et al. 2016) to answer some basic questions regarding gut flexibility in the framework of CRT. *Danio rerio* are model organisms with extensive literature regarding their stomachless gut morphology and digestive tract development, especially in disease models, yet, how these fish respond to changes in dietary fiber and protein across their lives remains to be studied (e.g., Ulloa et al. 2011; Liu and Leach 2011; Jing et al. 2011; Watts et al. 2012; Wiwgeer et al. 2012; Sadler et al. 2013; Cheng et al. 2016; Brugman 2016). Specifically, we investigate the effects of protein and fiber nutritional content on the phenotypic flexibility of *D. rerio* gut structure and function. The *D. rerio* used in this

Table 1 Previous studies that have examined flexibility of gut structure and function in response to diets with varying levels of protein

Publication	Organism	Diet type	Body size	Gut length	Gut mass	Carbohydrases	Proteases	Lipases
Reimer (1982)	*Brycon melanopterus*	High protein	–	–	–	Low	High	No difference
Linder et al. (1995)	*Dicentrarchus labrax*	High protein	Increase	–	–	–	High	–
Kramer and Bryant (1995)[a]	Tropical freshwater fishes	High protein	–	Shortest	–	–	–	–
Sabat et al. (1998)	*Zonotrichia capensis* and *Diuca diuca*	High protein	–	–	–	High	High	–
Levey et al. (1999)	*Dendroica pinus*	High protein	–	–	–	Low	High	No difference
Caviedes–Vidal et al. (2000)	*Passer domesticus*	High protein	No difference	No difference	No difference	No difference	High	–
German et al. (2004)	Stichaeidae	High protein	–	–	–	High	High	No difference
Hakim et al. (2006)	Tilapias	High protein	Increase	–	–	High	High	–
German and Horn (2006)	*Xiphister atropurpureus*	High protein	–	Shortest	Low	–	–	–
Horn et al. (2006)[a]	*Atherinops affinis*	Low protein	–	–	–	–	–	–
Hernandez et al. (2007)	*Diplodus puntazzo*	Low protein	Decrease	–	–	–	–	–
Olsson et al. (2007)	*Perca fluviatilis*	Low protein	–	Longest	–	–	–	–
Santigosa et al. (2008)	*Oncorhynchus mykiss* and *Sparus aurata*	Low protein	Decrease	Longest	–	No difference	Low	–
Wagner et al. (2009)[a]	Cichlid fishes	Low protein	–	Longest	–	–	–	–
Perez–Jimenez et al. (2009)	*Dentex dentex*	Low protein	–	–	–	High	High	High
Lin and Luo (2011)	*Oreochromis niloticus* and *O. aureus*	Low protein	Decrease	–	–	No difference	Low	–
Berumen et al. (2011)[a]	Chaetodontidae	Low protein	–	Longest	–	–	–	–
Li et al. (2014)	*Lateolabrax japonicus*	Low protein	–	–	–	Low	Low	Low
Zandona et al. (2015)[a]	*Poecilia reticulata*	Low protein	–	Longest	–	–	–	–
Ribeiro et al. (2015)	*Argyrosomus regius*	Low protein	No difference	–	–	–	Low	–
Kohl et al. (2016)	*Liolaemus ruibali*	Low protein	–	Longest	–	–	–	–
Yaghoubi et al. (2016)	*Sparidentex hasta*	Low protein	Decrease	–	–	Low	Low	Low
Magalhaes et al. (2016)	*Diplodus sargus*	Low protein	No difference	–	–	High	Low	High
Gisbert et al. (2016)	*Mugil cephalus*	Low protein	No difference	–	–	High	No difference	High

[a]Denotes a non-experimental investigation. Additionally, note that "high-protein" and "low-protein" distinctions are relative to the other diets used in each individual study

study will serve as the "parental generation" in a longer-term experimental evolution study during which multiple generations are exposed to varying diets. Thus, we reared *D. rerio* (from a controlled genetic background to ensure that any flexibility observed is drawn from the same population) on three experimental diets: high-protein (from casein and soy bean meal)/low-fiber (hereafter "carnivore" diet), moderate-protein/moderate-fiber (hereafter "omnivore" diet), and low-protein/high-fiber (hereafter "herbivore" diet). We compared experimental outcomes of fishes on these diets with *D. rerio* consuming the "ancestral diet" (a commercial feed with 55% protein from fish meal and poultry sources, and different ingredients than the experimental diets), on which the fish have been reared for hundreds of generations at University of California, Irvine. This study is novel in that both phenotypic flexibility of gut structure (mass, length, intestinal epithelial surface area, and enterocyte volume), and gut function (activity levels of digestive enzymes: amylase, maltase, trypsin, aminopeptidase, lipase, β-glucosidase, and cellobiohydrolase) are analyzed in response to variation in dietary protein and fiber content in the context of CRT. The aforementioned enzymes were chosen for our analyses because they cover the degradation of our two nutritional components of interest (fiber and protein) as well as lipids and carbohydrates, which were kept constant among our experimental diets. Additionally, these enzymes are commonly used in studies evaluating digestive physiology, and therefore, we can make informed predictions about how dietary changes will impact their activity levels. We hypothesize that diet composition will correlate with phenotypic changes to gut structure and function. Based on this hypothesis, we made a series of predictions about how gut size, gut structure, digestive enzyme activity levels, and terminal body size will vary among fish reared on the different diets (Table 2). Given the conflicting results of some of the studies discussed previously, these predictions are based solely on nutritional concentrations of the diets, as suggested by CRT. While we recognize that terminal body size is not included in the CRT model, it is worth noting that body size can be an indicator of the overall health status of vertebrates. This generally leads to overall improved performance in energy-heavy demands such as gonad maturation, reproductive success, tissue maintenance, migration, further energy uptake, etc., (e.g., Garcia-Correno et al. 2002; Gonzalez 2012; Karasov and Douglas 2013).

Materials and methods

Fish and feeding experiments

Two hundred and forty *D. rerio* larvae were obtained from a brood stock of "wild type" *D. rerio* maintained at University of California, Irvine. At 15 days post hatch (DPH), the fish were divided into four diet categories (60 fish per diet): ancestral (which is the diet they had been consuming in captivity for > 100 generations), "carnivore," "omnivore," and "herbivore" (Table 3). At this stage the fish were fed a mixture of rotifers and their respective diets. By 20 DPH, the fish were only fed their respective diet, and by 50 DPH, fish were transferred to the same re-circulating system of 75.6-L aquaria (30 fish per aquarium, two tanks per diet type) connected to common filtration, including a sump, biological, particulate, activated carbon, and UV filtration. Each tank had the same lighting conditions, and because the water in the system was re-circulating through a shared sump, all fish experienced the exact same conditions (except for diet) regardless of tank. Furthermore,

Table 2 Predictions for how body size, gut structure, and enzyme activities of *D. rerio* will be impacted by the ancestral, carnivore, omnivore, and herbivore diets

Characteristics	Ancestral	Carnivore	Omnivore	Herbivore
Terminal body size				
Body mass	Moderate/largest	Largest	Moderate	Smallest
Gut structure				
Relative intestinal length	Short/moderate	Short	Moderate	Long
Digestive somatic index	Smallest/moderate	Smallest	Moderate	Largest
Epithelial surface area	Least/moderate	Least	Moderate	Largest
Enterocyte volume	Least/moderate	Least	Moderate	Largest
Enzyme activities				
Amylase	Moderate	Moderate	Moderate	Moderate
Maltase	Moderate	Moderate	Moderate	Moderate
Trypsin	Moderate/High	High	Moderate	Low
Aminopeptidase	Moderate/High	High	Moderate	Low
Lipase	Moderate	Moderate	Moderate	Moderate
Cellobiohydrolase	Low/moderate	Low	Moderate	High
β-Glucosidase	Low/moderate	Low	Moderate	High

Table 3 Percent, on a mass basis, of nutrients in the four diets fed to *Danio rerio* in the laboratory

Dietary component	Ancestral diet[a]	Carnivore diet	Omnivore diet	Herbivore diet
Protein (%)	55	40.51 ± 4.05	31.88 ± 3.19	8.46 ± 0.85
Lipid (%)	15	12.04 ± 1.20	11.80 ± 1.18	11.75 ± 1.18
Carbohydrate (%)	7.5	42.03 ± 4.20	52.24 ± 5.22	76.26 ± 7.63
Cellulose (%)	1.50	15.00	30.00	60.00
Ash (%)	9.71 ± 0.10	3.18 ± 0.07	4.31 ± 0.07	2.18 ± 0.06
Organic matter (%)	90.29 ± 0.10	96.82 ± 0.10	95.69 ± 0.07	97.82 ± 0.06
Energy (kJ g^{-1})		16.37	16.51	16.59

Values are mean ± SEM ($n = 3$, except energy, which was calculated based upon nutrient proportions). Cellulose value is the percentage of the total mass of each diet composed of cellulose, and thus, these values do not have errors associated with them, and they were not compared statistically. Organic matter and ash were determined for each diet, including the ancestral diet

[a]We did not perform proximate analysis on the ancestral diet because it is a commercial feed (Ziegler Brothers Adult Zebrafish Complete Diet) with known nutrient concentrations. Thus, no errors are reported

we found the fish performed better and grew faster when housed in groups as opposed to individually; indeed, housing conditions (individual vs. group) affect experimental results with *D. rerio* (Parker et al. 2012). This design precluded us from measuring digestibility of the different diets in individual fish. The system contained deionized water supplemented with appropriate salts, and fish were under a 12 L:12 D light cycle. The water temperature was maintained at 23 °C with a submersible heater for the duration of the experiment and the temperature and chemical conditions (pH, ammonia concentrations) of the tank system was monitored daily to confirm that they did not vary during the experimental period. The tanks were scrubbed, debris and feces siphoned out, and 20% of the water changed every 3 days.

The ancestral diet was Adult Zebrafish Complete Diet (Zeigler Brothers, Gardners, PA, USA), which was high in animal by-product protein sources and low in fiber. The variable protein and fiber diets created in the laboratory (carnivore, omnivore, and herbivore) were composed of varying concentrations of protein sources (casein and soybean meal), carbohydrates (wheat flour, corn starch, rice bran, and cellulose), lipids (corn oil, menhaden oil, and cod liver oil), vitamins, minerals, and methyl cellulose as a binder (Table 4). The ingredients were mixed with water to make a paste, then pressed through a pasta maker (Newsome et al. 2011), dried at 60 °C, and ground back down to a particle size (~ 1 mm) suitable for zebrafish. The variable diets were designed to be nearly isocaloric, but vary mostly in the protein: fiber ratio. The fish were fed twice daily to satiation. Once they reached adulthood (> 5 months), 26 individuals fed the ancestral diet and 20 individuals from each of the variable protein:fiber diets were collected from the separate tanks haphazardly after a feeding event and were used for analyses. Any remaining fish that were not used for analyses were used as mates to generate lines of zebrafish reared on the different diets to be used for future studies.

Table 4 Experimental diets fed to *Danio rerio* in this study

Ingredient	Percent (by mass) of each ingredient in each diet		
	Carnivore	Omnivore	Herbivore
Casein	27.5	20.0	5.0
Soybean meal	27.5	20.0	5.0
Wheat flour	6.0	6.0	6.0
Corn starch	5.5	5.5	5.5
Rice bran	5.0	5.0	5.0
Corn oil	3.3	3.3	3.3
Menhaden oil	3.3	3.3	3.3
Cod liver oil	3.4	3.4	3.4
Cellulose	15.0	30.0	60.0
Methyl cellulose	1.5	1.5	1.5
Vitamin premix	1.0	1.0	1.0
Vitamin C	0.4	0.4	0.4
Mineral premix	0.6	0.6	0.6

Individual fish were euthanized in buffered water containing 1 g L^{-1} tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA), measured [standard length (SL) ± 1 mm], weighed [body mass (BM) ± 0.5 g], and dissected on a chilled (~ 4 °C) cutting board. Whole GI tracts were removed by cutting at the esophagus and at the anus and processed in a manner appropriate for specific analyses (see below). For each fish, the whole GI tract was weighed, and the intestine length was measured [intestine length (IL) ± 1 mm]. Relative intestine length ($RIL = IL \times SL^{-1}$) and digestive somatic index ($DSI = \text{intestine mass} \times \text{body mass}^{-1}$) were determined. Due to the fragility and small size of the fish at the start of the experiment, we did not measure initial body size and, therefore, could not calculate growth rate. However, terminal fish size at the conclusion of the experiment was recorded and compared amongst the fish on the different diets.

Dietary composition

The proportions of nutrients in the diets fed to *D. rerio* in this study are presented in Table 4. Proximate analyses were performed following the methods of the Association of Official Analytical Chemists (AOAC International 2006). Total fat was determined by acid hydrolysis followed by extraction in petroleum ether, and total protein was determined by Kjeldahl extraction. Ash was determined by drying the diets at 105 °C (dry matter), and then combusting them at 550 °C for 3 h. The remaining content was ash (the proportion that combusted was organic matter, OM). Soluble carbohydrate was calculated as the nitrogen-free extract, or the proportion of the diet that was not analytically determined as moisture, protein, fat, crude fiber, or ash.

Histological analyses

Upon removal from the body, the digestive tracts of six individuals representing each diet were gently uncoiled and divided into three equal sections: proximal, mid, and distal intestine. Three 1-mm sections were excised from each of the proximal, mid, and distal intestine regions (German 2009a) and were placed in their own individual vials containing fresh Trump's fixative [4% formaldehyde, 1% glutaraldehyde, in 10 mM sodium phosphate (monobasic) and 6.75 mM sodium hydroxide; (McDowell and Trump 1976), pH 7.5]. These tissues were then allowed to fix overnight (12 h) at 4 °C. Following fixation, the tissues were removed from the fixative and rinsed in 0.1 M phosphate buffered saline (PBS), pH 7.5, for 3 × 20 min, and a final rinse overnight at 4 °C. Following rinsing in PBS, the tissues were rinsed for 40 min in running DI water, and prepared following German (2009a). Intestinal tissues embedded in paraffin wax were serially sectioned at 7 µm, stained in hematoxylin and eosin (Presnell and Schreibman 1997), and photographed at 40×, 60×, and 120× with a Cannon EOS Rebel T6i digital camera attached to a Zeiss Axioskop2 plus light microscope. Images ($n = 2$ per intestinal region, per individual fish; 36 images per diet) were used to quantify the intestinal surface area of the fish on the different diets. The circumference of the intestinal sections [IC (mm)] was measured along the serosa using Image J analytical software (Abrámoff et al. 2004). We then used the same software to measure the length of the mucosal lining of the intestine (ML), and calculated the epithelial surface magnification (ESM) as the ratio of ML to IC ($ESM = ML \cdot IC^{-1}$; German 2009a; Hall and Bellwood 1995). ESM allows one to observe how much the mucosal folds increase the inner surface area of the intestine. The intestinal epithelial surface area of each region of the intestine was calculated as IL/3 × regional IC × ESM (Frierson and Foltz 1992). Because we have defined the proximal, mid, and distal intestine as equal length sections (German 2009a), the length of each intestinal region was estimated as IL/3. The sum of these surface areas provided an estimate of total intestinal epithelial surface area for the entire intestine of fish on each diet. Enterocyte number per intestinal fold was counted for three separate folds from proximal intestine sections of each individual fish. The height (H, µm) and width (W, µm) of ten proximal intestine enterocytes were measured from individual sections of each individual fish and used to calculate cell volume, using the equation for a cylinder where volume = $(0.5\ W)^2 \pi H$ (Day et al. 2014).

Tissue preparation for digestive enzyme analyses

For fishes designated for digestive enzyme analyses (ancestral diet $n = 20$, 10 males and 10 females; all other diets $n = 14$, seven males and seven females), the guts were dissected out, placed on a sterilized, chilled (~4 °C) cutting board, and uncoiled. Following length and mass measurements, each entire intestine was placed in a separate sterile centrifuge vial and frozen in liquid nitrogen. All of the samples were then stored at −80 °C until prepared for analysis (within 1 month). Intestinal homogenates were performed as described by German and Bittong (2009).

Assays of digestive enzyme activity

All assays were carried out at 25 °C in duplicate or triplicate using a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). All assay protocols generally followed methods detailed in German and Bittong (2009), unless otherwise noted. All pH values listed for buffers were measured at room temperature (22 °C), and all reagents were purchased from Sigma–Aldrich Chemical (St. Louis). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from the zebrafish. Each enzyme activity was measured in each individual fish, and blanks consisting of substrate only and homogenate only (in buffer) were conducted simultaneously to account for endogenous substrate and/or product in the tissue homogenates and substrate solutions.

α-Amylase activity was measured using 1% potato starch dissolved in 25 mM Tris–HCl containing 1 mM $CaCl_2$. Previous work had shown that low concentrations of Tris are suitable for the measurement of amylase and maltase (German and Bittong 2009). The α-amylase activity was determined from a glucose standard curve and expressed in U (µmol glucose liberated per minute) per gram wet weight of gut tissue.

Maltase activities were measured following Dahlqvist (1968), as described by German and Bittong (2009). We used 112 mM maltose dissolved in 200 mM phosphate buffer, pH 7.5. The maltase activity was determined from

a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

Trypsin activity was assayed using a modified version of the method designed by Erlanger et al. (1961). The substrate, 2 mM Nα-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA), was dissolved in 100 mM Tris–HCl buffer (pH 7.5). Trypsin activity was determined with a *p*-nitroaniline standard curve, and expressed in U (μmol *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase activity was measured using 2.04 mM L-alanine-*p*-nitroanilide HCl dissolved in 200 mM sodium phosphate buffer (pH 7.5). Aminopeptidase activity was determined with a *p*-nitroaniline standard curve, and activity was expressed in U (μmol *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Lipase (non-specific bile-salt activated) activity was assayed using 0.55 mM *p*-nitrophenyl myristate (in ethanol) in the presence of 5.2 mM sodium cholate dissolved in 25 mM Tris–HCl (pH 7.5). Lipase activity was determined with a *p*-nitrophenol standard curve, and expressed in U (μmol *p*-nitrophenol liberated per minute) per gram wet weight of gut tissue.

Cellobiohydrolase and β-glucosidase activities were measured following German et al. (2011), but activity of neither enzyme was detected, so results are not reported.

Statistical analyses

Appropriate sample sizes were determined via power analysis based on previous studies. A sample size of 8 or greater was deemed appropriate for enzyme assays (German et al. 2004; German and Bittong 2009) and a sample size of 5 or greater was deemed appropriate for histological analyses (German et al. 2010b). Comparisons of intestine length and mass were made among fishes on the different diets with ANCOVA (with body length or mass, respectively, as covariates), followed by a Tukey's HSD with a family error rate of $P = 0.05$. Homogeneity of slopes was confirmed by the lack of significance of the diet by body size interaction term. Comparisons of RIL, DSI, intestinal epithelial surface area, ESM, and intestinal enterocyte volume were similarly made among fishes on the different diets with ANCOVA (with body mass as a covariate) as done by German and Horn (2006) and German et al. (2014). Body mass and the activity levels of each enzyme were compared among fish on the different diets with ANOVA, also followed by a Tukey's HSD. Prior to all significance tests, a Levene's test for equal variance was performed to ensure the appropriateness of the data for parametric analyses. If the data were not normal, they were log transformed, and normality confirmed prior to analysis. All tests were run using SPSS (24.0) statistical software. Additionally, a principal components analysis (PCA) was run using Rstudio software (version 1.0.136) on six dependent variables: gut length (RIL), amylase, maltase, trypsin, aminopeptidase, and lipase. These six variables were chosen to represent both function and structure of the gut. RIL is used as the only metric of gut structure in the PCA because the other metrics (DSI, ESA, and enterocyte volume) exhibit results comparable to RIL (the vectors overlap). The first two principal components were plotted and the 95% confidence ellipses were plotted around the four dietary groups (ancestral, carnivore, omnivore, and herbivore). An analysis of similarities test (ANOSIM) was also performed on the PCA variables in Rstudio.

Results

The fish consuming the carnivorous diet were significantly heavier than the fish on the other diets (ANOVA diet: $F_{3,61} = 9.432$, $P < 0.001$), which did not differ from one another (Fig. 1). The fish reared on the carnivorous diet were also longer than the other fish (see Supplemental Table S1 in the online version of this manuscript). The fish consuming the herbivorous diet had the longest intestines among the four tested diets, and the fish consuming the other diets did not differ from one another (Fig. 1; ANCOVA diet: $F_{3,61} = 10.07$, $P < 0.001$; body mass: $F_{1,57} = 11.43$, $P = 0.001$). In terms of gut mass, the fish consuming the herbivore and omnivore diets had significantly lighter guts than fish consuming the ancestral and carnivore diets (Fig. 1; ANCOVA Diet: $F_{3,61} = 4.69$, $P = 0.005$; body mass: $F_{1,57} = 2.86$, $P = 0.096$). Similar results were observed for RIL and DSI (see Supplemental Table S1). The fish consuming the herbivorous diet had the largest ESM in their proximal intestines, followed by fish consuming the omnivorous and ancestral diets, which did not differ from one another, and the fish on the carnivorous diet had the smallest ESM (Figs. 2, 3.; ANCOVA Diet: $F_{3,23} = 14.307$, $P < 0.001$; body mass: $F_{1,19} = 0.098$, $P = 0.758$). Similar results were observed for the mid intestine (ANCOVA Diet: $F_{3,23} = 12.169$, $P < 0.001$; body mass: $F_{1,19} = 0.1198$, $P = 0.735$) and distal intestine (ANCOVA Diet: $F_{3,23} = 6.346$, $P < 0.001$; body mass: $F_{1,19} = 0.743$, $P = 0.402$). The herbivorous diet fish had significantly larger total intestinal epithelial surface area than fish on the other diets, followed by fish fed the ancestral diet and omnivorous diet, which did not differ from one another (Fig. 3; ANCOVA Diet: $F_{3,23} = 15.256$, $P < 0.001$; body mass: $F_{1,19} = 0.146$, $P = 0.707$). The carnivorous diet fish had significantly lower total intestinal epithelial surface areas than fish on the other diets (Fig. 3). In terms of average enterocyte number per proximal intestinal fold, there were no significant differences among fish fed the different diets (ancestral: 93.2 ± 8.7, carnivorous: 94.6 ± 8.2, omnivorous: 95.9 ± 10.2, and herbivorous: 95.3 ± 8.3; ANOVA $F_{3,24} = 0.314$, $P > 0.5$). The herbivorous

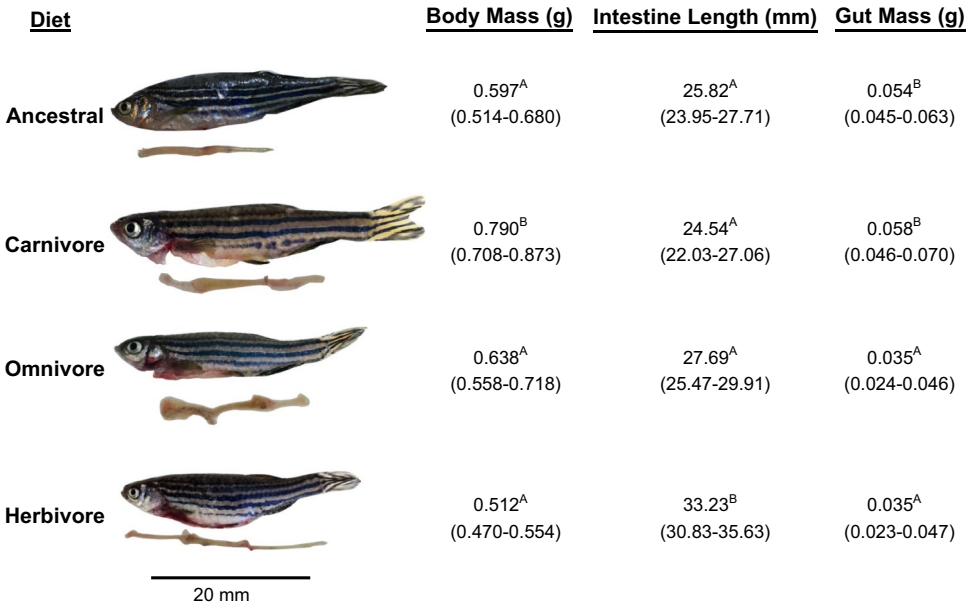

Fig. 1 Actual size images of *D. rerio* with their intestines, showing variation in intestinal size as a function of diet. Values (mean, with 95% confidence interval in parentheses below each mean) for final body mass, intestine length, and gut mass are also shown. The fish fed the carnivorous diet were heavier than the fish on the other diets (ANOVA Diet: $F_{3,61}$ = 9.43, $P<0.001$). The fish fed the herbivorous diet had larger intestines than fish on the other diets (ANCOVA Diet: $F_{3,62}$ = 10.07, $P<0.001$; body mass: $F_{1,57}$ = 11.43, $P=0.001$), whereas the fish consuming the ancestral and carnivorous diets had heavier guts than those on the omnivorous or herbivorous diets (ANCOVA Diet: $F_{3,62}$ = 4.69, $P=0.005$; body mass: $F_{1,57}$ = 2.86, $P=0.096$). Post hoc tests were Tukey's HSD with a family error rate of $P=0.05$. Values that share a superscript letter for a particular measurement are not significantly different

diet fish had significantly larger proximal intestine enterocyte volumes than fish on the other diets, which did not differ from each other (Fig. 3; ANCOVA Diet: $F_{3,23}$ = 19.533, $P<0.001$; body mass: $F_{1,19}$ = 0.279, $P=0.604$). Thus, the fish consuming the herbivorous diet had longer guts, larger mucosal area in each gut region, larger total intestinal epithelial surface areas, and larger enterocyte volumes than fish on the other diets (Figs. 2, 3).

The fish consuming the omnivorous and herbivorous diets had the highest amylase activities, although the fish consuming the herbivore diet did not possess significantly higher amylase activities than fish on the ancestral diet (Fig. 4; ANOVA $F_{3,61}$ = 12.997, $P<0.001$). The fish on the carnivorous diet had the lowest amylase activities, although they were not significantly lower than the fish on the ancestral diet (Fig. 4). For maltase, the fish on the omnivorous diet had significantly higher activities than the fish on the other diets, which did not differ from one another (Fig. 4; ANOVA $F_{3,61}$ = 29.900, $P<0.001$). Fish on each of the diets possessed significantly different trypsin activities in comparison to the other diets (omnivorous diet was the highest), with the exception of the ancestral and herbivorous diets, which did not differ (Fig. 4; ANOVA $F_{3,61}$ = 42.743, $P<0.001$). For aminopeptidase, the ancestral diet fish possessed significantly lower activities than fish on the omnivorous and herbivorous diets, but not lower than those on the carnivorous diets (Fig. 4; ANOVA $F_{3,61}$ = 15.517, $P<0.001$). In turn, the fish consuming the carnivorous diet were not different from those consuming the herbivorous diet (Fig. 4). The herbivorous diet fish had significantly elevated lipase activities in comparison to fish on the other diets (Fig. 4; ANOVA $F_{3,61}$ = 15.130, $P<0.001$), which did not differ from one another with the exception of the omnivorous diet fish having greater lipase than the carnivorous diet fish. Cellobiohydrolase and β-glucosidase activities were measured, but activity of neither enzyme was detected, so results are not reported.

To investigate the relationships between gut function and diet type, data were also analyzed with a principal components analysis (Fig. 5; PCA). Data for the first two principal components (PC) explained 50.5% (PC1) and 20.8% (PC2) of the variation in the data (totaling 71.3% of all variation when combined). This shows a clear distinction of the herbivorous diet fish from the other dietary groups with the herbivores having longer gut length and higher lipase levels. This also shows that the omnivorous diet fish exhibited higher levels of trypsin, maltase, aminopeptidase, and amylase when compared to the other diet types. An analysis of similarities (ANOSIM) test on the PCA variables revealed that all dietary groups are significantly different from each other ($P<0.001$).

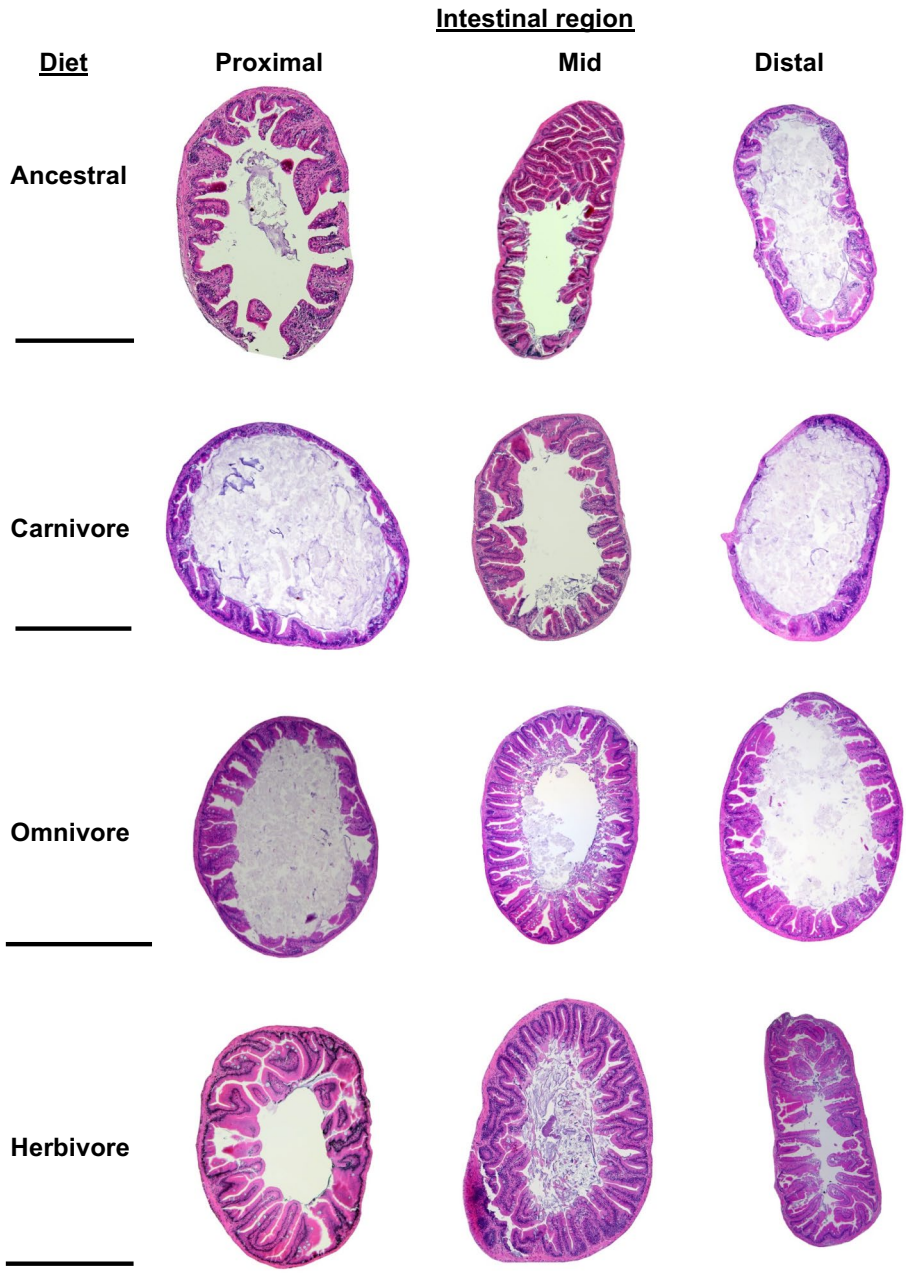

Fig. 2 Histological cross-sections of proximal, mid, and distal intestinal tissue of *D. rerio* consuming different diets. Tissues were stained with hematoxylin and eosin. Scale bars are 500 µm for each row of images

Discussion

The data gathered in this study support the hypothesis that zebrafish can adjust their gut structure and function in response to diets of varying biochemical composition, but these plastic adjustments are not necessarily in accordance with CRT. The predictions made about each characteristic from Table 2 will be discussed in detail below.

Gut structure

As predicted, fish consuming the herbivorous diet had the longest guts with the most intestinal epithelial surface area and largest enterocyte volume. However, the fact that these fish also had some of the smallest digestive somatic indices indicates that these individuals are exhibiting digestive flexibility by lengthening their guts without adding additional gut tissue (although the omnivore diet fish had the smallest DSI). Similar lengthening of the gut without an increase in mass was observed in herbivorous minnows in the genus *Campostoma* in comparison to carnivores in the genus *Nocomis* (German et al. 2010a). Fishes possess a transitional epithelium, which largely changes through cellular hypertrophy as opposed to increased cellular turnover or cell number (i.e., hyperplasia; Starck 2005). Indeed, the fish on the different diets did not have different enterocyte counts

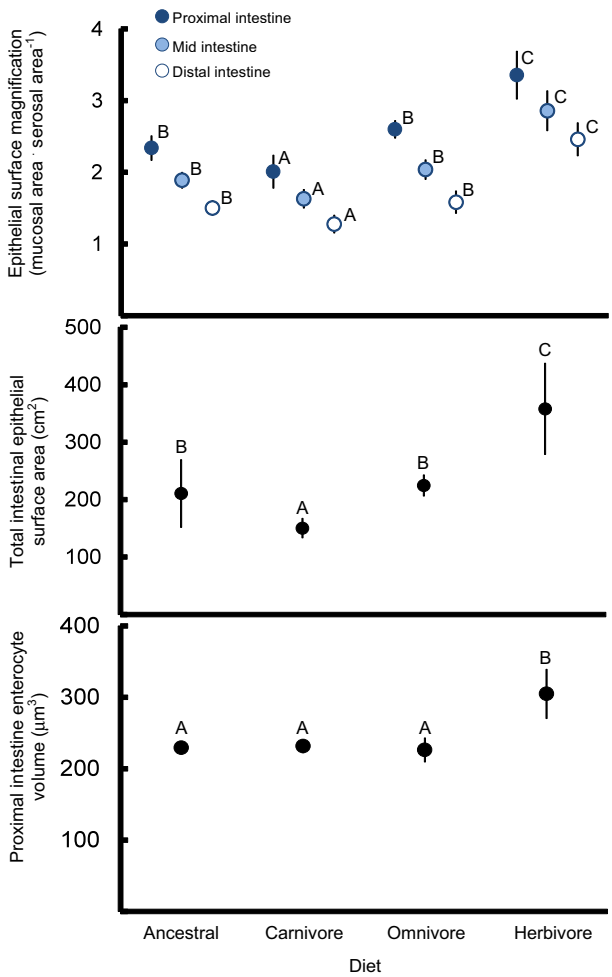

Fig. 3 Epithelial surface magnification of three intestinal regions (top), total intestinal epithelial surface area (middle), and proximal intestine enterocyte volume (bottom) in *D. rerio* consuming different diets. Values are means and error bars represent standard deviation. Inter-diet comparisons of epithelial surface magnification (top) in each gut region were made with ANCOVA (using body mass as a covariate) followed by a Tukey's HSD with a family error rate of $P=0.05$. Symbols for a specific gut region sharing a letter are not significantly different among the feeding groups. Intra-feeding group comparisons of epithelial surface magnification among gut regions were made with ANOVA followed by a Tukey's HSD with a family error rate of $P=0.05$. All intra-feeding group comparisons among gut regions were highly significant ($P<0.001$; not symbolized on graph). Inter-diet comparisons of total intestinal epithelial surface area (middle) and proximal intestine enterocyte volume (bottom) were made with ANCOVA (using body mass as a covariate) followed by a Tukey's HSD with a family error rate of $P=0.05$. Symbols not sharing a letter are significantly different

per proximal intestinal fold, but fish fed the herbivore diet had the largest proximal intestine enterocyte volumes, fitting the hypertrophy model that it is more energetically costly to produce a higher number of cells than it is to enlarge existing cells (Starck 2005). In fishes without stomachs, pyloric caeca, or elaborations of the hindgut, which can slow digesta transit, a lengthening of the intestine accommodates higher intake and maintains some minimum transit time of food through the alimentary canal, thereby increasing absorptive surface area and maintaining efficient digestion (German and Horn 2006; Horn et al. 2006; Wagner et al. 2009; Berumen et al. 2011; Zandona et al. 2015; German et al. 2015; Kohl et al. 2016). A longer gut means that more digesta can be processed per unit time, which is consistent with CRT predictions for high-intake.

Our results also fit well within the rate vs. yield continuum of Sibly (1981) and elaborated upon by German et al. (2015). Based on intake, fish fall along a continuum ranging from "rate-maximizers" with high-intake, rapid gut transit, long, active digestive systems, and lower overall digestibility, to "yield-maximizers" with lower intake, slower gut transit, shorter, less active digestive systems, with higher overall digestibility. Intake is largely determined by diet quality (here, we are defining this as protein quantity, since protein quality did not differ amongst the experimental diets), with lower quality foods requiring higher intake (Karasov and Douglas 2013; German et al. 2015). Thus, rate-maximizers have lower digestibility because of rapid gut transit, despite longer guts and relatively elevated digestive enzyme activities; the opposite is true for yield-maximizers with lower intake of high-quality food (German et al. 2015). Some herbivorous fishes, especially herbivorous cyprinids, can be described as rate-maximizers (which continually graze on low-quality foods), and thus having a longer gut would be ideal to process a continuous stream of digesta (Sibly 1981; German 2009b; German et al. 2010a; Karasov and Douglas 2013). *Danio rerio* are stomachless, possessing a straight, relatively short gut (Ulloa et al. 2011) without any elaborations (e.g., hindgut chambers), and therefore, are likely to be rate-maximizers (German 2011; German et al. 2015), similar to other cyprinid fishes (e.g., German et al. 2010a). Thus, lengthening of the gut on an herbivorous diet is a logical solution in response to the need to have increased intake of a relatively low-quality food to meet metabolic demands (Sibly 1981; Horn et al. 2006). We did not measure intake by individual fish on the different diets, but did anecdotally notice that fish fed the omnivore and herbivore diets spent more time foraging than the fish fed the carnivore diet. Thus, we assume that intake varied with diet, in the order herbivore > omnivore > carnivore, and our data on gut size and surface area support this supposition. To meet their metabolic demands on the low-quality diet, fish fed the herbivore diet needed to increase their likelihood of nutrient uptake by increasing absorptive surface area. Additionally, since the guts of the herbivorous diet fish exhibited overall lengthening, it makes sense that mucosal surface area would increase as well. However, this increase in mucosal surface area was

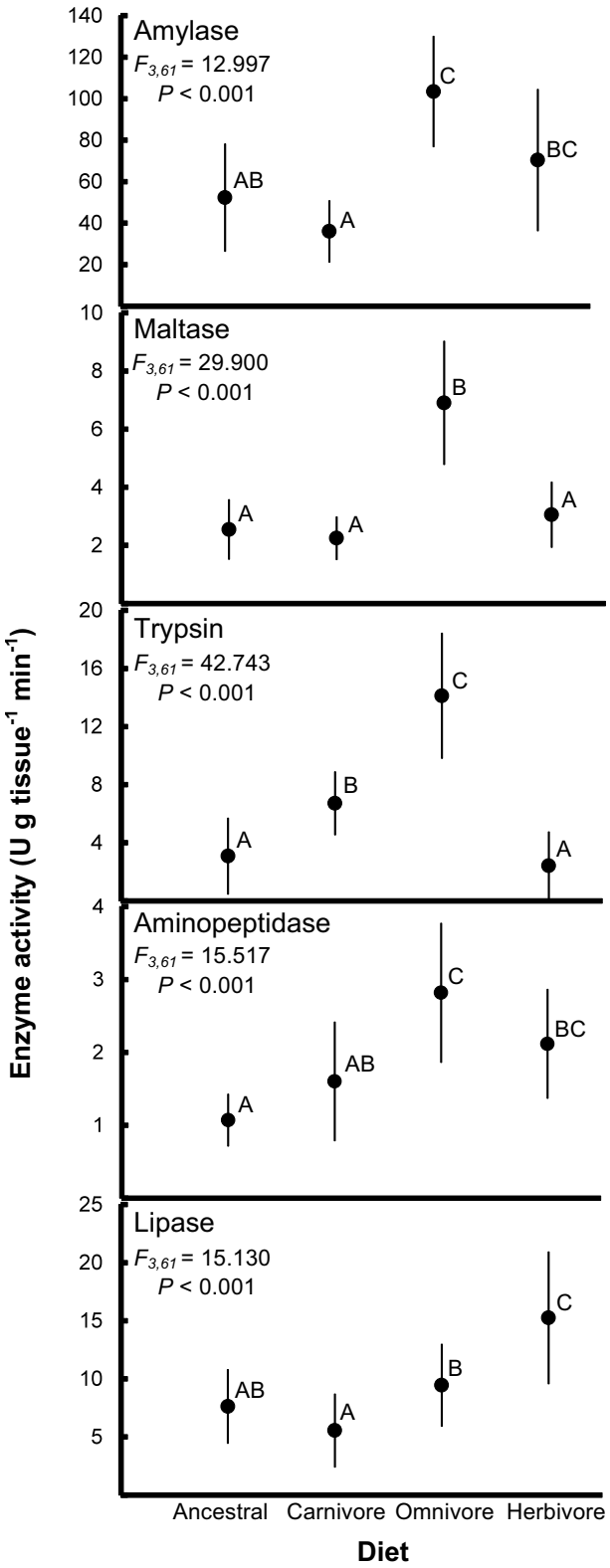

Fig. 4 Amylase, maltase, trypsin, aminopeptidase, and lipase (top to bottom) activities in the digestive tracts of *D. rerio* fed different diets. Values are means and error bars are standard deviation. Activity levels of each enzyme were compared among the fish fed the different diets with ANOVA, followed by a Tukey's HSD with a family error rate of $P = 0.05$. Symbols sharing a superscript letter for a specific enzyme are not different from one another

not only caused by the overall lengthening of the gut, but also by increasing the epithelial surface magnification, which may have been enhanced by enterocyte hypertrophy (Fig. 3). Changes in epithelial surface area (and overall gut length) as a response to fiber intake has been observed to occur rapidly in birds (*Coturnix japonica*, Starck and Kloss 1995; and *Gallus gallus domesticus*; Rahmatnejad and Saki 2016). It has also been shown to be reversible when fiber availability returns to original levels (Starck 1996). In mammals, intestinal cell proliferation and other intestinal maintenance activities account for 20–30% of total basal metabolic rate, making it an energetically costly process (Stevens and Hume 1995). As such, flexibility of the intestine to manage cell proliferation rate in accordance with food availability and diet type is metabolically critical (Boza et al. 1999; Dunel-Erb et al. 2011; Samuelsson et al. 2016). In snakes that feed infrequently, cellular hypertrophy following ingestion of a meal can be costly (Secor 2009), but, costs of intestinal epithelial maintenance, and enterocyte hypertrophy in particular, remains unknown in fishes. Overall, the intestinal epithelial surface areas that we observed in the zebrafish on all of the diet types are consistent with fishes of similar body masses (i.e., < 1 g; Karasov and Hume 1997).

One aspect of our diet design that cannot be overlooked is the inclusion of soybean meal (SBM) as a protein source for the fish fed the experimental diets. Soybean meal has been shown to induce inflammatory responses in the distal intestines of fishes, but the responses vary by species and dose of SBM (Bakke-Mckellep et al. 2000; Refstie et al. 2000; Krogdahl et al. 2003; Bakke-McKellep et al. 2007; Urán et al. 2008; Hedrera et al. 2013; Brugman 2016; Ulloa et al. 2016; Perera and Yúfera 2016). For example, in carnivorous salmonids, the effects of SBM are extreme distal intestine enteritis (e.g., Refstie et al. 2000; Krogdahl et al. 2003), however, in omnivorous common carp (a cyprinid), enteritis symptoms wane after about 5 weeks of consumption of a high SBM diet (Urán et al. 2008). In our study, which featured the carnivore-diet-fed fish consuming a moderate SBM diet across their lives (and the other fish consuming even less), we did not see many of the tissue-level problems of enteritis (e.g., lamina propria expansion; Refstie et al. 2000; Krogdahl et al. 2003; Urán et al. 2008) in the fish's intestines (Fig. 2), nor did fish on any of the diets display any other health problems. Hence, we are confident that our gut surface area measurements reflect dietary differences relating to protein and fiber contents (which affect intake) and not the effects of SBM on gut structure.

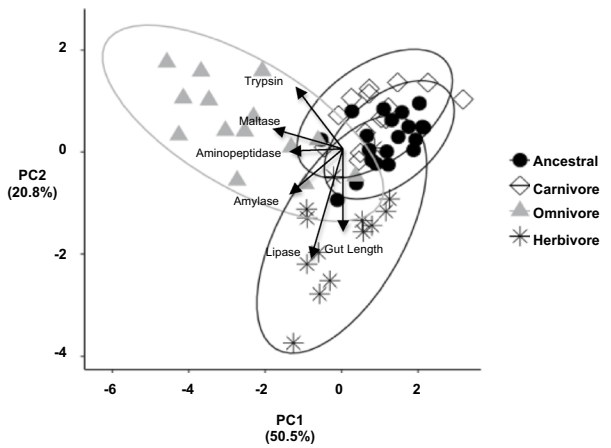

Fig. 5 Scatter plot of principal components analysis of enzyme activities (trypsin, maltase, aminopeptidase, amylase, and lipase) and gut length (RIL) for each diet type (ancestral, carnivorous, omnivorous, and herbivorous). Ellipses are 95% confidence intervals around diet types. Loading vectors for each variable are labeled. An analysis of similarities test determined all variables to be significantly different from each other ($P < 0.0001$)

Enzyme activities

Since carbohydrate concentration was kept constant for all of the diets, it was predicted that fish on all of the experimental diets would show comparable levels of amylase and maltase activities. However, the omnivorous diet fish had significantly higher amylase and maltase activity levels (and generally elevated trypsin and aminopeptidase activities) than any other dietary group (Fig. 4). Zebrafish are naturally omnivorous (Ulloa et al. 2011) and therefore, they may exhibit the highest digestive performance when consuming a diet that has the right balance of protein. Carbohydrate-degrading enzyme activities can be elevated in vertebrates on a high-protein diet, indicating that a high-quality diet can result in the non-specific increase of all enzymes, not just those that specifically relate to the degradation of proteins (Sabat et al. 1998; Russell et al. 1981; Galluser et al. 1988; Waheed and Gupta 1997; Timofeeva et al. 2009). There are limits to this generalization; however, a higher-protein diet than the omnivore diet did not lead to increased enzyme activities across the board, and in fact, the fish on the carnivore diet had some of the lowest digestive enzyme activities for all measured enzymes (Fig. 4). Hence, a protein concentration that is potentially closer to some optimum (~30%, Table 1) resulted in the most efficient gut performance in zebrafish, which one would predict would lead to the largest body size, yet the fish on the carnivore diet had the largest terminal size (Fig. 1).

Our data fit the rate vs. yield continuum well in a single species consuming different diets, and reflect patterns seen in larger comparative analyses (e.g., Sibly 1981; Fris and Horn 1993; Horn et al. 1995, 2006; German et al. 2010a, 2015). The one exception is that fish on the omnivorous diet generally possessed the greatest digestive enzyme activities, and this could better fit an "optimal protein" content model (Simpson et al. 2004). This was true even with respect to the carbohydrate-degrading enzymes amylase and maltase. Even though starch content did not vary among the diet types (Table 4), amylase and maltase activities were significantly higher for the omnivorous diet fish, suggesting that intake (determined by diet quality) may be an important driver of digestive enzyme expression (German et al. 2010a), in addition to dietary biochemical composition (Karasov and Martínez del Rio 2007). How changes in digestive enzyme activities are achieved, whether it be increased expression of the same genes, expression of different isoforms with different biochemistry, or even post-translational modifications (e.g., Gawlicka and Horn 2006; Kim et al. 2014) is unknown in *D. rerio*. For instance, *D. rerio* possesses three amylase genes in its genome, and two of them are on a different chromosome from the other gene, a characteristic that appears to be unique to the Ostariophysi, which includes catfishes, carps and minnows, and characins (Gawlicka and Horn 2006; Kim et al. 2014; German et al. 2016). Hence, how changes in digestive enzyme activities are achieved in *D. rerio* warrants further investigation.

Because we varied the cellulose contents of the different diets, we did attempt to measure two cellulose-degrading enzymes in the guts of the fish: cellobiohydrolase and β-glucosidase, with an expectation of higher activities in the fish consuming the omnivorous and herbivorous diets (Table 2). However, consistent with a rate maximizing strategy operating in *D. rerio*, neither enzyme, which would be of microbial origin (German 2011), was detectable in any of the fish on the different diets. This does not mean that *D. rerio* do not have active enteric microbial populations (they do; Roeselers et al. 2011; Semova et al. 2012), but rather that this community does not seem to be engaged in cellulose digestion and does not necessarily respond biochemically in a dose-dependent manner to changes in cellulose concentrations, which does not align with the predictions of CRT.

Lipid content was kept constant for all of the experimental diets, with only protein and fiber varying. Since lipid was constant, we predicted that the activity level of lipase would also remain constant for the three different diets. However, we found that lipase activity was highest in the fish consuming the herbivorous diet, which is not what we would expect according to CRT. This has been previously recorded in *Atherinops affinis, Brycon guatemalensis, Labeo rohita* and others where herbivores produced higher lipase activity levels than carnivores or omnivores (Horn et al. 2006; Drewe et al. 2004; Nayak et al. 2003; German et al. 2004). Clearly, this phenomenon is not unique to any one species of fish and may reflect lipid-scavenging by fishes consuming low-lipid

foods (German et al. 2004). Moreover, because herbivorous diets are generally high in fiber, and fiber binds fat in the digestive tract, thereby lowering lipid digestibility (German et al. 1996), lipase activities may need to be elevated in herbivores to ensure lipid digestion from their ingested food (Clissold et al. 2010; Sullam et al. 2015). In support of this, the bonnethead shark, *Sphyrna tiburo*, despite consistent lipase activity throughout its gut (Jhaveri et al. 2015), has lower lipid digestibility when fed a high-fiber diet in the laboratory (Leigh and German, unpublished data). Another possibility is that herbivores consuming low-protein diets use lipid as a "protein-sparing" energy source, thereby saving the protein that is assimilated for tissue maintenance (Watanabe 1982; German et al. 2004). However, coupled with lower lipid digestibility, using scant lipid as an energy source would leave less lipid for reproduction. In fact, we anecdotally observed lower fecundity in the zebrafish on the herbivorous diet. Reduced clutch size has been previously recorded in an herbivorous population of the lizard *Cnemidophorus murinus* that produced smaller clutch sizes compared to carnivorous populations (Dearing and Schall 1994). Hence, lipid may be a limiting nutrient for herbivores, especially for those that do not meet a large proportion of their energetic needs from microbial fermentation, and its impact on various aspects of vertebrate health and digestive efficiency should be explored further.

Body size

We acknowledge that body size is not considered in the CRT model; however, it is worth noting that body size can be an indicator of the overall health status of vertebrates. Larger body size is generally associated with a high-quality diet which can lead to overall improved performance in energy-heavy demands (e.g., Garcia-Correno et al. 2002; Karasov and Douglas 2013). These characteristics also impact the organism's role in the ecosystem via biomass, foraging, and excretion (German 2009a). The largest mean terminal body mass was exhibited by the fish consuming the carnivorous diet, which had the highest percentage of protein among the experimental diets. This is consistent with what was seen in studies by Garcia-Correno et al. (2002) and Horn et al. (1995, Table 1). Protein has been shown to provide an important structural component—fish bodies are ~70% protein on a dry mass basis (Horn 1989)—as well as an energy source to growing animals (Brett and Groves 1979). However, there may be a protein "threshold", above which body mass will reach a plateau and no longer continue to increase with increasing protein content (Horn et al. 1995). This indicates that an optimum protein concentration may exist, which would vary by species, diet specialization, and various phenotypic factors (Horn et al. 1995; Watts et al. 2012). For instance, the ancestral diet had more protein than our experimental carnivore diet (Table 3), yet the mean terminal body mass of the ancestral diet fish was significantly lower than the carnivore-diet-fed fish (Fig. 1). We offer this with the caveat that other parameters of the ancestral diet-fed fish (e.g., intestinal length, intestinal epithelial surface area) imply higher intake of this diet in comparison to the carnivore-diet-fed fish. The Adult Zebrafish Complete Diet has lower organic matter content than our experimental diets (Table 3); hence, the higher inorganic matter content of the ancestral diet may have led to greater intake in these fish, despite the apparent elevated protein content. Furthermore, the protein in the ancestral diet came from animal sources, as opposed to soybean meal in the experimental diets (but we also used casein in these diets). Plant-based protein sources like soybean meal generally lead to smaller terminal body sizes in comparison to animal-based protein sources (e.g., Biswas et al. 2007; Hernandez et al. 2007; Lin and Luo 2011; Li et al. 2014; Magalhaes et al. 2016; Yaghoubi et al. 2016; Król et al. 2016), again implying that the Adult Zebrafish Complete Diet functionally has less usable protein than our carnivore diet. Since the *D. rerio* in the present study received their prescribed diets throughout their entire lives (beginning at 15 DPH), we can conclude that extended exposure to a higher-protein diet results in an overall larger body mass.

In conclusion, the results of this project only conclusively support CRT in terms of gut structure, but there is only some support for CRT in terms of digestive enzyme activities. The results show that an omnivorous fish species can alter its digestive tract (structure) on levels usually seen in larger comparative analyses of different taxa that naturally have different diets. We observed that changes in gut length were associated with increases in intestinal epithelial surface area, but that this increased surface area was elaborated by increased mucosal folding, and was not just a function of increased intestinal length. Regarding gut function, we observed modulation of digestive enzyme activities that suggest the importance of protein and lipid intake rather than the simple CRT suggestion that an increase in a particular substrate concentration will result in the increase in the activity of the enzyme responsible for the degradation of that substrate (Clissold et al. 2010). This evidence, combined with evidence from previous studies as discussed in the introduction (e.g., Reimer 1982; Sabat et al. 1998, 1999; Levey et al. 1999; Caviedes-Vidal et al. 2000; German et al. 2004; Hakim et al. 2006, 2007; Kohl et al. 2016; Król et al. 2016) indicates that nutrient concentration is likely not the only factor controlling digestive enzyme activity levels. We acknowledge that dietary variations (particularly differences in fiber) can affect microbial diversity in the gut (Rawls 2012; Wong and Rawls 2012; Semova et al. 2012; Wong et al. 2013, 2015; Stephens et al. 2015). We did not measure microbial diversity in this particular study; however, it

is possible that enteric microbes are playing a role in the patterns of gut structure and function that we observed, which should be investigated in future studies (Nayak 2010; Stephens et al. 2015; Ghanbari et al. 2015). Future work will focus on experimental evolution of these phenotypic traits by including multiple generations of *D. rerio* reared on the experimental diets to observe whether permanent and irreversible changes to gut function and structure are possible on experimental evolutionary timescales (e.g., Herrel et al. 2008; Garland and Rose 2009). Investigations into the genetic underpinnings of these changes may also provide insight for how animals are able to evolve the ability to thrive on diets of varying quality.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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