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Maintenance of distal intestinal structure in the face of prolonged fasting: a comparative examination of species from five vertebrate classes

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Abstract

It was recently shown that fasting alters the composition of microbial communities residing in the distal intestinal tract of animals representing five classes of vertebrates [i.e., fishes (tilapia), amphibians (toads), reptiles (leopard geckos), birds (quail), and mammals (mice)]. In the current study, we tested the hypothesis that the extent of tissue reorganization in the fasted distal intestine was correlated with the observed changes in enteric microbial diversity. Segments of intestine adjacent to those used for the microbiota study were examined histologically to quantify cross-sectional and mucosal surface areas as well as thicknesses of mucosa, submucosa, and tunica muscularis. We found no fasting-induced differences in the morphology of distal intestines of the mice (3d), quail (7d), or geckos (28d). The toads, which exhibited a general increase in phylogenetic diversity of their enteric microbiota with fasting, also exhibited reduced mucosal circumference at 14 and 21 days of fasting. Tilapia showed increased phylogenetic diversity of their enteric microbiota, and showed a thickened tunica muscularis at 21 days fasting; but this morphological change was not related to microbial diversity or absorptive surface area, and thus, is unlikely to functionally match the changes in their microbiome. Given that fasting caused significant increases and reductions in the enteric microbial diversity of mice and quail, respectively, but no detectable changes in distal intestine morphology, we conclude that reorganization is not the primary factor shaping changes in microbial diversity within the fasted colon, and the observed modest structural changes are more related to the fasted state.

Keywords

starvation; nutrition; morphology; hindgut; malnutrition; microbiome

1. Introduction

The form and function of the digestive tract is evolutionarily tuned to an animal's diet and feeding habits. Diet is well known to differ widely among taxa (Stevens and Hume, 1995; Horn et al., 2006; German and Miles, 2010; Karasov and Douglas, 2013), and feeding frequency may also vary with some species eating nearly constantly and others experiencing

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long bouts between meals (Wang et al., 2006; McCue, 2010). Because the digestive tract is expensive to maintain—accounting for 20–25% of an animal's resting metabolic rate (Cant et al., 1996; Croom et al., 1999; Secor et al., 2012)—it is expected to be tightly regulated in the face of varying nutrient availability in response to varying diets and feeding schedules (Karasov and Diamond, 1988; Secor, 2001; Starck, 2005; Karasov and Douglas, 2013; Price et al., 2015).

Fasting tolerance is highly variable among animals, with some endothermic species expiring in less than two days without food and some ectothermic species lasting months to years without eating (Castellini and Rea, 1992; Navarro and Gutierrez, 1995; Wang et al., 2006; McCue, 2012). Despite various mechanisms to reduce energy expenditure (Secor and Carey, 2016), the fasted gut must be maintained in a state such that it retains the ability to quickly restore functionality in the event food is ingested.

The 'small' or 'proximal' intestine (hereafter: proximal intestine) is the most active region of the intestine, with high cellular turnover and activity, and it exhibits several adaptive responses to downregulate and conserve energy during times of fasting. Some of these changes include decreases in villi (or intestinal fold) size and number, a decrease in enterocyte cytoplasmic activity, and a decrease in microvilli length (Starck, 2003; Secor and Lignot, 2010; Lignot, 2012; Zaldua and Naya, 2014). The mechanisms of these reductions (e.g., changes in cell turnover *versus* cell volume) can vary among endothermic and ectothermic taxa (Starck, 2003, 2005).

The reduction in the surface area of the proximal intestine is concomitantly accompanied by decreases in membrane-bound enzyme activities (e.g., aminopeptidase, maltase) and nutrient transport rates (Secor et al., 1994; Ott and Secor, 2007; German et al., 2010). Although the mechanism of change varies among endothermic and ectothermic taxa, the general patterns of reduced activity and surface area in the proximal intestine in response to fasting are common among vertebrates having been documented in fishes (Gas and Noailliac-Depeyre, 1976; German et al., 2010; Zaldua and Naya, 2014), amphibians (Perez-Gonzalez and Robinson, 1971; Cramp and Franklin, 2003; Cramp et al., 2005), reptiles (Secor and Diamond, 2000; Starck and Beese, 2002; Secor and Lignot, 2010), birds (Hume and Biebach, 1996; Karasov et al., 2004; Smirnov et al., 2004), and mammals (Dunel-Erb et al., 2001; Habold et al., 2004; Habold et al., 2007).

The colons, hindguts, or distal intestines (hereafter: distal intestine) of vertebrate animals have received considerably less attention than the proximal intestine in terms of structural and functional responses to fasting and starvation (Gas and Noailliac-Depeyre, 1976; Baeverfjord and Krogdahl, 1996; German et al., 2010). Given that the distal intestine is responsible for absorbing nutrients, vitamins, electrolytes, and water (Savage, 1986; Stevens and Hume, 1995; Clements and Raubenheimer, 2006) a complete understanding of how fasting affects the gastrointestinal tract must consider the distal intestine (Okada et al., 2013).

The distal intestine is home to the largest enteric microbial population outside of the caeca in animals that possess them (Roediger, 1990; Caporaso et al., 2011; Kohl et al., 2014). These

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microbial communities subsist on nutritive digesta periodically pulsing through the gut, as well as host-produced glycans and mucins on the gut lining (Derrien et al., 2004; Sonnenburg et al., 2005). However, in fasted animals the nutrients in the distal intestine may become scarce (Okada et al., 2013) creating an ‘energy crisis’ for the intestinal microbes (McCue, 2012). This increase in phylogenetic diversity, appears to be driven by crashing populations of the species that predominate the gut in times of high nutrient availability when the hosts are nourished {Kohl, 2014 #4713}. Many of the enterocytes lining the distal intestine also obtain a significant proportion of their energy needs from the short chain fatty acids produced via microbial fermentation (Bjorndal, 1979; Troyer, 1984; Roediger, 1990; Bugaut and Bentejac, 1993; Crawford et al., 2009), and may require microbial metabolites (e.g., lactate) for proliferation (Okada et al., 2013).

Microbial communities often exhibit typical species-area relationships, such that larger habitats contain higher diversity (Bell et al., 2005; Godon et al., 2016), and this relationship has been demonstrated in vertebrate gut microbial communities (Bell et al., 2005; Godon et al., 2016). In times of fasting the enterocytes may also experience reduced nutrient uptake that may drive the atrophy of the proximal intestine thereby creating a ‘housing crisis’ for the microbiome (McCue, 2012). Thus, changes in the structure of the distal intestine, such as decreased volume or available membrane space, may result in changes to microbial diversity (Secor and Carey, 2016). However, as noted above, morphological changes to the distal intestine as a result of fasting have been largely overlooked, with one previous mammalian example showing no change in mucosal area of the distal intestine following fasting (Okada et al., 2013).

Recent studies of microbial diversity in the distal intestine demonstrate varying responses to fasting. Fasted animals show shifts in their microbial communities that range from increases, to no changes, to decreases in microbial diversity (Crawford et al., 2009; Sonoyama et al., 2009; Costello et al., 2010; Kohl et al., 2014; Xia et al., 2014). Although these changes are statistically significant there do not yet seem to be systematic changes that are generalizable among different groups of vertebrate animals (Kohl et al., 2014). Given the importance of the enteric microbiota in overall animal health (Jabbar et al., 2003; Backhed et al., 2004; Turnbaugh and Gordon, 2009; Caporaso et al., 2011; Mischke and Plosch, 2013), it is crucial to understand if and how fasting-induced structural changes in the distal intestine may drive the observed changes in the microbial community that it supports.

A recent study demonstrated that the diversity of the microbiomes within the distal intestines of animals representing five vertebrate groups (classes): a teleost fish (tilapia), an amphibian (toads), a reptile (leopard geckos), a bird (quail), and a eutherian mammal (mice) (Kohl et al. 2014; Table 1). Thus, within these organisms, we are presented with the unique opportunity to answer the following questions regarding the distal intestine in the face of fasting: 1) How do the distal intestines of disparate endothermic and ectothermic lineages respond to fasting, and, despite likely mechanistic differences, is there a common, generalizable response (e.g., reduction in surface area)? 2) How are fasting-induced structural changes of the distal intestine correlated with previously documented changes in enteric microbial diversity within this gut region?

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In order to answer these questions, we examined the distal intestines of tilapia, toad, leopard gecko, quail, and mouse subjected to different levels of fasting. The samples we examined were collected from adjacent regions of the distal intestine that we previously used to characterize fasting-induced changes in the microbiome. We prepared histological cross sections and measured several characteristics including the surface area of the mucosa and serosa, as well as thickness of the tunica muscularis, submucosa, and mucosa to characterize the effect of fasting on the structure of the distal intestine.

2. Materials and Methods

Animals

All experiments involving vertebrates were conducted at St. Mary's University (StMU) under the auspices of the StMU Institutional Animal Care and Use Committee (StMU #2010–4; 2011–5, 2012–1; 2012–2; 2012–3). The laboratory temperature (28±1°C) and photoperiod (14L:10D) remained constant during the experiments, and all animals experienced these temperatures whether in air or water. The only caveat is that the gecko cages were lit in one corner by an incandescent lightbulb, giving them one warm microclimate (~30°C) in which to bask.

Nile tilapia, *Oreochromis niloticus*, (males and females; fry ~1.5cm total length; TL) were obtained from a commercial breeder (Tilapia Depot, Saint Augustine, FL) and raised on the Tilapia Depot Standard High-Protein Pelleted Diet (>50% protein, >16% fat) for approximately 4 months in a 400-gallon aquarium until reaching ~20cm TL. Wild captured adult and subadult southern toads, *Anaxyrus terrestris*, (males and females; ~15–30g) were purchased from a commercial distributor (Gulf Hammock Herps, Dade City, FL) and maintained for 60 days on a diet of live crickets (Fluker Farms, Port Allen, LA). Captive bred, adult leopard geckos, *Eublepharis macularius*, (males and females; ~50–60g) were obtained from Leopardgecko.com (Boerne, TX) and maintained in the lab for approximately 9 months on a diet of live tenebrionid beetle larvae raised in the laboratory. Geckos were given supplementary heat lamps to permit voluntary basking. Common quail, *Coturnix coturnix*, hatchlings (males and females; 2 days old) were obtained from a commercial breeder (Diamond H Ranch, Bandera, TX) and raised in the lab for approximately 3 months on a standard quail diet (Nature Wise, Nutrena) until adulthood (~250g). Weanling mice, *Mus musculus*, (males; ~10–12g) were obtained from a commercial breeder (Alamo Aquatic Pets, San Antonio, TX) and raised in the lab on a standard rodent diet (Teklad, Harlan Laboratories) for approximately 2 months until adulthood (~26–30g).

Fasting and tissue sampling

On the first day of the fasting experiment, the toads and geckos were held without food for 24 hours, at which time they were sampled (e.g., fasting day 0; Table 2). Similarly, food was removed from the tilapia, quail, and mice 6 hours prior to the first sampling time point (e.g., fasting day 0). All other animals were maintained without access to food for prolonged periods of time (Table 2). Water was available at all times to fasting animals. Sample sizes are presented in Table 2.

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At predetermined time points, animals were euthanized (Table 2) in accordance with standard guidelines for euthanasia (AVMA, 2013). The ‘late-fasting’ time point was determined using preliminary data with the goal of achieving a 20–30% loss of body mass in the endotherms (Toth and Gardiner, 2000; Rowland, 2007), and a sufficient period (≥three weeks) in the ectotherms, which show variable fasting times. The fasting time frame was divided in thirds to determine the ‘early-fasting’ and ‘mid-fasting’ time points.

Within 10 minutes of euthanasia, the gastrointestinal tract from the distal esophagus to the rectum was removed intact. A central section (approximately 1cm in length) of large intestine was removed from each animal, placed in a tissue cassette, and fixed in Carnoy’s solution (v/v; 60% anhydrous methanol, 30% chloroform, and 10% glacial acetic acid) (Johansson and Hansson, 2012). Each sample was embedded in paraffin, sectioned and fixed onto a (1”×3”) slide with 6 to 9 tissue sections per slide (Texas A&M Department of Veterinary Pathobiology) and later stained with hematoxylin and eosin using standard protocols (Meyerholz et al., 2009).

Morphometrics

The stained slides were digitized into .tiff files using PathScan Enabler IV (Meyer Instruments, Houston, TX). The highest quality cross-section from each animal was selected for morphological measurements. We used Image J (NIH.gov) software to measure distances and areas. When measuring short distances (e.g., thickness of tunica muscularis or mucosal thickness) we made four measurements (randomly selected from each of the four quadrants of the cross-section) and averaged them (n=16 measures for each value). For more complex distances and areas (e.g., basement membrane length or lumen area) the values were based on polygons usually exceeding 200 landmarks – an approach used in other studies (Meyerholz et al., 2010; Risse et al., 2012). Cross section was not calculated for the toads because the sections were not circular.

In order to adjust for small differences in individual body size we divided the mucosal area by the total cross-sectional area to create a unitless relativized mucosal length for the endotherms. We used a similar approach for the relativized mucosal length in the ectotherms by dividing the mucosa length by the serosa perimeter. We used serosa perimeter in the ectotherms because we were less confident that the apparent cross sectional area of the ectotherm sections accurately represented the caliber of the intestine. All values were examined using the same statistical approaches for the other metrics (see next section).

Statistics

After confirming normality with a Shapiro-Wilk test, and homogeneity of variance with Levene’s test, we used a parametric one-way analysis of variance (ANOVA) to compare measured variables within a species across the four time points. We corrected p-values using the Benjamini-Hochberg False Discovery Rate correction (Benjamini and Hochberg, 1995), and a critical α of 0.05 to determine significance. In cases where the ANOVAs were significant (Bold values in Table 3 and 4) we ran Holm-Sidak *post hoc* tests comparing measurements at each fasting treatment (early, mid, late) with the control (nourished) values.

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ANOVAs were executed using JMP 12.0, while Holm-Sidak *post hoc* tests were executed using SigmaPlot 12.

Additionally, we were interested whether changes in gut morphology might correlate with previously documented changes in luminal microbial diversity. For each species, we tested for interactions between fasting state, anatomical measurements, and measurements of Faith's phylogenetic diversity, and relative abundances of microbial phyla, as determined by Kohl et al. (2014). Faith's phylogenetic diversity is determined using a phylogenetic tree of all microbial members, and is calculated as the cumulative branch lengths from a random sampling of many microbial members from a particular sample (Faith, 1992). For each species, we used the Response Screening function in JMP 12.0 to test for correlations between anatomical measurements and either Faith's phylogenetic diversity or relative abundances of microbial phyla while incorporating fasting state as an additional independent variable. Resulting P-values were corrected using the Benjamini-Hochberg False Discovery Rate correction (Benjamini and Hochberg, 1995), and a critical α of 0.05 to determine significance.

3. Results

None of the measured variables in the distal intestine of the endotherms (i.e., mouse or quail) changed in response to prolonged fasting (Table 3). Because the duration of fasting of the mice and quail was sufficient to elicit ~25% reductions in body mass (McCue et al., 2013; McCue and Pollock, 2013) any fasting-induced morphological changes in the distal intestines should have occurred within the observation periods.

The only significant fasting-induced changes that we observed in the distal intestines of the ectotherms occurred in the tilapia and the toads (Table 4). In tilapia, all six measured variables differed significantly among the fasting time points. The perimeter of the serosa significantly differed (ANOVA, df=3, F=4.09, FDR-corrected p=0.025), however *post hoc* comparisons did not identify any particular time points where the fasted values differed from the initial values. The thickness of the tunica muscularis differed across treatments (ANOVA, df=3, F=6.18, FDR-corrected p=0.019). *Post hoc* comparisons revealed that the tunica muscularis was significantly thicker at day 21 (Holm-Sidak$_{0-21}$; t=3.759, p<0.001), and this represents a 70% increase in thickness in comparison to day 0. The tunica muscularis is composed of an outer, longitudinal layer of muscle and an inner, circular muscle with fibers that run perpendicular to the longitudinal layer. We reexamined the slides of the tilapia from day 0 and day 21 and concluded that this difference appears to be driven by a relative enlargement of the inner, circular muscle layer (Fig. 1). Interestingly, each of the ectothermic taxa increased the thickness of the tunica muscularis by the end of the experiment (toad by 37%, gecko by 25%), but it was only a significant increase in tilapia (Table 4).

The total cross-sectional areas of the tilapia distal intestines differed across time in comparison to the time 0 sections (ANOVA, df=3, F=3.96, FDR-corrected p=0.025), but *post hoc* tests did not show that the values on day 0 significantly differed from any of those at other fasting time points. The mean thickness of the submucosa of the fish also

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significantly differed among the fasting treatments (ANOVA, df=3, F=3.63, FDR-corrected p=0.028). Comparisons among the time points revealed the submucosa was significantly thicker on day 7 (Holm-Sidak$_{0–7}$; t=3.150, p=0.004), but not at the later time points. Both the actual length of the mucosa (ANOVA, df=3, F=4.00, FDR-corrected p=0.025) and the relativized mucosa lengths (ANOVA, df=3, F=4.09, FDR-corrected p=0.025) differed among the fasting treatments. *Post hoc* comparisons revealed that the mucosa length significantly decreased by day 14 (Holm-Sidak$_{0–14}$; t=3.458, p=0.002), but that the relativized mucosa length was decreased only at day 7 (Holm-Sidak$_{0–7}$; t=3.148, p=0.005).

Three of the five variables in the toads exhibited significant differences among the fasting treatments (Table 4). Like the tilapia, the perimeter of the serosa differed (ANOVA, df=3, F=3.72, FDR-corrected p=0.044) and values were significantly greater on day 7 (Holm-Sidak$_{0–7}$; t=2.983, p=0.007) and day 21 (Holm-Sidak$_{0–21}$; t=2.449, p=0.023). The submucosa thickness also differed (ANOVA, df=3, F=5.53, FDR-corrected p=0.014) and was significantly greater at day 7 (Holm-Sidak$_{0–7}$; t=3.221, p=0.004).

The toads exhibited shorter relative mucosal lengths as a result of fasting. In particular, these lengths were shorter at day 14 (Holm-Sidak$_{0–14}$; t=2.810, p=0.010) and day 21 (Holm-Sidak$_{0–21}$; t=3.693, p<0.001) of fasting. This trend was not apparent in the absolute mucosal lengths likely because of variation in the body sizes of the toads. Of the species we studied the toads had the largest variation in body size (~2-fold range), and so the index values (see Materials and Methods) provide a better proxy for variation caused by fasting. We reexamined the slides of the toads from day 0 and day 21 of fasting and it appears that the basement membrane had contracted or otherwise become less convoluted during fasting. The mucosa thickness also appeared to be reduced at day 21 (Fig. 1).

Last, we investigated whether anatomical changes in the distal intestine correlated with previously documented changes in microbial diversity (Kohl et al., 2014). We did not detect any significant correlations in any host species and or any morphological measurement with measurements of Faith's phylogenetic diversity (Tables 3 and 4). Plots of microbial phylogenetic diversity as a function of mucosal area (endotherms) or mucosal length (ectotherms) can be seen (Fig. 2). To test whether the abundances of specific bacterial phyla (e.g., Bacteroidetes, Proteobacteria, and Fusbacteria; Table 1) may have varied with mucosal area or length, we also generated plots to test those relationships (Fig. 3). No significant relationships among the abundance of specific bacterial phyla and mucosal area or length were detected.

4. Discussion

In this study we asked two questions: 1) How the distal intestine of disparate endothermic and ectothermic species respond to fasting, and, despite likely mechanistic differences, is there a common, generalizable response (e.g., reduction in surface area)? And, 2) How are fasting-induced structural changes of the distal intestine correlated with previously documented changes in enteric microbial diversity within this gut region? The answers to the first question are "variable responses", and "no, there is not a common response". We observed no significant changes in the endothermic mouse and quail. Among the ectotherms

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the results were mixed with changes in the tilapia and the toads but no changes in the geckos. Thus, there is no common, generalizable response of the distal intestine to fasting. Several studies in fishes have observed changes in the distal intestine structure following extended periods of fasting. For instance, after ≥ six months of fasting, (Gas and Noailliac-Depeyre, 1976) and (German et al., 2010) showed decreased mucosal surface area in the distal intestines of *Cyprinus carpio* and *Pterygoplichthys disjunctivus*, respectively, whereas (Baeverfjord and Krogdahl, 1996) observed decreased surface area in salmon distal intestine after 21 days of fasting. The mucosal surface area reduction is more pronounced after six months (Gas and Noailliac-Depeyre, 1976; German et al., 2010), as would be expected, but the differences in 21 days (Baeverfjord and Krogdahl, 1996) are still measurable, and are similar to what we observed in tilapia in this study.

Difficult to explain is the apparent increase in the thickness of the tunica muscularis in the ectothermic organisms. Although this could be an artifact of decreased overall gut length in response to fasting (Weaver et al., 1991) making the muscle appear proportionally larger, correlations of muscularis thickness and serosal circumference are not strong ($R^2 = 0.0117$), suggesting this is not the case (we would expect a large negative correlation if this were true). Alternatively, muscle thickness may be maintained or even show a trophic increase in response to fasting in the ectothermic taxa [e.g., (do Nascimento et al. 2016)]. For example, it is clear that each of the specimens still had material in their distal intestine at the end of the food deprivation (Fig. 1), and fecal material was noted at time of dissection. This is consistent with an animal with low-intake (i.e., in a fasted state). For example, digestion in animals tends to operate in the rate *vs* yield continuum (Sibly, 1981; Hume, 2005; German et al., 2015). On one end, a rate maximizer has high-intake, rapid movement of digesta through the gastrointestinal tract, and little reliance on microbial digestion in the distal intestine. Rate maximizers tend to digest more of the soluble components of their ingested food, and have lower digestibilities overall, and especially of more fibrous material. On the other end, a yield maximizer has lower-intake, slower movement of digesta through the alimentary canal, and more of a reliance on microbial digestion in the distal intestine to result in higher overall digestibilities, especially of fibrous material (Sibly, 1981; Hume, 2005; German et al., 2015).

Some animals [e.g., rate-maximizing loricariid catfishes (German and Bittong, 2009); yield-maximizing ruminants (Hofmann, 1989)] can largely exist near extreme ends of the spectrum, whereas many animals likely switch back and forth depending on intake (e.g., (Klumpp and Nichols, 1983; Connor et al., 2016) because the presence of new ingesta in the stomach is a promoter of gut motility (Olsson and Holmgren, 2001). Because the animals in this study were deprived of food, we forced them into a yield maximizing situation (i.e., no intake means no new ingesta promoting more rapid gut motility). With reduced extraction rates, each of the animals, therefore, maintained material in their distal intestines to obtain as many nutrients as possible from what remained. This could include microbial metabolism of any nutrients (carbohydrates, proteins, lipids) in addition to fibrous components of ingesta remaining in the distal intestine. Not all distal intestine fermentation is of carbohydrates (Bergman, 1990; Clements et al., 2016). This microbial activity would require muscular contractions (e.g., segmental contractions; (Olsson and Holmgren, 2001)) of the distal intestine to mix the digesta, especially to allow contact of potentially fermentable substrates with microbial symbionts (Stevens and Hume, 1995). Indeed, all of the ectothermic animals

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apparently increased the thickness of the tunica muscularis, albeit only significantly so in tilapia. Interestingly, (do Nascimento et al., 2016) also observed a thickening of proximal intestine muscularis thickness in juvenile tegus (*Tupinambis merianae*), which they attributed to a potential anticipatory response for refeeding.

Tilapia are known to have active microbial fermentation and absorption of short chain fatty acids in their distal intestine (Titus and Ahearn, 1988, 1991), and intestinal microbes secrete more essential amino acids when tilapia are fed low-protein diets (Newsome et al., 2011). Hence, the alleged thickening of the muscle may be a trophic increase in response to a yield maximizing strategy imposed by fasting. Another possibility is that the thickening of the muscle could represent a pathology resulting from a change in the expression of specific genes (e.g., malfunctioning Cystic Fibrosis Transmembrane-conductance Regulator (CFTR) leads to muscle thickening in the intestine of pigs; Meyerholz et al. 2010) or an immune response (Sekirov et al., 2010) to fasting. For instance, an absence of resources on which enteric microbes can metabolize leads them to forage on host mucus, which causes inflammatory responses in the gut, and can allow for pathogen invasion (Desai et al., 2016). Either way, why a thickening of the smooth muscle doesn't also occur in the endothermic mouse and quail is unknown. (Okada et al., 2013) clearly showed that colonic cellular turnover explains the lack of changes in the mucosal thickness of mice: cell proliferation arrests during fasting, whereas elevated cell proliferation observed upon refeeding is matched by cellular shedding. The consistent mucosal thickness we observed in our mice matches this finding. Quail may also show similar alterations in cellular turnover, and thus, show little change in their distal intestine mucosal thickness.

With regards to changes in gut structure matching with changes in the microbiome, the answer is also *no*. Again, we predicted that decreases in microbial diversity would be matched by decreases in the size of the distal intestine, given that microbial communities (including those of the vertebrate gut) exhibit traditional species-area relationships (Bell et al., 2005; Godon et al., 2016). Tilapia and toad generally showed an increase in intestinal microbe diversity with fasting (Kohl et al., 2014), and here, we see different responses of the gut tissue to a lack of food (Table 4). For comparison, the Burmese python shows a decrease in distal intestine microbial diversity (Costello et al., 2010) with a decrease in distal intestine mass (Secor et al., 1994). The gecko showed no change in its enteric microbial diversity or distal intestine structure with fasting. The endothermic quail (decrease in microbiome diversity) and mouse (increase in intestinal microbial diversity) showed contrasting patterns (Kohl et al., 2014) with little change in their distal intestine structures. Thus, although we expected to see a decrease in microbial diversity match with a decrease in distal intestine structure [e.g., (Ott and Secor, 2007; Costello et al., 2010)], we did not observe such a pattern. We also did not observe any correlations between anatomical measurements and the relative abundances of microbial phyla, suggesting that changes in intestinal structure did not drive the community shifts observed in the studied taxa (Kohl et al., 2014). Though, it should be noted that our studies only conducted 16S rRNA sequencing, which only inventories the relative abundances of microbial taxa. It is likely that fasting alters absolute microbial abundance or microbial load, but additional techniques are required to determine these measurements (Stammler et al., 2016). Overall, changes in distal intestine microbial diversity and community structure as a result of fasting do not seem linked to structural

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changes in the distal intestine, and thus the notion of a ‘housing crisis’ is not supported. Rather, the changes in diversity may be driven by an ‘energy crisis’ as nutrients are no longer entering the system, and microbial metabolites likely play a key role in cellular proliferation of the distal intestine upon re-feeding (Okada et al., 2013). *In vitro* culture systems may be useful for testing the ‘energy crisis’ hypothesis in the future.

The next logical steps are to evaluate such changes over longer time scales, [*sensu* (Gas and Noailliac-Depeyre, 1976; German et al., 2010) (e.g., 6 months of food deprivation in fishes)], to observe whether starker changes in distal intestine morphology to prolonged fasting do indeed match with changes in microbiome diversity. Alternatively, there may be smaller-scale changes in host physiology that underlie these changes in diversity, such as alterations in host immune physiology (Fukatsu and Kudsk, 2011; Hodin et al., 2011) or mucus dynamics (Thompson and Applegate, 2006). This should then be followed by investigations of the potential roles played by microbes in the restructuring of distal intestine morphology (Scheppach, 1994; Sharma et al., 1995; Scheppach et al., 1997; Okada et al., 2013) in preparation for digestion after the resumption of feeding (Secor et al., 1994; Costello et al., 2010).

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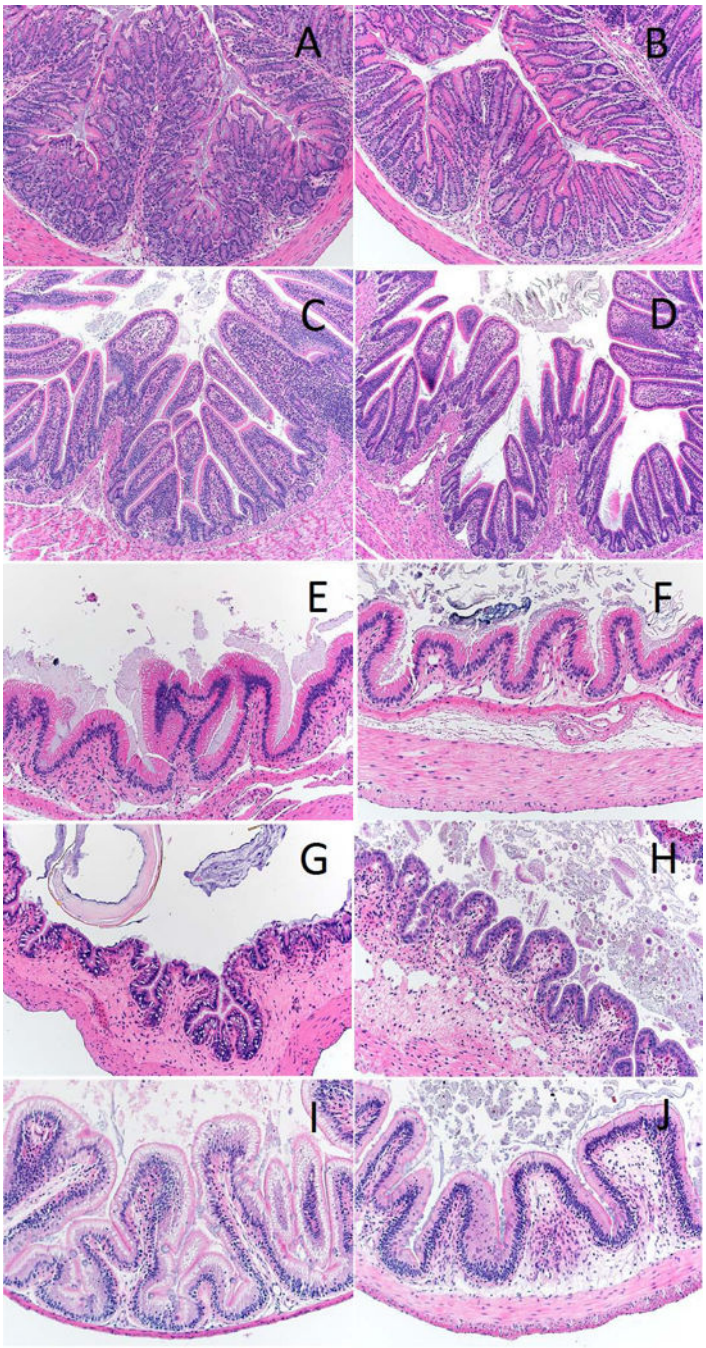

Figure 1.
Representative light micrographs of distal intestine cross sections of each taxon at the beginning and end of the fasting trials, respectively. (A-B, mouse; C-D, quail; E-F, gecko; G-H, toad; I-J, tilapia)

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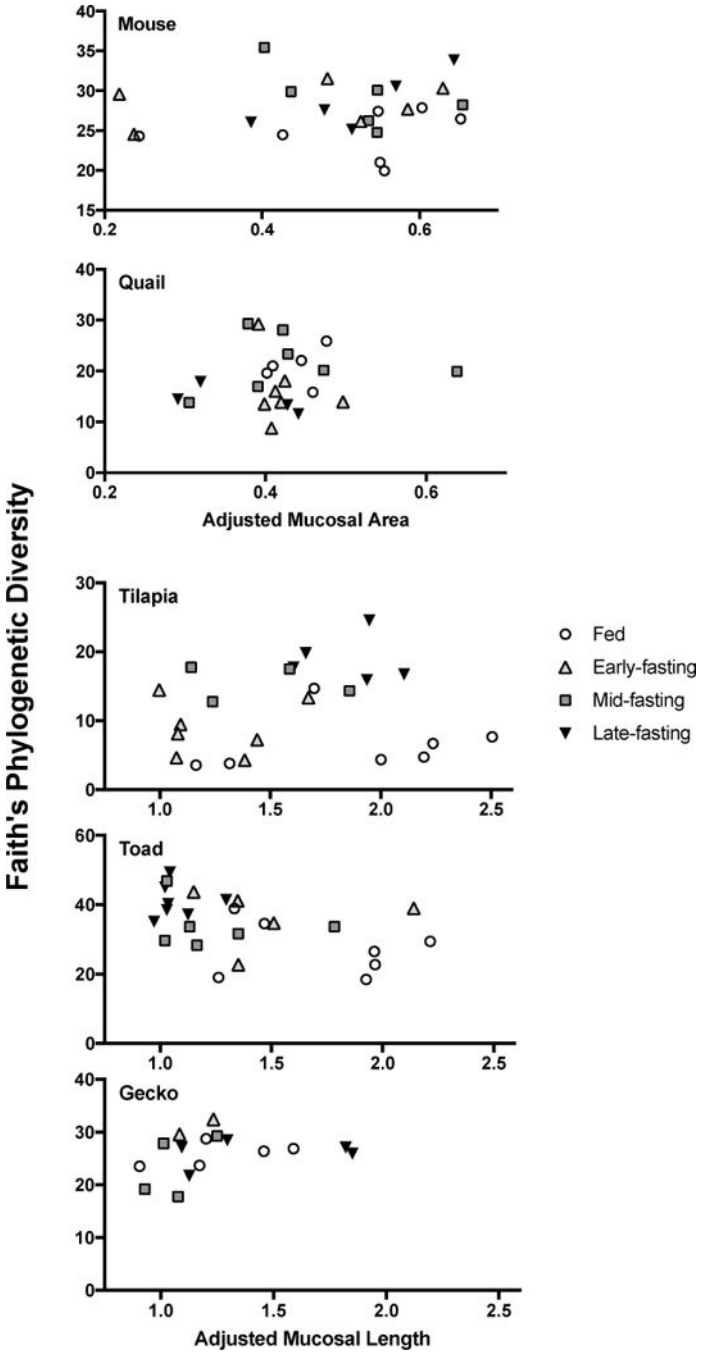

Figure 2.

Relationships between Faith's phylogenetic diversity and either adjusted mucosal area (for endotherms) or adjusted mucosal length (for ectotherms). No relationships were statistically significant.

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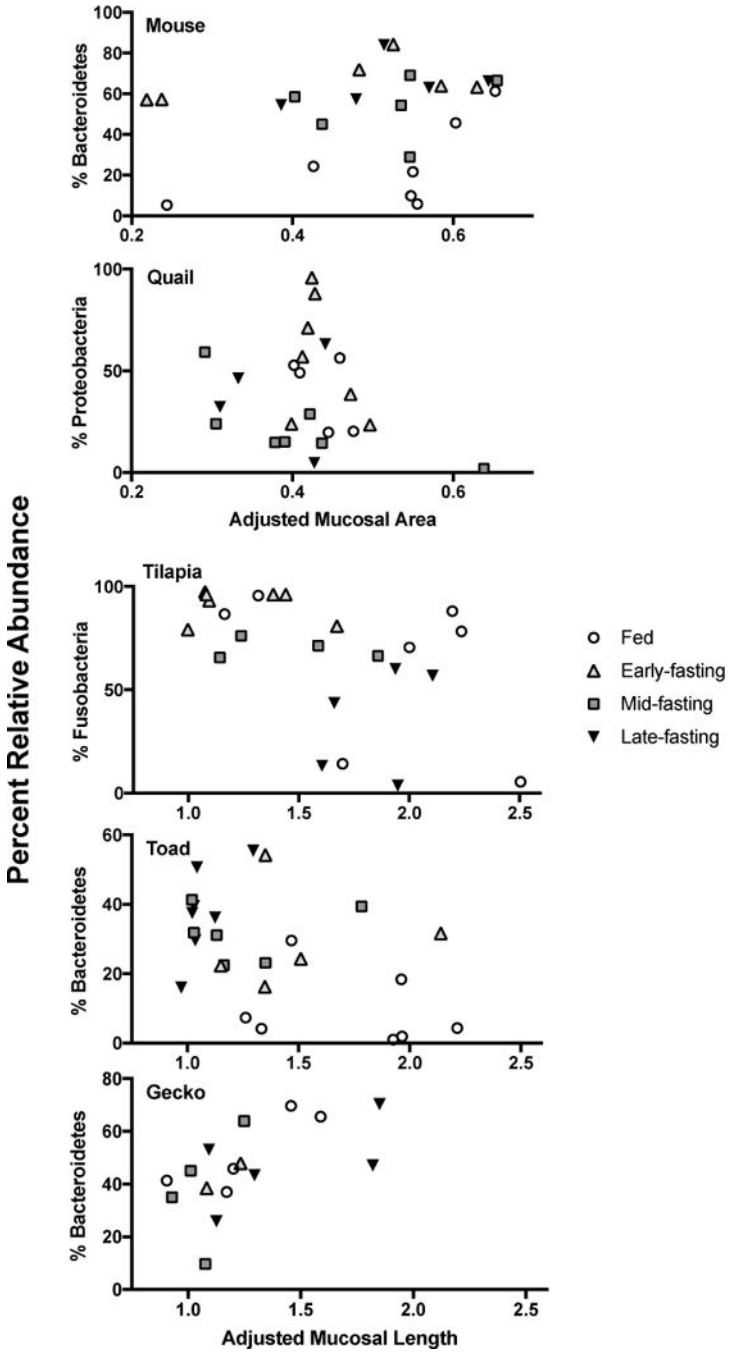

Figure 3.

Relationships between the relative abundances of dominant microbial phyla and either adjusted mucosal area (for endotherms) or adjusted mucosal length (for ectotherms). No relationships of any microbial phyla were statistically significant.

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Table 1

Phylogenetic diversity (PD) of microbial communities at various fasting time points. Numbers represent PD means ± standard error. Diversity was compared within a species using Tukey's HSD test. Values not sharing superscripted letters are significantly different. The last column presents the two most dominant microbial phyla in each species. More detailed analysis can be found in Kohl et al. (2014).

Taxon	Fed	Early-fasting	Mid-fasting	Late-fasting	Major Phyla
Mouse	24.49 ± 1.16^{a}	28.30 ± 1.09^{b}	29.97 ± 1.53^{b}	29.06 ± 1.20^{b}	Bacteroidetes Firmicutes
Quail	22.82 ± 1.69^{a}	16.21 ± 2.42^{bc}	21.65 ± 2.14^{ab}	14.31 ± 1.33^{c}	Proteobacteria Firmicutes
Tilapia	6.51 ± 1.48^{a}	8.81 ± 1.49^{a}	15.60 ± 1.22^{b}	18.95 ± 1.54^{b}	Fusobacteria Firmicutes
Toad	27.07 ± 2.92^{a}	36.25 ± 3.69^{b}	33.98 ± 2.71^{ab}	40.95 ± 1.83^{b}	Firmicutes Bacteroidetes
Gecko	26.43 ± 1.01	29.01 ± 0.97	24.74 ± 2.02	26.26 ± 0.97	Bacteroidetes Verrucomicrobia

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Table 2

Animals used in the fasting experiments. The first number is the number of days that postabsorptive animals were fasting. The value in parentheses refers to the sample size at each time point.

Taxon	Scientific name	nourished	early-fasting	mid-fasting	late-fasting
Mouse	*Mus musculus*	0 (7)	1 (6)	2 (5)	3 (8)
Quail	*Coturnix coturnix*	0 (7)	2 (7)	4 (7)	7 (4)
Tilapia	*Oreochromis niloticus*	0 (7)	7 (5)	14 (6)	21 (6)
Toad	*Anaxyrus terrestris*	0 (7)	7 (5)	14 (6)	21 (7)
Gecko	*Eublepharis macularius*	0 (7)	7 (7)	14 (4)	28 (5)

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Table 3

Measurements of the distal intestine in fasting endotherms. Values are means followed by standard deviations in parentheses. Mucosa areas are presented in absolute terms (i.e. mm^2) or in a unitless value (denoted by '–') that adjusts for body size by dividing the mucosa area by the cross sectional area. P-values were corrected using the Benjamini-Hochberg False Discovery Rate correction. When correlating large intestinal measurements with microbial diversity, fasting state and interaction terms were included in the model, though these were not significant in any models. Here, we only present the P-values for correlation of anatomical measurements with microbial diversity.

			Fasting day					
Mouse	**metric**	**units**	**0**	**1**	**2**	**3**	**Effect of Fasting P-value**	**Correlation with Microbial Diversity P-value**
Cross section	area	mm^2	6.45 (4.86)	9.14 (4.48)	5.43 (1.64)	4.39 (0.45)	0.32	0.87
Serosa	perimeter	mm	8.47 (2.80)	10.58 (2.56)	8.58 (1.47)	7.23 (0.35)	0.32	0.87
Tunica muscularis	thickness	mm	0.161 (0.052)	0.12 (0.032)	0.143(0.072)	0.134 (0.032)	0.69	0.87
Basement	perimeter	mm	13.698 (4.472)	17.668 (3.829)	15.38 (5.618)	11 (4.037)	0.32	0.87
Lumen	perimeter	mm	13.367 (4.855)	17.365 (3.28)	15.668 (5.485)	11.16 (5.084)	0.32	0.87
Lumen	area	mm^2	1.93 (1.29)	3.83 (1.60)	1.26 (0.56)	0.88 (0.21)	0.47	0.91
Mucosa	thickness	mm	0.24 (0.072)	0.212 (0.045)	0.234 (0.085)	0.266 (0.052)	0.70	0.87
Mucosa	area	mm^2	2.759 (0.725)	3.528 (0.856)	2.737 (0.533)	2.258 (0.354)	0.30	0.87
Mucosa	area	–	0.511 (.136)	0.447 (.177)	0.520 (0.090)	0.519 (.097)	0.74	0.87
			Fasting day					
Quail	**metric**	**units**	**0**	**2**	**4**	**7**	**Effect of Fasting P-value**	**Correlation with Microbial Diversity P-value**
Cross section	area	mm^2	6.020 (1.269)	6.638 (0.649)	6.329 (2.989)	6.650 (1.736)	0.95	0.95
Serosa	perimeter	mm	8.520 (0.801)	8.601 (0.440)	8.270 (1.753)	8.822 (1.017)	0.95	0.95
Tunica muscularis	thickness	mm	0.283 (0.056)	0.29 (0.061)	0.254 (0.059)	0.235 (0.031)	0.71	0.95
Basement	perimeter	mm	8.518 (1.284)	8.801 (0.536)	8.987 (2.073)	8.609 (1.103)	0.95	0.95
Lumen	perimeter	mm	5.346 (0.941)	5 (0.855)	5.977 (2.551)	5.961 (1.637)	0.95	0.95
Lumen	area	mm^2	0.820 (0.402)	0.989 (0.402)	1.391 (1.950)	1.989 (1.593)	0.80	0.95
Mucosa	thickness	mm	0.384 (0.040)	0.403 (0.08)	0.359 (0.086)	0.316 (0.043)	0.71	0.95
Mucosa	area	mm^2	2.626 (0.534)	2.786 (0.210)	2.534 (0.655)	2.346 (0.502)	0.80	0.95
Mucosa	area	–	0.438 (0.032)	0.421 (0.035)	0.435 (0.104)	0.365 (0.066)	0.71	0.95

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Table 4

Measurements of the distal intestine in fasting ectotherms. Values are means followed by standard deviations in parentheses. The lengths of the mucosa lengths are presented in absolute terms (i.e., mm) or in a unitless value (denoted by '–') that adjusts for body size by dividing the mucosa length by the serosa perimeter. P-values were corrected using the Benjamini-Hochberg False Discovery Rate correction. Unique subscripts indicate significant differences between fasting day 0 and other time points according to Holm-Sidak pairwise tests. When correlating large intestinal measurements with microbial diversity, fasting state and interaction terms were included in the model, though these were not significant in any models. Here, we only present the P-values for correlation of anatomical measurements with microbial diversity.

			Fasting day					
Tilapia	**metric**	**units**	**0**	**7**	**14**	**21**	**Effect of Fasting P-value**	**Correlation with Microbial Diversity P-value**
Cross section	**area**	**mm^2**	**2.912 (1.168)^a**	**4.411 (2.482)^a**	**1.883 (0.891)^a**	**2.063 (0.771)^a**	**0.025**	0.71
Serosa	**perimeter**	**Mm**	**6.31 (1.14)^a**	**7.51 (1.09)^a**	**5.04 (1.37)^a**	**5.26 (0.89)^a**	**0.025**	0.71
Tunica muscularis	**thickness**	**Mm**	**0.030 (0.007)^a**	**0.037 (0.009)^a**	**0.031 (0.010)^a**	**0.051 (0.011)^b**	**0.019**	0.71
Submucosa	**thickness**	**Mm**	**0.039 (0.014)^a**	**0.055 (0.009)^b**	**0.044 (0.007)^a**	**0.049 (0.007)^a**	**0.028**	0.71
Mucosa	**length**	**mm**	**11.49 (2.51)^a**	**9.22 (2.69)^a**	**7.34 (2.03)^b**	**9.33 (1.45)^a**	**0.025**	0.79
Mucosa	**length**	**–**	**1.87 (0.50)^a**	**1.25 (0.25)^b**	**1.49 (0.41)^a**	**1.79 (0.24)^a**	**0.025**	0.79
			Fasting day					
Toad	**metric**	**units**	**0**	**7**	**14**	**21**	**Effect of Fasting P-value**	**Correlation with Microbial Diversity P-value**
Serosa	**perimeter**	**mm**	**11.0 (4.4)^a**	**17.2 (4.8)^b**	**12.9 (3.0)^a**	**15.9 (2.0)^b**	**0.044**	0.87
Tunica muscularis	thickness	mm	0.086 (0.034)	0.113 (0.057)	0.143 (0.054)	0.118 (0.044)	0.26	0.82
Submucosa	**thickness**	**mm**	**0.12 (0.04)^a**	**0.26 (0.11)^b**	**0.20 (0.10)^a**	**0.17 (0.04)^a**	**0.014**	0.82
Mucosa	length	mm	19.548 (10.051)	24.830 (13.149)	15.621 (2.875)	17.024 (2.557)	0.26	0.87
Mucosa	**length**	**–**	**1.73 (0.37)^a**	**1.41 (0.40)^a**	**1.25 (0.29)^b**	**1.08 (0.11)^b**	**0.014**	0.82
			Fasting day					
Gecko	**metric**	**units**	**0**	**7**	**14**	**28**	**Effect of Fasting P-value**	**Correlation with Microbial Diversity P-value**
Cross section	area	mm^2	12.7 (5.9)	14.1 (1.91)	16.0 (4.9)	13.2 (6.7)	0.86	0.97
Serosa	perimeter	mm	13.0 (3.2)	14.2 (0.2)	14.4 (2.2)	13.1 (3.3)	0.86	0.97
Tunica muscularis	thickness	mm	0.17 (0.05)	0.13 (0.03)	0.18 (0.06)	0.21 (0.06)	0.84	0.97
Submucosa	thickness	mm	0.175 (0.119)	0.148 (0.027)	0.103 (0.037)	0.140 (0.068)	0.86	0.97
Mucosa	length	mm	16.8 (6.9)	16.4 (1.7)	15.3 (2.5)	18.1 (3.0)	0.86	0.97
Mucosa	length	–	1.27 (0.07)	1.16 (0.11)	1.07 (0.14)	1.44 (0.37)	0.84	0.97