Mechanical Properties of Alimentary Tissues in Teleostean Fishes

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Abstract

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Fishes are a diverse paraphyletic group that have evolved numerous abilities to occupy a diversity of niches. One successful feeding strategy that has independently evolved in many clades of fishes is commonly known as durophagy—specializing in eating hard prey by crushing protective shells of mollusks and crustaceans, or ingesting whole intact prey. The majority of studies on feeding in fishes have focused heavily on mechanistic and performance analyses of jaw mechanics and prey capture but few studies have examined the effects of ingesting hard or even soft prey on visceral alimentary tissues. Moreover, secondary loss of the stomach in fishes has also occurred independently in approximately 15-20% of extant species, and stomachless fishes must resist mechanical damage from the initial influx of hard prey structures. If the mechanical properties of the intestinal tissues are not able to withstand the varying degree of physical stress caused by the influx of hard structures (e.g. exoskeletal fragments and spines), especially without the aid of a distensible storage, then mechanical damage could lead to premature death. It would therefore be advantageous for the alimentary system to evolve the ability to withstand mechanical stresses acting on the intestinal wall, particularly as prey size and intake increase with body size, in order to maintain its proper function. The main objective of this dissertation is to evaluate the mechanical properties of intestinal tissues in teleost fishes. I address two distinct questions: (1) do tissue properties vary radially and spatially along the length of the alimentary tract? (2) do tissue properties of the alimentary tract differ over ontogeny? I test the hypothesis that the mechanical properties of the alimentary tract will differ along its length and over ontogeny, and predicted that the proximal region of the gut should be the strongest and most extensible in stomachless fishes that lack a storage depot. I used two stomachless species of surfperches (Family: Embiotocidae), *Cymatogaster aggregata* and *Embiotoca lateralis* to gain insight into these questions. I developed a custom pressure inflation technique to measure the passive mechanical properties of the intestinal tract. I showed that in *Cymatogaster aggregata* the mechanical properties differ significantly at three relative positions (25%, 50%, and 75%) along the length of the alimentary tract, with 25–46% greater ultimate strength, strain, extension ratio, and toughness at the proximal (25%) position compared to the more distal (50% and 75%) positions. I also found find that the alimentary tissues are highly extensible and anisotropic, with the proximal (25% position) capable of a radial expansion of 360% percent from the initial state. I next quantified the allometric changes in alimentary morphology and mechanical properties of the striped perch, *Embiotoca lateralis*, at three relative positions along the intestinal tract in fish ranging from 4-22 cm in length. My results showed that gut mass and length grow isometrically whereas fish mass, gape area, outer gut circumference and gut wall thickness showed positive allometric growth relative to body length. The mechanical properties changed along the intestinal tract and were 5% stronger, 34% more extensible and 29% tougher at the proximal (25%) position of the intestine compared to the more distal (75%) position. Tissues in fish within large and medium size classes were similar and were 18% stronger, 20% tougher, and 49% more extensible than small size classes. These studies provide insight into the mechanistic and

developmental variations in mechanical properties along the gastrointestinal tract and over ontogeny. My results also corroborate the suggestion that the proximal section of the intestinal tract acts as a "stomach" in these agastric fishes. These data contribute to our knowledge of the mechanical properties of alimentary tissues and guide future studies of factors that may not only influence possible selective pressures driving the evolution of intestinal tissues but also the evolution of teleostean fishes.

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Acknowledgments

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Dedication

To all the haters, naysayers, and obstacle placers, thank you for boosting my energies and pushing me to reach higher limits.

The *unfortunate* SARS-CoV-2 (COVID-19) pandemic *certainly* made things interesting at the very end.

Chapter 1

A review of durophagy and the gastrointestinal tract in teleostean fishes

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1.1 INTRODUCTION

1.1.1 Fishes

Fishes are a diverse paraphyletic group comprising over 29,000 species which span nearly every geographical niche across the globe, from hydrothermal vents to high-elevation mountains (Nelson 2006). Fishes have evolved numerous structures, behaviors, and abilities to occupy a diversity of niches with remarkable success and are the ancestors of all tetrapodamorpha— animals with four limbs. The vertebrate jaw is arguably the most important evolutionary achievement, allowing for the exploitation and occupancy of new niches that contributed to the diversification of this group. As a result, the day-to-day feeding strategies of fish are fairly diverse; some eat only detritus, some are opportunistic and gorge or consume seasonally available prey, some are strict herbivores, and some have the ability to consume hard prey, a strategy called durophagy.

1.1.2 Durophagy

The term durophagy (from the Latin: *durus* "hard" and Greek: *phagia* "to eat") is used to describe the eating behavior of organisms that consume hard prey composed mainly of exoskeletons and shells. This feeding strategy has evolved independently numerous times in both cartilaginous and bony fishes dating back in the fossil record to the early Cambrian; there was a marked increase in durophagy, along with the successful innovation of the vertebrate stomach, during the Mesozoic marine revolution (Oji et al. 2003; Zaton and Salamon 2008; Koelz 1992). Hard prey consumption is used by many vertebrate groups, including some mammalian carnivores, reptiles and birds, however, this term is generally associated with fishes. Durophagous fishes are found across the full spectrum of this paraphyletic group, and most predators that specialize in eating hard prey do so by crushing protective shells of mollusks and crustaceans (Nelson 2006). Fish usually process captured prey by fragmenting shells before they are ingested, but may also swallow whole intact prey (Lauder

1987; Norton 1988); very few regurgitate indigestible material (Hattin 1996; Hulsey 2006). Durophagy and other modes of feeding require specific adaptations to successfully process food, and the majority of studies on feeding in fishes have focused heavily on mechanistic and performance analyses of bite force mechanics (Huber et al. 2005), cranial morphology (Dean 2007), prey capture behavior and kinematics (Pietsch 1978; Sasko et al. 2006), and dentition specializations (Norton 1988). Despite the fact that most fishes swallow minimally masticated prey, very few studies have examined the effects of ingesting hard or even soft prey on visceral alimentary tissues.

1.1.3 Gastrointestinal Tract

The gastrointestinal tract (GIT) of fishes undergoes several morphological and physiological transformations during development, with the most dramatic changes occurring during hatching and early larval development as gut differentiation and enzymatic activity increases (Kawai and Ikeda 1973; Segner et al. 1994; Kamisaka et al. 2001). This dynamic and multifunctional system functions primarily to breakdown food (mechanically and chemically) into usable micromolecules that can be absorbed easily to provide the body with energy and nutrients (Cyrino et al. 2008). The GIT is composed of four basic morphofunctional divisions: the *headgut* that secures and mechanically process prey, the *foregut* that chemically breaks down ingested material, the *midgut* that absorbs nutrients, and the *hindgut* that stores and excretes waste (Harden 1975). The foregut and midgut show the greatest morphological diversity and their shape changes depending on diet; for example, intestines are longer in herbivorous fishes to increase nutrient absorption (Suyehiro 1942). In addition, the basic radial wall structure of the GIT consists of four layers: the *tunica mucosa,* mucosal epithelium and vascularized connective tissue, the *submucosa,* connective tissue, the *tunica muscularis,* circular and longitudinal muscle, and the —*tunica serosa*, mesothelial cells and vascularized connective tissue (Harden 1975). Collagen (stiff) and elastin (stretchy) fibers serve as the main structural components that maintain gut wall integrity as smooth muscle provides minimal structural support (Krafka 1939; Wainwright et al. 1982). The GIT also performs a variety of homeostatic and physiological functions other than nutrient absorption, such as non-digestible waste excretion, osmoregulation (Yuge et al. 2006), hormonal regulation (Taylor and Grosell 2006), immune defense (McGhee and Kiyono 1999), predation defense (Brainerd 1994), gas exchange (Wu and Chang 1945; Yadav and Singh 1980), and withstands degradation in the presence of digestive enzymes, such as lipase and trypsin (Sklan et al. 2004).

Typically, the primary structure of the GIT is the stomach, a distensible pouch-like structure in the foregut where consumed food is stored before chemical breakdown by a variety of pepsinogens and HCL (Smit 1968). However, secondary loss of the stomach in fishes has occurred independently numerous times, in approximately 5-8% of fish families and 15-20% of extant species (Suyehiro 1942; Nelson 2006). Fishes without stomachs must not only absorb nutrients but also handle the initial influx of consumed prey without the aid of a distensible storage depot. However, the alimentary tissue must also resist mechanical damage from indigestible material passing through this complex system; particularly in stomachless fishes. Although mucus serves an important role in protecting the epithelia from mechanical injuries or bacterial invasion (Humbert et al. 1984; German 2010), if the mechanical properties of the intestinal tissues are not able to withstand the potential damage caused by the influx of large prey, gorging during acute or sudden food availability, or sharp foreign bodies (e.g. shell shards, organismal spines and bones, exoskeletal fragments, or calcite pellets), then mechanical damage could result, which could reduce digestive efficiency and even lead to death (Walsh et al. 1991; Compton and Kerfoot 2004).

Soft biological tissues exhibit a vast array of structural and functional diversity. The mechanical performance of these pliant composites is largely due to the assemblage of relatively few structural elements, such as collagen and elastin fibers. Variation in fiber ultrastructure—from the molecular composition to whole tissue orientation—can lead to broad-spectrum changes in the mechanical properties of the tissue (Shadwick et al. 1992; Gosline et al. 1999; Gosline 2018). The functional range of these structures is fundamentally dependent on the deformation experienced by the tissue. As a bolus travels down the gut in fishes, the multifunctional alimentary system experiences a range of deformations due to the diverse set of prey, and varying degree of physical stress imposed at various stages of digestion. Therefore, it would be advantageous for the alimentary system to evolve the ability to withstand these mechanical stresses in order to maintain its proper function.

1.1.4 Objectives

The main objective of this dissertation is to evaluate the material properties of intestinal tissues in select teleost fishes. The general structures of fish alimentary tissues are well known, as are the more specialized structures used in specific feeding strategies, such as cytological structures that have enabled the consumption of soft, large, and hard prey (Al-Hussaini 1949). The vast majority of research on the alimentary tract of fishes within the last 20 years mainly focuses mainly on systems other than tissue mechanics (Figure 1.1); though Burnstock (1959) was one of the first to question the extensibility of fish stomachs. In this study, I focused on the ontogenetic and spatial variation in mechanical properties along the length of the alimentary tract in two species of surfperches (Family: Embiotocidae): *Cymatogaster aggregata* (shiner perch) and *Embiotoca lateralis* (striped perch). The majority of prey consumed by these fish are small zooplankton, amphipods, mollusks, and crustaceans (Fritzsche and Hassler 1989). In addition, these fishes feature an alimentary tract that is agastric (lacking a true stomach) and is completely cylindrical lengthwise. The absence of the stomach is particularly interesting because there is no ‘storage’ compartment, i.e. a true stomach, where large prey can accumulate before being chemically broken down into smaller components before travelling down the gastrointestinal tract. I address two distinct questions: (1) do tissue properties vary along the length of the alimentary tract? (2) do the mechanical properties of the alimentary tissues differ over ontogeny? These studies detail the mechanistic and transformational changes in gut tissues over ontogeny, and the variation and basis of change in mechanical properties along the gastrointestinal tract, thereby providing insight into the evolution and mechanical design of alimentary tissues in teleostean fishes.

1.1.5 Pressure System

To measure the variation in mechanical properties along the length of the alimentary tract, I used a custom pressure apparatus modified from inflation techniques established by Gosline and Shadwick (1996). I use the stomachless teleost fish *Cymatogaster aggregata* to investigate the strength, extensibility, toughness, and extension ratio along the alimentary tract at three relative positions along the length of the tract (25%, 50%, 75%). Whole intact digestive tracts were carefully removed to ensure the tissue fiber network remained intact and marked at the relative positions. The proximal end was then connected to the pressure apparatus, flushed, and the distal end was then sealed. A high-definition camera was mounted, and inflation behaviors at incremental pressures for each tissue were recorded until failure. I used the percent wall thickness from histological cross-sections and a set of equations, which assumed constant tissue volume during inflation, to generate stress-strain curves in order to quantify the mechanical properties of the alimentary tissue. The results showed there was no significant difference in the mean outer radius, wall thickness, or mean percent wall thickness among the three relative positions (25%, 50%, 75%; proximal, midpoint, distal, respectively) along the alimentary tract and that the load-bearing wall was approximately 5% of the radius. Mechanical properties were assumed to be independent of fish length over the small range of body sizes tested. Mean breaking strength decreased distally, with the proximal locale 46%

stronger than the more distal locale. Mean breaking strain and extension ratio both decreased significantly along the alimentary tract, with the proximal locale 25-39% more extensible compared to and the more distal positions. The proximal portion of the alimentary tract had a maximal expansion of 360% percent and was also 45.0% tougher than the other two locales. Strain ratios were 3.25 times higher than the expected strain-ratio value of 2 for the inflation of a standard cylinder, an indication of the anisotropic properties of these tissues. These results suggest that the proximal section of the intestinal tract acts as a "stomach" in this agastric fish.

I next assessed the allometric relationship between morphometric traits (e.g. fish mass, gape diameter) and alimentary mechanical properties (e.g. strength, extension ratio, toughness) over a broad size range (4-22cm SL) of striped perch, *Embiotoca lateralis*, to provide insight in ontogenetic changes alimentary tissue mechanics. The established pressure techniques were used to inflate tissue segments from the three locations (25%, 50%, 75%) along the length of the intestine for each individual. I found a significant and positive correlation between all fish and gut morphometric variables, with both gut mass and gut length exhibiting isometric growth relative to fish length, while fish mass exhibited 6% positive allometric growth relative to fish length. Fish gape became proportionally shorter and wider (-12% and +24%, respectively) and gape area showed an allometric increase of 9%. The outer gut circumference and wall thickness exhibit a positive and significant correlation that were 17% and 23% percent higher than expected, while the relative percent wall thickness did not differ from an expected slope of zero and was approximately 7% of the structural radius.

The alimentary mechanical properties depended strongly on fish length as well as location. Strength, extensibility, and toughness decreased along the intestinal tract, with at the 25% locale 5% stronger, 34% more extensible and 29% tougher than the 75% locale. The mechanical properties of the smallest sized fish were markedly lower than the medium and large sized fish and did reveal clear linear scaling relationships with respect to fish length like those seen in for the morphological and histological parameters. Strength differences between the large and medium groups along the length of the alimentary averaged 2% between the three positions, whereas an average positional difference of 20% occurred between large and small groups. Similar positional trends were found in toughness and extension ratio, where average differences between large and small groups of 20.0% and 37%, respectively.

Altogether, I developed a custom quasi-static pressure system to quantify the relative changes in the passive mechanical properties along the length of the alimentary tract and over ontogeny of the stomachless embiotocids, *Cymatogaster aggregata* and *Embiotoca lateralis*. These data show that mechanical properties differ along the length of the alimentary tract for both species, and exhibit strong scaling relationships for the morphological and histological parameters with respect to fish length. Surfperches are viviparous, so they are more developed, and even sexually mature at birth but eat smaller, less complex prey items than adults. Therefore, similarities in alimentary tissue architecture are not surprising given digestive capabilities and maturity of this fish at birth. The shift in alimentary mechanical properties once gape and body length increase in size giving middle and larger fish the ability to exploit larger prey. Mechanical capabilities would be highly adaptive in order to accommodate larger prey and resist potential damage associated with processing structural complex prey. The ultimate strength, strain, extension ratio, and toughness were significantly greater at the proximal (25%) position than the more distal (50% and 75%) positions along the alimentary. Additionally, the tissues were highly extensible and anisotropic at the initial 25% position, where it takes more energy per unit tissue volume to cause tissue failure and withstands large-scale energy absorption and circumferential deformations (pressure stress) associated with the influx of prey items. The maximum extension ratio (λ) of the intestine at full rupture was 3.6 in the shiner perch and 2.8 in the striped perch, with the former substantially higher than approximate values for the stomach of humans and guinea pigs, 2.4 and 2.0, respectively (Egorov et al. 2002, Storkholm et al. 1998). Al-Hussaini (1946) and Wallace et al. (2005) referred to the anterior portion of the intestinal tract of stomachless fishes as an intestinal bulb that likely functions as a reserve, and these data support their hypothesis. This may also be the mechanism adopted by other stomachless fishes to deal with the forces generated with primary ingestion of diverse prey.

1.1.6 Future Studies

Although we did not investigate tissue composition and ultrastructure, there was no distinguishable difference in the percent wall thickness of 5-7% along the alimentary tract at the three relative positions (25%, 50%, 75%), suggesting differences in mechanical properties are due to fiber composition and orientation as these factors can affect tissue load responses (Clark and Cowey 1958; Gosline 1971; Wainwright et al. 1978). As the stiffer collagen and more extensible elastin fibers create a system of cross-linkages that traverse the length of the intestinal tract, fiber orientation may differ in the area of the "intestinal bulb" which could explain the observed material differences and anisotropic distensibility at the 25% locale. Therefore, changing the fiber orientation relative to the long axis of the structure could influence and alter the regional mechanical properties, and this could be advantageous for at gut that experiences differential stresses as non-uniform prey items decrease in size as they are processed and travel down the gut. In addition, it is now possible to 3-D print tubes using various fibrous tissue types (e.g. elastin, collagen, or comparable materials) and modify the orientation and wall thickness to better understand how subtle changes in these parameters affect tissue behaviors and mechanical properties. The synthesis of man-made materials specifically designed for optimal compatibility with biological systems has the potential to fundamentally change the field of medicine, with the development of artificial veins, arteries, values, fascia, etc. The bio-inspired design of flexible piping could have useful applications in manufacturing and construction industries.

In addition to ultrastructure studies, a detailed phylogenetic perspective would shed light on the complex role of mechanical properties in an evolutionary context. There are several cases where a single genus expanded to new niches through adaptation of feeding and digestion modes to exploit novel food resources in relatively short evolutionary time (Campbell et al. 2005; Olsson et al. 2007; Baliga and Mehta 2016). Also, a specific trophic strategy may influence the intestinal length, longer versus shorter for herbivores and carnivores, respectively (Al-Hussaini 1949; Fange and Groove 1979; Lobel 1981; Buddington and Diamond 1987). This is likely due to the plastic nature of intestinal tissues that adapt to geographic and dietary availability over time. However, the extent to which alterations in the mechanical properties of alimentary tissues contributed to the exploitation of these niches remains unknown. Future studies could test if the mechanical properties of phylogenetically related groups remain similarly constrained, or change based on the dietary specificity of a given species, as several studies showed strong relationships between morphological and histological parameters with respect to evolutionary history (Elliott and Bellwood 2003; German and Horn 2006; German et al. 2010b). German and Horn (1996) have shown that gastrointestinal mass and length of prickleback fishes are influenced more by ontogeny and phylogeny than diet, suggesting genetic adaptations over plasticity. However, it is unknown whether selection has altered the mechanical properties of the alimentary system (i.e. associative fiber network and ultrastructure) to optimize mechanical function in order to accommodate the range of physical stresses placed on this tissue during the consumption of different prey types, or whether intraspecific mechanical differences are simply due to phylogenetic constraints. I used sequences from GenBank to infer a preliminary phylogenetic relationship of pricklebacks (Figure 1.2). Sequences were aligned using the software program Muscle (v. 3.18), and phylogenetic inferences were determined using MrBayes (v. 3.1.2). Combining the phylogenetic knowledge of a group (pricklebacks) with mechanistic function can provide insight into the evolutionary change of these vital properties, and would eventually allow us to determine if mechanical properties differ among closely related species with different diets.

Overall, this study used whole and segmented tissue inflation testing to establish that the mechanical properties differ along the length of the alimentary tract of a stomachless fish, and change over ontogenesis. This study provides insight into the various selective pressures driving the evolution of intestinal tissues; the mechanical properties of alimentary tissues should be a considered in our quest to understand the evolutionary and ecological importance of intestinal morphology, gut development, feeding behaviors, and digestion in fishes.

1.2 TABLES AND FIGURES

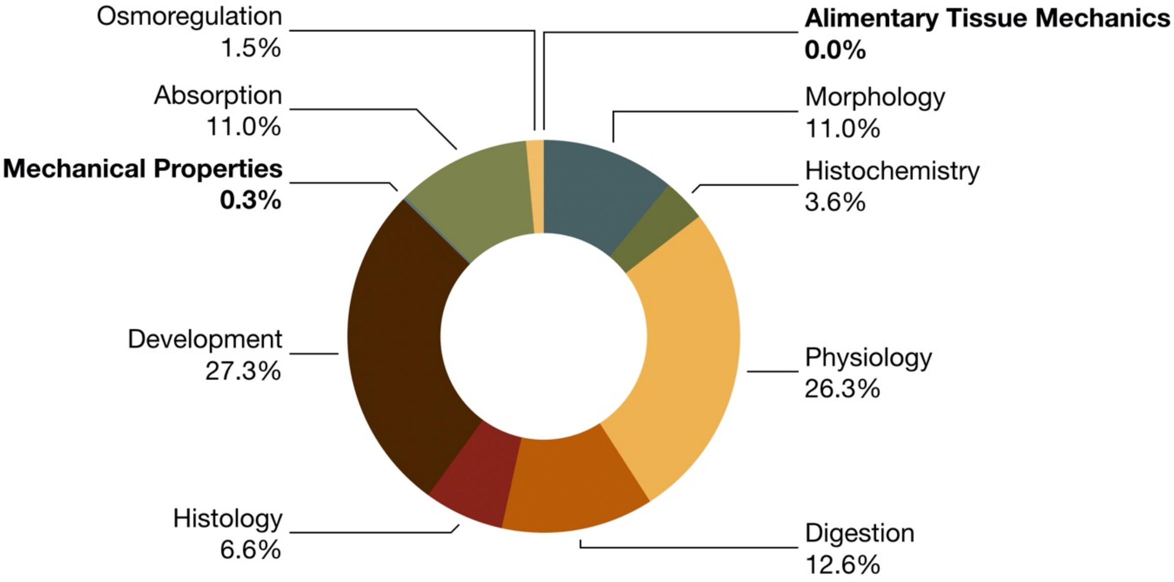

Figure 1.1 Main topics of study on the gastrointestinal tract. Results from a literature search using the Scopus database. Note: Mechanical properties returned 26 articles but none where specifically related to studies on mechanical properties of alimentary tissues

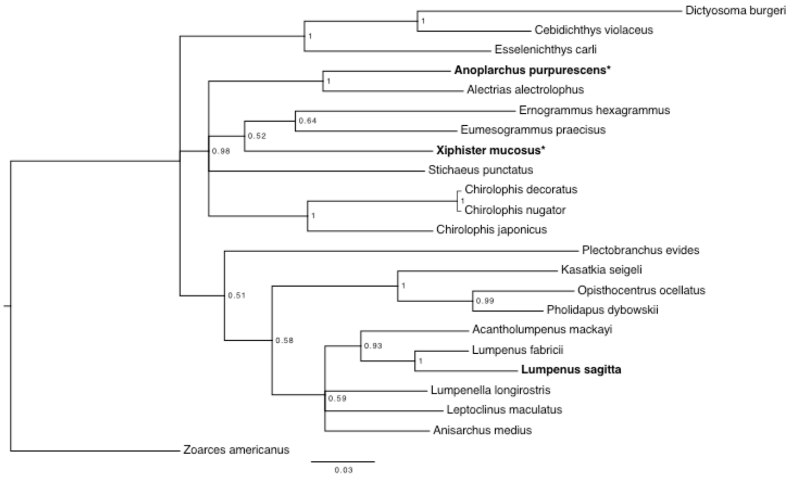

Figure 1.2 Phylogenetic relationships among 22 species of stichaeid (prickleback) fishes, using cytochrome c oxidase subunit I (CO1) sequences from GenBank. Values at nodes are posterior probabilities calculated using a Bayesian analysis. The CO1 nucleotide gene sequences for three study prickleback species were not found in the GenBank database and therefore would need to be sequenced before final phylogenetic analyses. The two species with asterisks are sister taxa to *Anoplarchus insignis* and *Xiphister mucosus*, respectively.

Table 1.1 Scopus literature search total results and for each search topic, accessed in March 2020. The *Search Terms* were: Title-Abstract-Keywords "gastrointestinal tract" OR "alimentary tract" OR "intestinal tract" AND "fish" OR "fishes" OR "teleost" AND Limit to "Articles" AND Limit to years "1999 to 2019" AND "*Study Topic*" each added separately.

Study Topic	**Total Number of Articles (n = 4911)**
	Number of Study Articles
Morphology	1103
Histology	661
Physiology	2646
Osmoregulation	146
Digestion	1261
Development	2742
Absorption	1102
Histochemistry	358
Mechanical Properties	**26**
Alimentary Mechanical Properties	**0**

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Chapter 2

Tough and stretchy: Mechanical properties of the alimentary tract in a fish without a stomach

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2.1 ABSTRACT

The mechanical properties of intestinal tissues determine how a thin-walled structure exerts forces on food and absorbs the force of food as it enters and travels down the gut. These properties are critically important in durophagous and stomachless fishes, which must resist the potential damage of foreign bodies (e.g., shells fragments) in their diet. We test the hypothesis that the mechanical properties of the alimentary tract will differ along its length. We predict that the proximal region of the gut should be the strongest and most extensible to handle the large influx of prey often associated with stomachless fishes that lack a storage depot. We developed a custom inflation technique to measure the passive mechanical properties of the intestine of the stomachless shiner perch, *Cymatogaster aggregata*. We show that mechanical properties differ significantly along the length of the alimentary tract, with 25–46% greater ultimate strength, strain, extension ratio, and toughness at the proximal (25%) position compared to the more distal (50% and 75%) positions. We also find that the alimentary tissues (excluding the heavily muscular rectum) are generally highly extensible and anisotropic, and do not differ in wall circumference or thickness along the alimentary tract. These findings contribute to our knowledge of the mechanical properties of fish intestinal tissues and guide future studies of factors influencing the evolution of fish alimentary systems.

2.2 INTRODUCTION

Durophagous fishes are predators that specialize in eating hard prey, generally by crushing the protective shells of mollusks and crustaceans (Nelson 2006). These fishes generally process captured prey by fragmenting shells before they are ingested, or at times swallow intact prey items (Norton 1988); few regurgitate indigestible material (Hattin 1996; Hulsey 2006). This feeding ecology has been found in the fossil record dating back to the early Cambrian, with a marked increase in durophagy during the Mesozoic marine revolution (Oji et al. 2003; Zaton and Salamon 2008).

Numerous studies have investigated the feeding mechanisms and performance of durophagous fishes, focusing on bite force mechanics (Huber et al. 2005), cranial morphology (Dean, 2007), pharyngeal jaw structures (Grubich 2003), prey capture behavior and kinematics (Pietsch 1978; Sasko et al. 2006), and dentition specializations (Norton 1988). All of the previous studies examine aspects associated with mechanisms and processes that enable these fishes to consume hard prey. Substantially less is known, however, about what happens after hard prey enter the digestive tract and more specifically, the effect of ingesting hard prey on intestinal tissue mechanics.

The alimentary system of fishes is a dynamic system that performs a variety of functions, such as nutrient absorption (Cyrino 2008), osmoregulation (Yuge et al. 2006), hormonal regulation (Taylor and Grosell 2006), immune defense (McGhee and Kiyono 1999), ion and water transport (Scott at al. 2006; Taylor and Grosell 2006), gas exchange (Wu and Chang 1945; Yadav and Singh 1980), even defense (Brainerd 1994). These structures must also resist tissue degradation in the presence of digestive enzymes, such as lipase and trypsin (Sklan et al. 2004). In addition, if hard shell or exoskeletal fragments are ingested, as in most durophagous fishes, then the intestinal tissues must also resist mechanical damage as indigestible material passes through this complex system.

Many teleost fishes lack a true stomach (e.g., Cyprinidae, Cyprinodonitdae, Labridae), which potentially is a plesiomorphic or secondary neotenic character (Barrington 1957; Kobegenova 1988; Harada et al. 2003a). A stomachless state has been hypothesized to be advantageous for its ability to provide an alkaline intestinal tract for fishes both in freshwater environments (Williams and Eddy 1986), and those with calcium carbonate rich diets such as durophagous fishes (Lobel 1981), as well as to reduce energetic costs (Day et al. 2011). Moreover, there are also known differences in the structural morphology of the alimentary tract, e.g., thickness, folding, stomach and gut distention (Suyehiro 1942; Al-Hussaini 1949; Burnstock 1959; German et al. 2010). Despite the lack of distinct structural compartmentalization in stomachless fishes, there must be greater proximal expansion of the gut in order to accommodate the initial influx of whole-food items, or bolus, as they enter and travel along the digestive tract. However, how the material properties and mechanical function change along the length of the alimentary tract it is not well understood. If the intestinal tissues do not sufficiently distend, large foreign bodies or prey items (e.g., shell fragments) exerting forces on this thin-walled structure, could lead to mechanical damage, which in turn could ultimately lead to death (Compton 2004). Given the necessary expansion and body confinement of the alimentary tract, knowledge of the ultimate stress, strain, extensibility, and toughness of these tissues are needed to fully understand the evolutionary history and function of alimentary tissues.

The relative symmetry and agastric nature of the alimentary system of the shiner perch *Cymatogaster aggregata* (Gibbons 1854) offers an ideal system to investigate the mechanical properties of gut tissues. In this study, we test the hypothesis that the mechanical properties of the shiner perch alimentary tract differ spatially along the length of the alimentary tract, with stronger, tougher and more extensible values near the proximal end to handle high load forced in by swallowing. We develop an inflation technique to measure the passive mechanical properties of whole alimentary tissues in fishes under quasi-static pressure testing to (1) quantify alimentary tissue breaking strength, breaking strain, toughness, and extension ratio at three relative spatial positions (25%, 50%, 75%) along the length of the tract; and (2) assess whether tissue properties at the three relative positions exhibit anisotropic characteristics (i.e., radial extension is different than longitudinal extension). This study establishes a new method that shows how the spatial variation in the mechanical properties of alimentary tissues in a stomachless and durophagous teleost fish can accommodate a complex and mechanically challenging diet.

2.3 METHODS

2.3.1 Specimens

The shiner perch, *Cymatogaster aggregata* (Gibbons 1854) is a stomachless, viviparous, and demersal fish that inhabits coastal areas rich in eelgrass beds along the eastern Pacific from Baja, Mexico to Wrangell, Alaska (Eschmeyer 1983; Figure 2.1A). Twenty *C. aggregata* (82–96 mm SL; 11.4–17.9 g) were collected by seine at Jackson Beach, San Juan Island, Washington, USA (Lat 48º31'12"N; long 123º00'36"W). Fish were maintained in flow-through seawater tanks (128 L x 76 W x 24 H cm) at Friday Harbor Laboratories (FHL), University of Washington, for a period of 4–6 days to eliminate preexisting digestive material. All animal experiments were performed in accordance with the University of Washington Institutional Animal Care and Use Committee (IACUC).

2.3.2 Fish and Alimentary tissue preparation

Fish were euthanized with 350mg/L of buffered Tricaine Methanesulphonate (MS–222) in seawater. Fish body mass (BM) was weighed (± 0.1 g), and standard length (SL), gape height (GH), and gape width (GW) measurements were taken using digital calipers (± 0.1 mm). Gape area (GA, in mm^2) was calculated using the area of an ellipse:

$$GA = \pi * (GH/2) * (GW/2) \qquad \text{Equation (2.1)}$$

Fish were then separated into two groups with similar morphological traits (i.e., total length and mass). Complete alimentary tracts were then meticulously dissected from all individuals and unfurled to avoid mechanical tissue damage (Figure 2.1B). The total length of each alimentary tract was then measured and immediately placed in a teleost Ringer's solution at 12ºC until the cessation of smooth muscle activity was observed (approximately 40 to 60 min); this procedure was repeated for all individuals in both groups. Upon removal from the Ringer's solution, the lengths of each alimentary tract were blot dried and marked at three relative positions (i.e., 25%, 50%, and 75%) with red recorder ink. Approximately five mm of tissue was trimmed from the proximal end of the alimentary tract (to the bile duct) and one cm from the distal rectal region (RR), which had a distinct brown color and firmness when handled. The alimentary tracts from one group were then marked again with red recorder ink at five mm increments to measure longitudinal expansion during materials testing. These tissues (approximately 23 mm in length) was then returned to Ringer's solution until the inflation test was performed. The alimentary tracts from the other group were then sectioned into three segments of similar length, and approximately six mm of tissue from the relative midpoint at the three marked positions (25%, 50%, 75%) were then removed for histological analyses.

2.3.3 Histological preparation & analysis

Tissue segments designated for histological analyses via light microscopy were placed in Trump's fixative (4% formaldehyde, 1% glutaraldehyde in 10 mM monobasic sodium phosphate and 6.75 mM sodium hydroxide) (McDowell and Trump 1976) for 24-32 hours at 4ºC; the solution was buffered to a pH of 7.5 to prevent degradation of tissue ultrastructure. Tissue segments were removed from the fixative and rinsed four times in 0.1 M Phosphate buffered saline (PBS) at pH 7.5

for 20 min and stored in PBS overnight at 4ºC. Samples were then rinsed with DI water, dehydrated in a graded ethanol (EtOH) series, and stored in 70% EtOH. In preparation for paraffin embedding (Paraplast X-Tra, Oxford labware; cat#8889-503002), tissues were run through an additional ethanol series of 80%, 90%, and two changes of 100% EtOH for 45 min. Gut tissue segments were then placed in a 1:1 100% EtOH/Xylene solution for 45 min, transferred to Xylene for 45 min (twice), and moved into a 1:1 Xylene/Paraffin solution for 45 min. Segments were then infiltrated in three changes of paraffin for 45 min in a vacuum oven at 60ºC. Tissues were transferred into an embedding mold with heated forceps, and the resulting paraffin blocks were stored at room temperature until sectioned.

Paraffin blocks were trimmed to the midpoint of the tissue segment and then serially sectioned at 7 μm using a mechanical microtome (model 820, American Optical Co., Buffalo New York, USA). Sections were mounted on slides and stained with a modified Milligan's Trichrome (Milligan 1946). Photographs of tissue cross-sections from three relative position segments (25%, 50%, and 75%) along the alimentary tract were taken with a Zeiss Axiocam HR camera (Axiovision Software) mounted on an Olympus SZX-12 microscope. Images were then analyzed in ImageJ (v. 1.46r) to identify gut perimeters and measure the perimeters of the outer and inner wall in order to quantify the wall radii and relative wall thickness used for data analysis (Figure 2.2).

2.3.4 Pressure Inflation

A high-definition camera was mounted over the testing area to record the inflation behavior of a whole alimentary tract (Figure 2.3). For the first group of fish, one end of an intestinal tract was sutured onto a two cm piece of polyethylene (PE) 40 tubing with human hair and sealed with a small drop of cyanoacrylate. The PE tubing was then affixed to a syringe linked to a custom pressure device (Figure 2.3). Static pressure was determined using the equation $P=\rho hg$, where P is pressure, ρ

is the density of sea water, h is the height the water container was raised, and g is the acceleration of gravity.

System pressure was slowly increased from 0 to 200–400 Pa to flush out excess lumen material and fill the tract with seawater. The pressure was then lowered back to a resting pressure of zero before testing began. The distal end of the intestinal segment was then sealed shut with hair sutures and an additional drop of cyanoacrylate was added; segments were then submerged under 5 cm of seawater. The pressure of the system was increased by 200 Pa increments to 1000 Pa, and then increased by increments of 500 Pa thereafter. Pressure equilibrium was reached within the alimentary tract before each incremental increase, generally within 30–60 seconds. This procedure was repeated until structural failure (tissue rupture) occurred.

2.3.5 Data & Computational Analysis

For all individuals, still photographs from each incremental pressure during the test run were labeled and transferred to NIH ImageJ (v. 1.46r). The total length of the alimentary tract and the diameter at three relative positions along the length of the structure (25%, 50%, 75%) were measured for all individuals.

We used the following equations to quantify the inner radius at a given relative position and pressure, assuming a constant alimentary wall volume and circular in cross-section. First, constant volume (CV, in m^3) was calculated as:

$$CV = L_o * \pi \left(r_{outer,\,initial}{}^2 - r_{inner,\,initial}{}^2\right) \qquad \text{Equation (2.2)}$$

where L_o is the initial length (in m) of the alimentary segment and $r_{outer,\,initial}$ and $r_{inner,\,initial}$ are the outer and inner radius (in m), respectively, of the intestinal lumen at zero pressure. The outer radius was measured at the relative position, and corresponding mean histological percent wall thickness (Eqn. 2.10) was used to find the inner radii. The total, wall, and lumen areas (in m^2) were calculated as follows:

$$\text{Area}_{\text{total}} = \pi * r_{\text{outer}}^2 \qquad \text{Equation (2.3)}$$

$$\text{Area}_{\text{wall}} = CV / L \qquad \text{Equation (2.4)}$$

$$\text{Area}_{\text{lumen}} = \text{Area}_{\text{total}} - \text{Area}_{\text{wall}} \qquad \text{Equation (2.5)}$$

where L is the instantaneous length of the specimen. At each pressure, the outer radius (r_{outer}, half the alimentary tissue diameter, in m), inner radius (r_{inner}), and mid-wall radius ($r_{\text{mid-wall}}$) associated with alimentary tissue distension were determined by using the following formulae:

$$r_{\text{inner}} = (\text{Area}_{\text{lumen}} / \pi)^{1/2} \qquad \text{Equation (2.6)}$$

$$r_{\text{mid-wall}} = (r_{\text{outer}} + r_{\text{inner}}) / 2 \qquad \text{Equation (2.7)}$$

The thickness (t, in m) of the intestinal wall at a given pressure was the difference between the outer and inner radii:

$$t = r_{\text{outer}} - r_{\text{inner}} \qquad \text{Equation (2.8)}$$

To assess the relative expansion, or extensibility, of the inner lumen at a given pressure, the extension ratio (λ_{inner}) was calculated as the ratio of the inner radius to the initial inner radius:

$$\lambda_{\text{inner}} = r_{\text{inner}} / r_{\text{inner, initial}} \qquad \text{Equation (2.9)}$$

The inner radius was used for all extension ratio calculations because this size determines the maximum size of an object that can move through the system within a specific region. The initial cross-sectional wall thickness was measured from the mean histological cross-sections from similar pairs, and calculated as a relative percent (%) of the overall radius of the structure using the following:

$$\text{Percent wall thickness (\%)} = (t_{\text{initial}} * 100) / r_{\text{inner}} \qquad \text{Equation (2.10)}$$

There is a very small constant pressure differential acting between the outer and inner walls of thin-walled structures (as strain varies across the wall thickness), so the mid-wall radius was used to calculate the circumferential stress for each cross-sectional tissue segment at a given incremental pressure (P). Thus, using the mid-wall extension ratio in determining the material properties provides average properties for the wall of the alimentary tract segment. The circumferential, or engineering, hoop stress σ (in Pascals, derived using Laplace's Law for a closed cylinder; Vogel 2003, 51) was calculated as:

$$\sigma = (P * r_{\text{mid-wall}}) / t \qquad \text{Equation (2.11)}$$

Whereas strain, ε, was determined from the mid-wall extension ratio:

$$\varepsilon = \lambda_{\text{mid-wall}} - 1 \qquad \text{Equation (2.12)}$$

From stress versus strain relationships, we quantified several alimentary mechanical properties at each position: ultimate stress (MPa) as the maximum force required to cause tissue failure, breaking strain (ε) as the relative change in deformation during loading per unit length, stiffness or modulus of elasticity (E) as the region of the pressure trace where force is proportional to strain, toughness (MJ/m^3) as the area under the curve that equals the energy the tissue can absorb before tissue failure, and the extension ratio or extensibility (λ) as the maximal radial extension of the gut relative to the initial no-load state (Figure 2.4). Maximal radial extension provides a rough estimate of the maximal bolus or prey size that can be passed down the alimentary tract.

We calculated the circumferential to longitudinal strain ratio of each pressure trace to determine whether alimentary material conformed to Laplace's Law, which states that in a simple closed cylinder the hoop stress is twice the longitudinal stress, i.e., radial growth is twice the increase in length. Circumferential strain was found to be substantially higher, so we focused our statistical analysis on circumferential expansion for this study.

2.3.6 Statistical analyses

Data were analyzed using the statistical program R (v. 2.15.3 GUI 1.53; R Foundation for Statistical Computing, Vienna, Austria). Fish of similar lengths were used, and data were normally distributed.

A Student's t-test was used to evaluate morphological differences between the two fish groups. An analysis of variance (ANOVA) was used to evaluate the effect of relative position (25%, 50% and 75%) along the length of the alimentary tract on each mechanical property (e.g., ultimate strength, toughness). If the effect of relative position was significant, a post-hoc Tukey HSD test was used to compare between pairs of positions.

2.4 RESULTS

2.4.1 Fish and alimentary morphometrics

All morphometric measurements are listed in Table 2.1. The length range of the whole alimentary tract for the fish used in the inflation and histology analyses were 69–94 mm and 73–91 mm, respectively. There were no significant differences in any of morphological metrics between the two analyses groups (P= 0.33–0.87; Table 2.1). There was a distinct rectal region (RR) in each tract that was typically 13% of the total intestinal length. Additionally, there was no significant difference in the mean outer radius, wall thickness, or mean percent wall thickness among the three relative positions (25%, 50%, 75%) along the alimentary tract (P= 0.39–0.62; Figure 2.5, Table 2.2); a similar finding by Young and Fox (1936). Overall, the load-bearing gut wall is approximately 5% of the structural radius.

2.4.2 Alimentary mechanical properties

The mechanical properties along the length of the alimentary tract at three relative positions (25%, 50%, and 75%) were assumed to be independent of body length over the small range of body sizes tested (Table 2.1). Mean breaking strength depended significantly on relative position and exhibited a decreasing trend distally ($F_{2,27}$=8.634; P<0.01; Figure 2.6A). Relative to the 50% position, the more proximal 25% position was significantly stronger (by 46%) while the more distal

75% position was not significantly different (Table 2.3).

A similar pattern was observed in all of the other material properties we measured (Table 2.3). Mean breaking strain decreased significantly along the alimentary tract ($P<0.01$; Figure 2.6B). Relative to the gut midpoint (50% position), the more proximal 25% position was 39% more extensible while the more distal 75% position was not significantly different. Mean extension ratio (λ) decreased significantly along the alimentary tract ($P<0.01$; Figure 2.6C). The gut at the proximal position (25% position) was approximately 25% more extensible than at the midpoint (50% position), and there was no significant difference in extension ratio between the 50% and 75% positions. This result translates to a total gut distension, from the initial no-stress state to maximal expansion, of approximately 360% percent in the proximal portion of the alimentary tract. The mean toughness (MJ/m^3) also decreased significantly along the alimentary tract ($P<0.01$; Figure 2.6D). Compared to the midpoint position (50%), the proximal position was 45.0% tougher, while the distal position was not significantly different.

There was a significant difference in mean strain-ratio among the three relative positions of the alimentary tract ($P<0.01$, Table 2.3, Figure 2.7 and Figure 2.8). Moreover, the mean strain ratios were 3.25 times higher than the expected strain-ratio value of 2 for the inflation of a standard cylinder.

2.5 DISCUSSION

We developed a custom quasi-static pressure system to quantify the relative changes in the passive mechanical properties along the length of the alimentary tract of a stomachless fish, *Cymatogaster aggregata*. These data support our hypothesis that the mechanical properties differ along the length of the alimentary tract. The ultimate strength, ultimate strain, extension ratio, and toughness were significantly greater at the proximal (25%) position than the more distal (50% and

75%) positions along the alimentary tract (Table 2.3). In addition, the alimentary tissues were highly extensible and anisotropic (Figure 2.6 and Figure 2.8). Essentially, the initial 25% position takes more energy per unit tissue volume to break and withstands a larger force per unit area (stress). We found no distinguishable difference in the wall thickness along the alimentary tract at the three relative positions (25%, 50%, 75%) (Figure 2.5), suggesting these differences in mechanical properties are due to differences in the alimentary tissue structure and/or composition.

Durophagous fishes differ in their approach in capturing prey items and few regurgitate indigestible material (Norton 1988; Hattin 1996; Hulsey 2006). Regardless of the potential evolutionary advantages associated with a lack of a "true" compartmentalized stomach, the alimentary tract must accommodate the initial influx of minimally processed hard prey items of durophagous fishes. Al-Hussaini (1946b) and Wallace et al. (2005) referred to the anterior portion of the intestinal tract of stomachless fishes as an intestinal bulb that likely functions as a reserve. Our data are consistent with this hypothesis; we show the initial pressure associated with the influx of prey items effect a greater distension at the proximal (25%) region of the alimentary tract, indicating this position is capable of large-scale energy absorption and circumferential deformations (Figure 2.6 and Table 2.3). This may also be the mechanism adopted by other stomachless fishes to deal with the forces generated with primary ingestion (Sklan 2004).

As discussed above, there were no significant differences in circumference or wall thickness at the three relative positions (Figure 2.5A, Figure 2.5B and Table 2.2). However, the mechanical properties differed significantly at the proximal, 25% position of the alimentary tract; with increases of 25–45% relative to the mid-section (Figure 2.6, Table 2.3). These results suggest that the organization of the composite materials contribute to the differences in mechanical properties at the 25% position. The main structural components that maintain gut wall integrity are elastin and collagen, with smooth muscle providing minimal structural support (Krafka 1939). Elastin enables distension of the gut wall while the stiffer collagen fibers maintain structural integrity (Fung 1981; Wainwright et al. 1982; Storkholm 1998). Therefore, changing the fiber orientation relative to the long axis of the structure could alter mechanical properties and cause anisotropy—where the properties differ depending on the direction of the applied load (Wainwright et al. 1978). In a closed, thin-walled pressurized cylinder, reinforcing fibers wound at 55° relative to the long-axis maintain its proportions and a strain ratio equal to 2, whereas fibers less than or greater than 55° produce shorter fat tubes or thinner elongate tubes, with strain ratios greater than or less than 2, respectively (Clark and Cowey 1958). Gosline (1971) showed that the circumferential elastic modulus in mesoglea is an order of magnitude greater than the longitudinal modulus due to an increase in reinforcing collagen fibers in the circumferential direction. While not investigated in this study, it is likely that variation in tissue ultrastructure and composition (i.e., collagen, elastin, smooth muscle) along the alimentary tract influences regional and anisotropic mechanical properties. This may be advantageous because the gut will experience differential stresses as non-uniform prey items, or bolus, decrease in size as they are processed and digested while travelling down the gut.

The extension ratio (λ) of the small intestine at full rupture was 3.6 in the shiner perch, substantially higher than in humans and guinea pigs (2.4 and 2.0, respectively; Egorov et al. 2002, Storkholm et al. 1998). The maximum extension ratio provides some guidance for estimating the maximal bolus size that can be ingested, which could ultimately dictate prey size and preference. Similarly, maximal gape area can serve as a proxy for the maximal cross-sectional area of prey a fish might ingest. This is a rough approximation, because presumably the pharyngeal jaws could assist in compressing the prey, and some materials and structures like fish fins, or segmented shells composed of chitin may be resistant to mechanical breakdown and simply compress and expand again once moved past the pharyngeal jaws (personal observation). If the gape area is smaller than the proximal gut area (a Gut-to-Gape Index, or GGI > 1), then the gut can safely accept prey and even allow for the expansion of prey once it enters the proximal part of the alimentary tract. However, if the gape area is larger than the proximal gut area (GGI <1), then some prey item packaging or processing would need to take place before entering the gut or moving down the alimentary tract in order to decrease the size and/or activity level of the prey item. The mean maximal gape area of the shiner perch in this study was 28.3 mm^2, which corresponds to a GGI of 1.39 at the proximal position of the alimentary tract. This value is above the critical 'safety factor" of 1, indicating the fish are likely not at risk for gut failure during the initial influx of prey items. In comparison, the GGI of the midpoint and distal positions was 0.62 and 0.45, respectively. These GGI values indication the bolus must compress as food is broken-down and digested as it moves along the alimentary tract. In addition, the gut is a dynamic tissue so smooth muscle control, creep, or strain softening could be implemented if necessary, to help resist tissue failure.

We pressurized whole intestinal tissues to maintain the interwoven cross-linking of the fibers throughout these structures (Orberg et al 1983; Gabella 1987). Thin-walled tubes are often cut into ring segments for testing properties (e.g., Lillie and Gosline 2007), which has the potential to destroy adjacent cross-links and destabilize the structural integrity of the tissue and alter the radial geometry. This damage and instability could lead to inaccurate measurement of tissue mechanical properties. However, a future study testing shorter relative segments (i.e., cut alimentary tract into thirds with the focal relative position in the center of the segment) may be warranted. This may determine if any adjacent histological structures at a given position, particularly connective tissues that run the length of the alimentary tract (e.g., submucosa), are providing indirect structural support to an upstream or downstream part of the alimentary tract. There is a possibility that tissue fibers run the entirety of the structure, which may add residual strength and influence the structural dynamics of these tissues. In addition, while puncture testing would be interesting, mucus production and increased knowledge of prey diversity would be needed in order to determine an appropriate test method (e.g., probe size, probe shape, puncture rate). Although our time dynamics were appropriate, considerations of creep and stress relaxation could also be addressed in future studies. The findings of this study lay the groundwork for such tests of the distension limits of the alimentary tissues in fishes.

Overall, this study used whole tissue inflation testing to establish that the mechanical properties differ along the length of the alimentary tract of a stomachless fish. To develop this method, we focused on a narrow size range of a single species. An interesting avenue for future studies would be to determine if the mechanical properties differ during development, i.e., over an ontogenetic size range. Ferraris and Cruz (1987) have shown that histological characteristics are similar across developmental stages, yet the geometry of these regions change during development. In addition, ontogenesis leads to changes in intestinal length, prey preference, and trophic shifts in fishes (Stoner and Livingston 1984; Kramer and Bryant 1995a; Tibbetts and Carseldine 2005). There is therefore the potential for changes in mechanical properties across developmental stages. What remains to be determined is whether ontogenetic changes in alimentary tract morphology lead to associated changes in the mechanic properties of these structures, which will ultimately increase our understanding of the various selective pressures driving the evolution of intestinal tissues.

2.6 LITERATURE CITED

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2.7 TABLES AND FIGURES

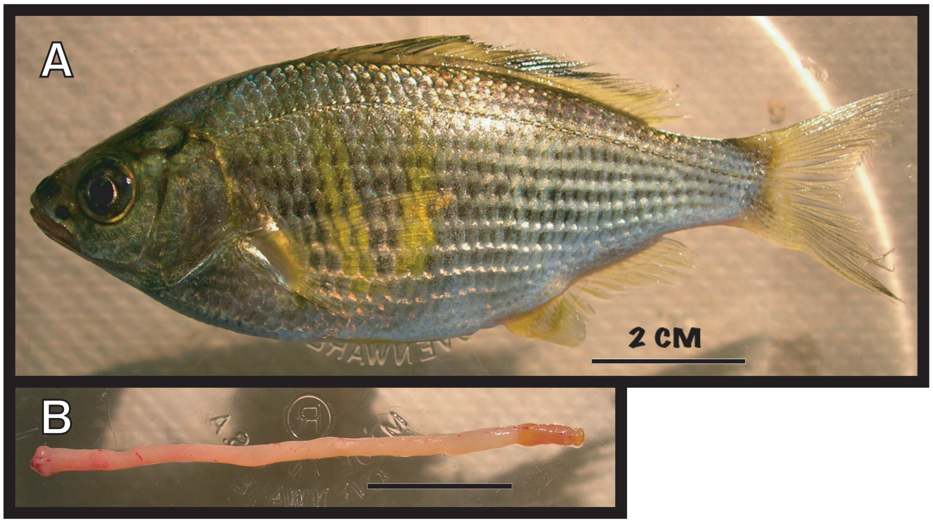


Figure 2.1 Subject fish and intestinal images. (A) Photograph of the demersal, durophagous, and stomachless shiner perch, *Cymatogaster aggregata*. (B) Extracted and unfurled "S-shaped" alimentary tract in the zero-stress state. The anterior portion begins just distal to the pharyngeal apparatus and posterior segment ends just proximal to the cloaca; scale bar = 2 cm.

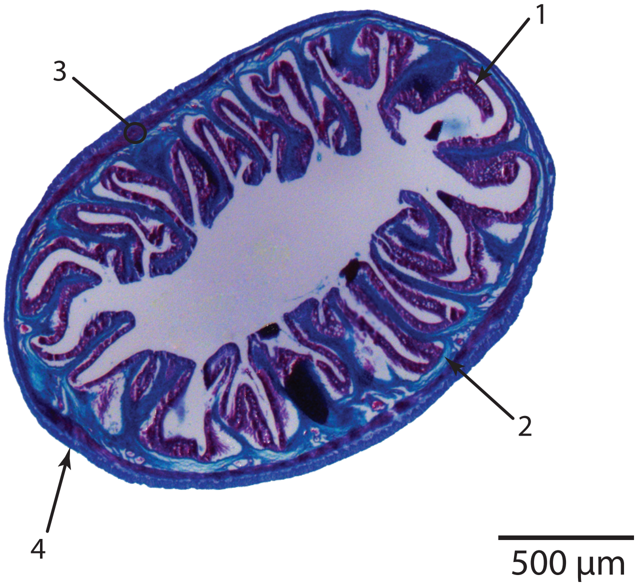


Figure 2.2 Representative radial cross-section showing the general wall structure of the alimentary system of the shiner perch, *Cymatogaster aggregata*. Wall consists of four layers: (1) tunica mucosa (mucosal epithelium and vascularized connective tissue), (2) submucosa (connective tissue), (3) tunica muscularis (muscle tissue), and (4) tunica serosa (mesothelial cells and vascularized connective tissue).

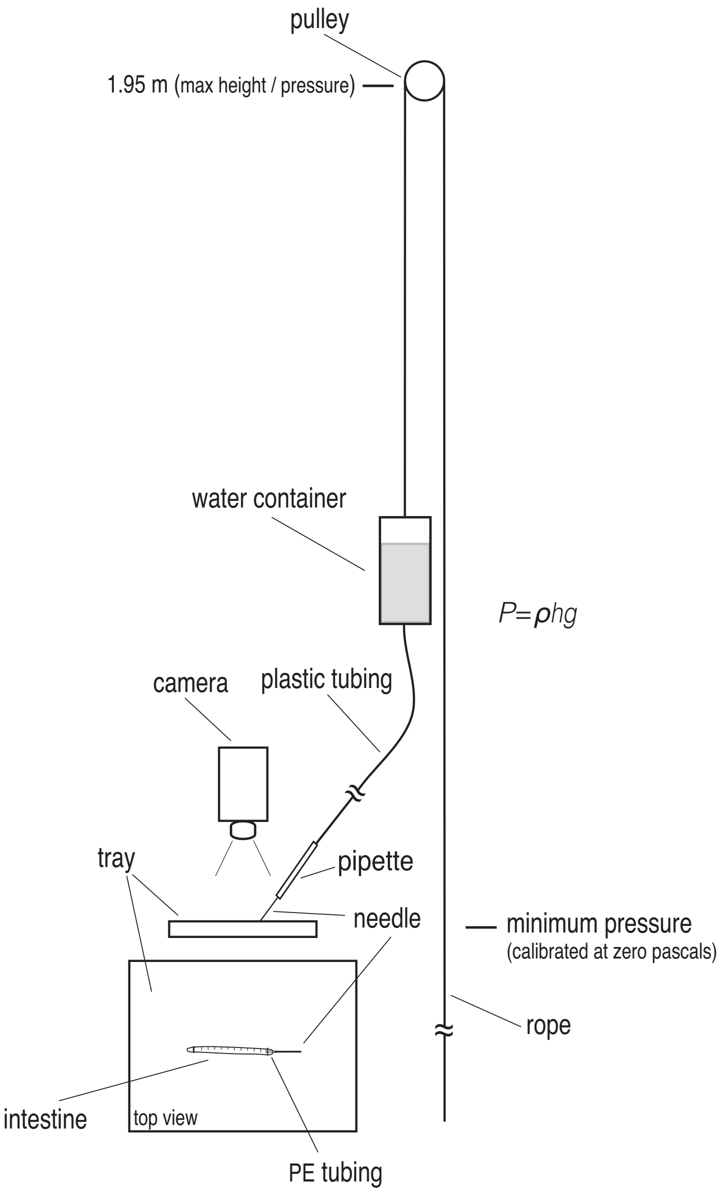


Figure 2.3 Schematic of the pressure device used for inflation tests. See detailed description in the methods section. The break in the illustrated plastic tubing and rope indicates length changes of the material depending on height of water container, and the tray containing the intestinal segment is shown in side and top view.

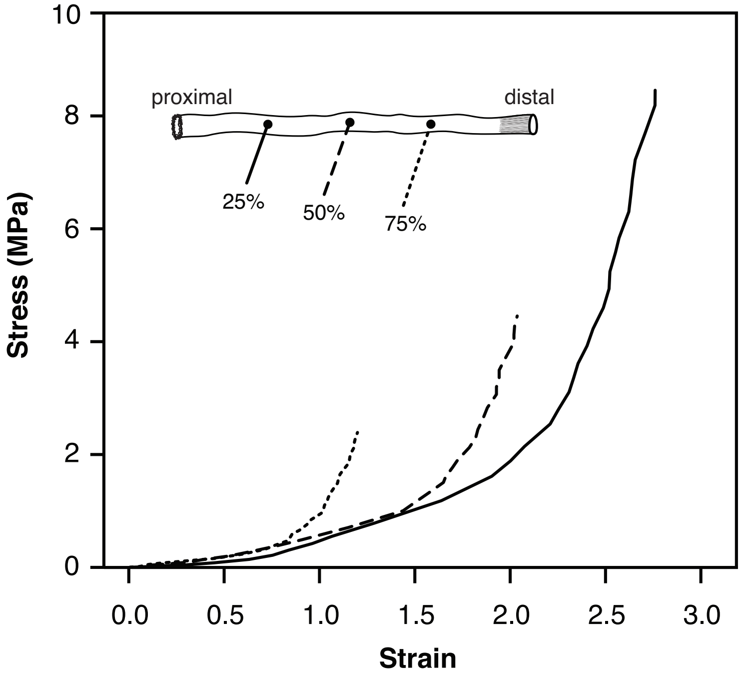
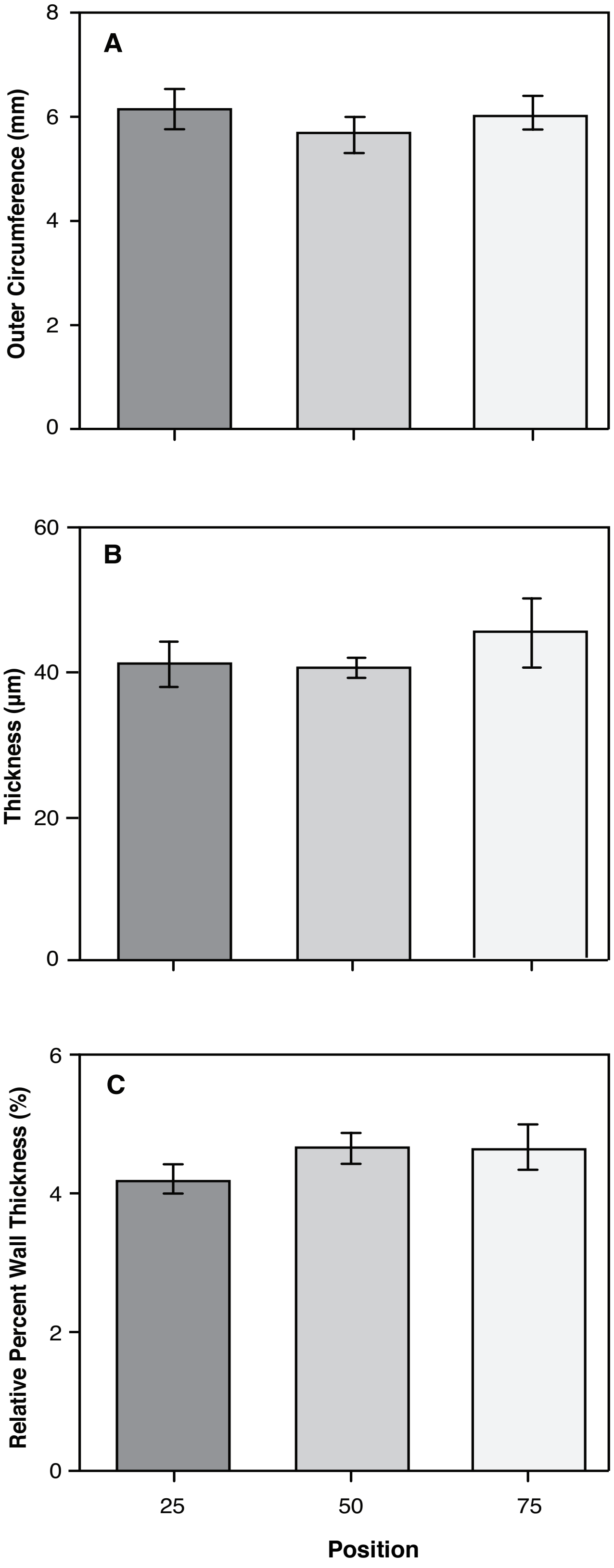


Figure 2.4 Representative stress-strain curve of the alimentary tract at three relative positions. (25%—solid line, 50%—large dash, 75%—small dash) Curves exhibit the typical non-linear J-shaped stress-strain relationship of many pliant biological materials. The alimentary tissues are highly extensible under low pressures and then become increasingly stiff with increasing pressure. Note the 25% position is overall more extensible and stronger compared to the two more distal positions.

Figure 2.5 Histological parameters. Outer radius (**A**), wall thickness (**B)** and relative radial percent thickness (**C**) at three relative positions (25%, 50%, 75%) along the length of the alimentary tract, measured from histological cross-sections. Bars are means ± SE, n=30. There were no significant differences among groups (Tukey HSD, P<0.05).



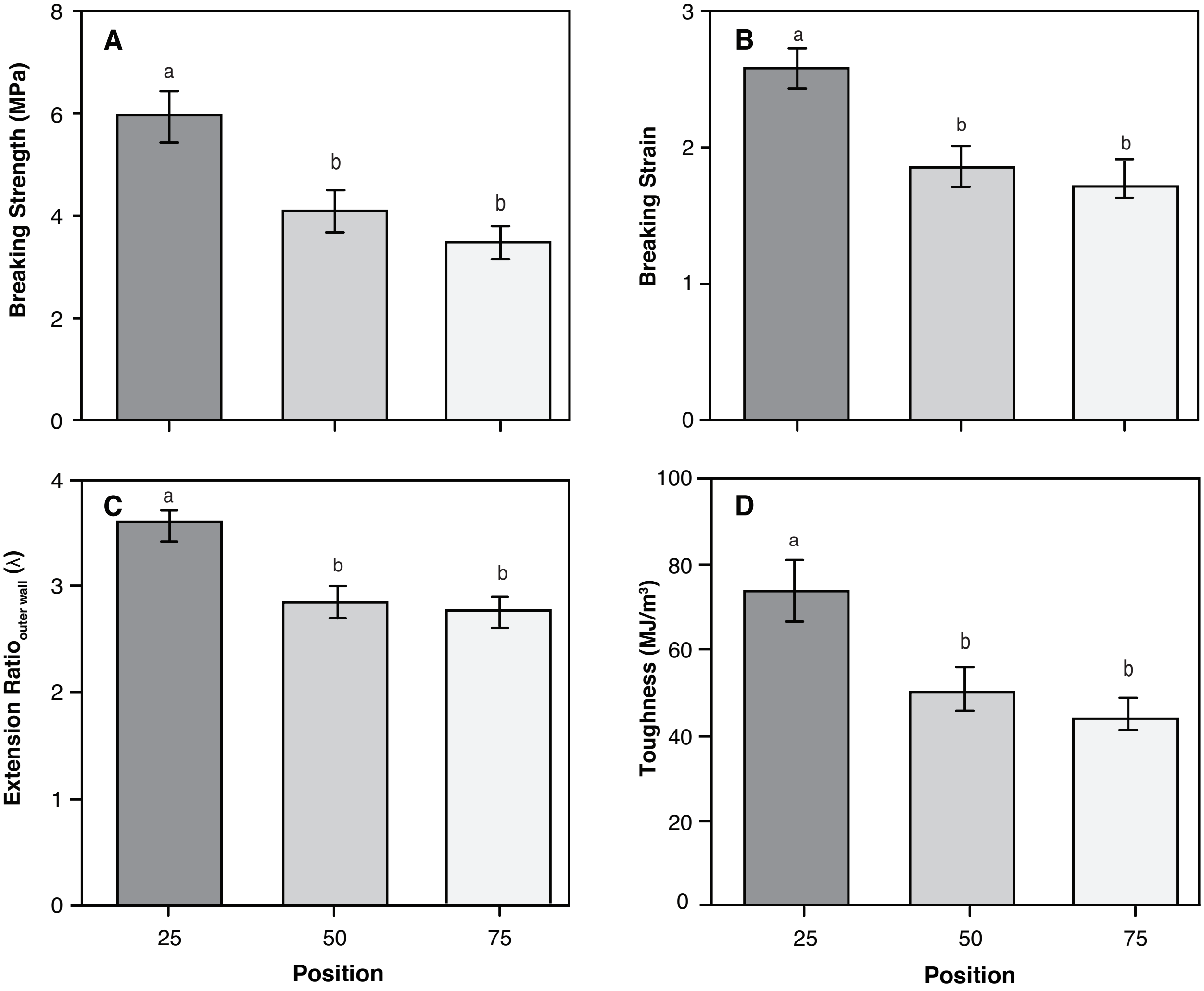


Figure 2.6 Mechanical properties at three relative positions (25%, 50%, 75%) along the length of the alimentary tract. All values are mean ± SE, n=30. (**A**) Breaking strength (**B**) Breaking strain (**C**) Mid-wall extension ratio (λ) and (**D)** Toughness. Different letters above bars indicate significant different among groups (Tukey HSD, $P<0.05$).

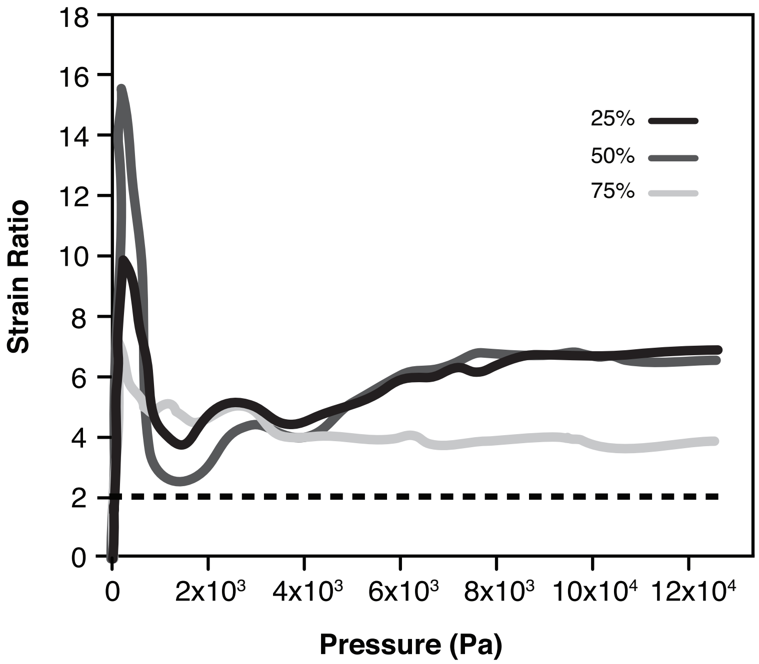


Figure 2.7 Representative traces of strain ratio as a function of inflation pressure for each of the three relative positions (25%, 50%, 75%) of the alimentary tract. Each line exhibits the characteristic high-pressure spike near zero inflation, i.e., an aneurism, needed to overcome the initial tension of a thin walled tube. The ratio drops down to the predicted value of 2 (horizonal dotted line) but with increasing pressure increases to almost three-fold at maximum pressure.

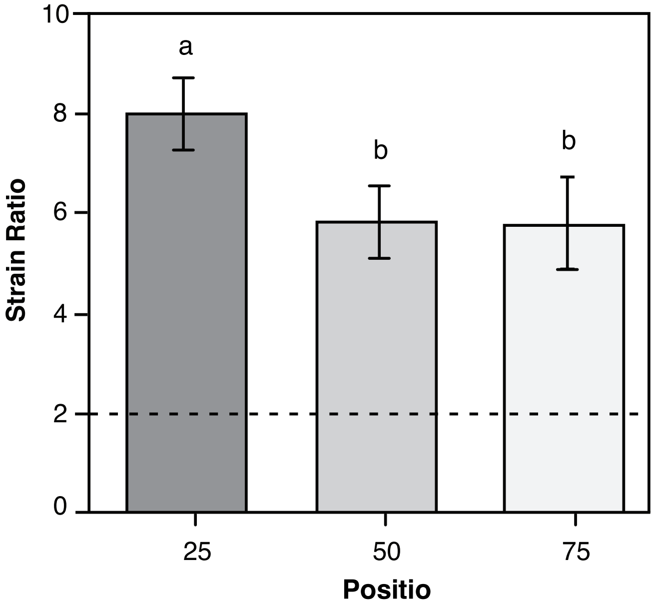


Figure 2.8 Strain ratio at three relative positions (25%, 50%, 75%) along the length of the alimentary tract. All values are mean ± SE, n=30. Different letters above bars indicate significant different among groups (Tukey HSD, P<0.05). All values are substantially higher than the expected strain ratio value of 2 (dotted line) for a standard cylinder.

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Table 2.1 Summary of morphometric measurements. Fish were used for either inflation or histological analyses. Values are mean (± SE) and range; n = 10 for each analysis group. A Student's *t*-test was used to evaluate differences in morphology between the two analysis groups.

Analysis	**Standard length** (SL, mm)	**Body mass** (BM, g)	**Gut Length** (GL, mm)	**Gape Height** (GW, mm)	**Gape Width** (GW, mm)	**Gape Area** (GA, mm^2)
Inflation						
mean + SE	86.9 ± 1.5	13.6 ± 0.6	83.3 ± 2.8	7.0 ± 0.2	5.1 ± 0.1	28.3 ± 0.8
range	81.5 - 96.7	11.4 - 17.9	69.2 - 94.3	6.0 - 8.0	4.9 - 5.5	23.6 – 32.4
Histology						
mean + SE	85.5 ± 1.0	13.4 ± 0.3	82.4 ± 1.8	7.0 ± 0.2	5.3 ± 0.1	28.9 ± 1.2
range	80.6 - 93.3	12.2 - 14.5	73.0 - 91.0	6.0 - 7.8	5.0 - 5.5	23.6 – 33.7
Student's *t*-test *t* (df = 18)						
p-value	0.865	0.700	0.738	0.760	0.339	0.779

Table 2.2 Summary of statistical analyses of histological parameters at three relative positions along the length of the alimentary tract. Values are mean (± SE) and range; n = 30. There was no significant difference among the three (25, 50, 75%) relative position; ANOVA, P< 0.01. Overall, the load-bearing gut wall is roughly 5% of the structural radius.

Histological parameter	Mean ± SE for each position along length of alimentary tract			
	25%	50%	75%	ANOVA
outer radius (μm)	967.9 ± 58.4	889.5 ± 59.6	951.6 ± 60.5	$F_{2,27}=0.483$; $P=0.622$
wall thickness (μm)	41.1 ± 3.24	40.5 ± 0.15	45.6 ± 0.15	$F_{2,27}=0.578$; $P=0.568$
percent wall thickness (%)	4.25 ± 0.22	4.67 ± 0.24	4.71 ± 0.32	$F_{2,27}=0.970$; $P=0.392$

Table 2.3 Summary of statistical analyses of the mechanical properties. Three relative positions along the length of the alimentary tract and percent change relative to the midpoint (50%) position (n=30). Mean (± SE) values are shown for each mechanical property. Only the 25% position differed significantly from midpoint (50%) and distal (75%) positions (ANOVA, $P < 0.01$; Tukey HSD, $p<0.05$; bold font). There was a significant difference in strain-ratio among the three relative positions and all values were considerably greater than the expected strain-ratio value of 2 for the inflation of a standard cylinder.

Mechanical Property	**Mean ± SE at each position along length of alimentary tract**				**Percent change relative to midpoint position**		
	25%	50%	75%	ANOVA	25%	50%	75%
strength (MPa)	**5.92±0.50**	4.07±0.42	3.58±0.31	$F_{2,27}=8.634$; $P<0.01$	↑ 45.4%	-	↓ 13.7%
strain (ε)	**2.59±0.14**	1.87±0.15	1.77±0.15	$F_{2,27}=9.426$; $P<0.01$	↑ 38.4%	-	↓ 5.3%
extension ratio (λ)	**3.59±0.14**	2.87±0.15	2.77±0.15	$F_{2,27}=9.426$; $P<0.01$	↑ 25.0%	-	↓ 3.3%
toughness (MJ/m^3)	**73.36±7.05**	50.58±4.89	44.70±3.39	$F_{2,27}=8.097$; $P<0.01$	↑ 45.0%	-	↓ 11.6%
Strain Ratio *(c/l =2)*	**7.93±0.71**	5.83±0.72	5.74±0.92	$F_{2,27}=8.713$; $P<0.01$	↑ 36.0%	-	↓ 1.5%

2.8 ACKNOWLEDGMENTS

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Chapter 3

Ontogenetic changes in morphology and intestinal mechanical properties in an agastric fish

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Keywords: mechanical properties, ontogeny, allometry, soft tissues, stomachless, intestine, fish

3.1 ABSTRACT

The alimentary system of fishes undergoes physiological and morphological changes during ontogenesis. As prey size and intake increase with body length, intestinal tissues must cope with the mechanical stresses acting on the intestinal wall. Severe limitations on food acquisition, assimilation and overall performance may result if the alimentary tract is not capable of stretching and accommodating the bolus and associated structures of consumed prey. In this study, we quantify the allometric changes in alimentary morphology and mechanical properties of the striped perch, *Embiotoca lateralis*, at three relative positions (25%, 50%, 75%) along the intestinal tract for fish ranging from 4-22 cm in length. We test the following hypotheses: 1) fish and gut morphology will show isometric growth with respect to body length; and 2) gape area and alimentary mechanical properties will exhibit positive allometric growth over development. The results confirm our hypotheses that gut mass and length grow isometrically. However, fish mass, gape area, outer gut circumference and gut wall thickness showed positive allometric growth relative to body length. The mechanical properties changed along the intestinal tract and were 5% stronger, 34% more extensible and 29% tougher at the proximal 25% position of the intestine compared to the more distal 75% position. Contrary to our hypothesis, mechanical properties did not scale allometrically; tissues in fish in the large and medium size classes were similar and were stronger, tougher, and more extensible (by 18%, 20%, and 49%, respectively) than in the smallest sized class. This study provides new insight into ontogenetic changes in the mechanical properties of alimentary tissues in a stomachless fish, and may serve to guide future studies on factors influencing the evolution of fish alimentary systems.

3.2 INTRODUCTION

The body plans of fishes undergo many changes from their larval to adult forms (Osse et al. 1997, Reis et al. 1998; Loy et al. 2001). Some adaptive and functional modifications leading to mature body plans are the development of hardened fish scales to protect from predation (Khayer et al. 2015; Yang et al. 2019), changes in gape morphology that allow for capturing diverse prey or exploitation of new food sources (Hegrenes 2001; Winkler et al. 2017), or changes in body morphology that facilitate the exploitation of new niches (Leis et al. 2009; McMenamin and Parichy 2013). At times rapid larval development can be detrimental to adult performance (da Silvav et al. 2019), however, some organ systems can secondarily adapt to changing ecological environments or demands (Ke et al. 1970; Osse 1990; Gagnat 2016).

The differential growth of anatomical traits relative to body length over the course of development is known as allometric growth, which can significantly affect the trajectory and properties of shapes and structures (Huxley 1932; Gould 1966; Calder 1984; Schmidt-Nielsen 1984; Emerson and Bramble 1993; Brown et al. 2000; Arendt et al. 2001). Morphological structures or characters that change in proportion to length are considered isometric, whereas traits that disproportionately increase or decrease are considered to have positive or negative allometry, respectively. Fuiman (1983) shows that the anterior structures of larval fish, specifically the head region that includes the eyes and feeding apparatus, grow at a faster rate than the midbody and tail regions. This relative growth differential accommodates increased development of sensory systems and feeding structures, enabling the acquisition of oxygen and food needed for sustained growth. Moreover, the disproportionate increase in snout length was shown to enhance substrate suction, an adaptation some neotropical fishes exploit for survival (Cassemiro et al. 2008). However, negative allometry of eye growth has been shown in other fish species, shifting energy resources to other sensory systems that enhance prey and predatory detection (Lima-Junior and Goitein 2003; Ward-

Campbell and Beamish 2005). Therefore, both positive and negative allometric trade-offs can increase survival by allowing adaptations to specific environmental influences.

The digestive tract is an important system that undergoes numerous morphological and physiological transformations during ontogenesis. The most dramatic changes occur during hatching and early larval development, where gut differentiation and enzymatic activity increases (Kawai and Ikeda 1973; Segner et al. 1994; Kamisaka et al., 2001). Moreover, the majority of teleost fishes lack a functional stomach during the larval stage and rely mainly on the enzymatic breakdown of zooplankton (Hunter, 1981); this larval feeding mode is independent of the adult feeding type. Some fish that undergo dietary shifts have associated increases in gut length to optimize digestion (Benavides et al. 1994). However, numerous teleost families lack a stomach throughout their life history, *i.e.* adult forms, and have persisted over evolutionary time.

As food intake and prey size typically increase with body size (Fahy 1980; Werner and Gilliam 1984; Rose 1986; Ghan and Sprules 1993), the alimentary tissues must cope with the mechanical stress resulting from larger bolus sizes traveling down the alimentary tract once a dietary shift to larger prey occurs, or rapid ingestion during binging periods when prey are abundant. If these structures are not capable of stretching and accommodating the bolus of consumed prey, they may pose limitations on prey acquisition or transport. In this manner, the mechanical properties of the intestinal tissues may play a role in dietary choice over ontogeny and evolution of feeding mechanisms.

The goal of this study is to describe how alimentary mechanical properties change with body size in the striped perch, *Embiotoca lateralis* (Agassiz 1854). This species is a stomachless, viviparous, and demersal fish that inhabits coastal areas rich in eelgrass beds along the eastern Pacific from Baja, Mexico to Wrangell, Alaska (Eschmeyer and Herald 1983). *E. lateralis* offers an ideal system to investigate the ontogenetic changes in alimentary mechanical properties because the larval forms are similar to adult body plans and, unlike most incomplete functional systems at hatching, this species is sexually mature at birth (Eschmeyer and Herald 1983). In addition, previous studies have described how both dietary specializations and patch choice change during development, as well as the role of gape morphology on prey selection (Haldorson and Moser 1979; Holbrook et al. 1985; Holbrook and Schmitt 1992). These dietary differences and restrictions can potentially be limited by the mechanical properties of alimentary tissues.

In this study, we use techniques established in Horton et al. (2020) for the shiner perch *Cymatogaster aggregata* (Gibbons 1854), to quantify the material properties of the alimentary tissues of its close phylogenetic relative, *E. lateralis*. These species offer an interesting comparison because they both have a uniform alimentary tract and a similar body form throughout their life history (larval and adults are shaped similarly), which ultimately will allow for future understandings of the mechanical properties in an ecological context. The aims of this research were to: (1) determine scaling relationships of fish body, gape, and gut morphology with respect to body length; and (2) assess ontogenetic changes in alimentary tissue mechanical properties. We test the hypotheses that: (1) fish mass, gut morphology will scale isometrically with respect to body length; (2) gape morphology will show positive allometry as fish increase in size; (3) outer circumference and wall thickness of intestinal tissues will exhibit positive allometry while percent wall thickness will scale isometrically with body length; and (4) strength, extension ratio, and toughness will increase over developmental growth. This study provides new insight into ontogenetic changes in the mechanical properties of alimentary tissues in a stomachless fish.

3.3 METHODS

3.3.1 Specimens

Eighteen *Embiotoca lateralis,* ranging in size from 55 – 255 mm standard length (SL) and 2.8 –

459.4 g, were collected by seine at Jackson Beach, San Juan Island, Washington, USA (48º31'12"N 123º00'36"W; Figure 3.1). Fish were maintained in flow-through seawater tanks (128 L x 76 W x 24 H cm) at the Friday Harbor Laboratories (FHL), University of Washington, for a period of 4 to 6 days to eliminate preexisting digestive material (J. Jenson, *personal communication*). All animal experiments were performed in accordance with the University of Washington Institutional Animal Care and Use Committee (IACUC).

3.3.2 Fish and Alimentary tissue preparation

We used a modified method from (Horton et al., 2020) to prepare samples for mechanical testing, as follows. Fish were euthanized with 350mg/L of buffered Tricaine Methanesulphonate (MS-222) in seawater. Fish body mass (*BM*, ± 0.1 g) was weighed, and morphometric measurements of standard length (*SL*), gape height (*GH*), and gape width (*GW*) were measured with digital calipers (± 0.1 mm); gape area (*GA,* in mm^2) was also calculated as the area of an ellipse, with GH and GW as major and minor axes, respectively.

The complete alimentary tract was meticulously dissected from all individuals and unfurled to avoid mechanical tissue damage (Figure 3.2A). The total length of each alimentary tract was then measured and immediately placed in a teleost Ringer's solution at 12 ºC until the cessation of smooth muscle activity was observed (~40-60 min). Upon removal from the Ringer's solution, the lengths of each alimentary tract (length range: 42 mm – 166 mm) were blot dried and marked at three relative positions (*i.e.* 25%, 50%, and 75%) with red recorder ink. Approximately 5 mm of tissue was trimmed from the proximal end of the alimentary tract (to the bile duct), and 1 cm from the distal rectal region (*RR*). The alimentary tracts were then marked again with red recorder ink at 5 mm increments to measure longitudinal expansion during materials testing. Whole tracts from each specimen were then cut at the midpoints between marked relative positions (25%, 50%, and

75%) into three tissue segments of similar length. Approximately 6 mm from the proximal end of each segment was removed for histological analyses. Tissue segments were filled with Ringer's solution until the inflation test was performed; this procedure was repeated for all individuals.

3.3.3 Histological preparation & analysis

Tissue segments designated for histological analyses via light microscopy prepared following the methods of Horton et al. (2020). Briefly, tissue segments were fixed (Trump's fixative; McDowell and Trump 1976) and embedded in paraffin blocks and sectioned at 7μm. Images of tissue cross-sections were then analyzed in ImageJ (v. 1.46r) to quantify gut wall circumference and thickness. We assumed that these morphological features do not differ significantly along the alimentary tract for a given individual, as has been shown for a related species (Horton et al. 2020), and used the highest quality image for each individual for these analyses.

3.3.4 Inflation tests

For the remaining tissue segments, those from the three positions of each individual, we measured the passive mechanical properties using the custom inflation technique developed in Horton et al. (2020). We used a high-definition camera to record the inflation length and diameter behavior of an alimentary tract segment that was affixed to a custom pressure device. Static pressure was slowly increased from 0 to 200-400 Pa to flush out excess lumen material and fill the tract with seawater. The pressure was then lowered back to a resting pressure of zero, and the distal end of the intestinal segment was sealed shut and submerged under 5 cm of seawater; this procedure was repeated for successive segments. System pressure was increased by 200 Pa increments to 1000 Pa, and then increased by increments of 500 Pa thereafter. Pressure equilibrium—cessation of flow and tissue expansion—was reached within the alimentary tract before each incremental increase, generally within 30-60 seconds. This procedure was repeated until structural failure (tissue rupture) occurred. Any segments that exhibited premature failure (e.g. leakage at suture site) during pressure testing were excluded from analyses.

3.3.5 Data & Computational Analysis

For all tissue segments, still photographs from each incremental pressure during the test run were labeled and transferred to NIH ImageJ (v. 1.46r) and the total length and diameter of each segment (25%, 50%, 75%) was measured. We assumed a constant alimentary wall volume and circular cross-section for each segment. The outer radius was measured on tested intestinal segments, and corresponding histological percent wall thickness for a given individual was used to calculate the inner radii, as well as the total, wall, and lumen areas (for details see Horton et al., 2020). There is a very small constant pressure differential acting between the outer and inner walls of thin-walled structures (as strain varies across the wall thickness), so the mid-wall radius was used for further calculations. We used *Laplace's Law* to derive circumferential—or engineering—hoop stress (σ), and strain (ε) for each cross-sectional tissue segment at a given incremental pressure. The *in vivo* behavior of intestinal tissues in the constrained abdominal cavity lead to our exclusive focus on circumferential expansion. These data were used to plot stress versus strain quantify the following mechanical properties: ultimate stress (σ, MPa)—the maximum force required to cause tissue failure, breaking strain (ε)—the relative change in deformation during loading per unit length, toughness (MJ/m^3)—area under the stress-strain curve that equals the energy the tissue can absorb before tissue failure, and the extension ratio or extensibility (λ)—the maximal radial extension of the gut relative to the initial no-load state; which provides a rough estimate of the maximal bolus size that can travel down the alimentary tract. In addition, fish were binned into three size classes, 0 – 80 mm, 81 – 160 mm, and 161 – 240 mm; which for simplicity we refer to as small, medium, and large, respectively. These groupings overlap generalized size classes determined by time of year (e.g. YOY, Y1, Y2) though environmental conditions can greatly affect categorical assumptions (Ong et al. 2015).

3.3.6 Statistical analyses

The program R (v. 3.6.2 GUI 1.70; R Foundation for Statistical Computing) was used for statistical analyses. We used the general allometric equation:

$$y = a * x^b \qquad \text{(Equation 3.1)}$$

where y is the response variable, x is the explanatory variable, a is the proportionality coefficient, and b is the scaling coefficient. Log transformed data were fit to a linear model:

$$\log y = \log a + b \log x \qquad \text{(Equation 3.2)}$$

An ordinary least-squares regression analysis was then used to determine relationships between all morphometric and gut parameters, and a Pearson's correlation was used to determine the strength of these linear relationships. Slope tests using 95% confidence intervals and one-tailed *P*-values were performed to determine if the observed isometric slope differed from predicted slope—which is based on dimensionality (*i.e.* mass scales to the power of three) (Schmidt-Nielsen 1984); a $P<0.05$ value indicates slopes are different. An ANCOVA was run to determine the effect of relative position (25%, 50%, 75%) along the length of the alimentary tract on each mechanical property (e.g. ultimate strength) with body length as a covariate. Tissue mechanical properties did not scale linearly with size using Eqn 3.2. To determine if the mechanical properties differed over ontogeny, we therefore used a two-way ANOVA with size class and position as fixed effects. A Shapiro-Wilks' test was used to test for normality, and a Leven's test was used to test homogeneity of variance for all positions. We used post-hoc pairwise comparisons performed with a Bonferroni adjustment to identify differences between positions.

3.4 RESULTS

3.4.1 Organismal and ontogenetic allometry

The standard length (SL), body mass, gut length, and gut mass of fish used in this study ranged from 58 – 227 mm, 3.5 – 256.9 g, and 42 – 166 mm, 0.7 – 48 g, respectively. We found a significant and positive correlation between all fish and gut morphometric and variables (Figure 3.3 and Table 3.1). Gut mass and gut length both exhibited isometric growth relative to body length, with estimated slopes that were not significantly different from expected slopes of three and one (p=0.399 and p=0.451), respectively. However, fish mass exhibited positive allometric growth relative to body length, with an estimated slope that was 6% higher than expected for isometry (p<0.001; Figure 3.3 and Table 3.1). Gut mass also showed the isometric growth relative to gut length, with a slope that was not significantly different from three (p=0.736; Figure 3.3 and Table 3.1).

There was a positive and significant correlation between all gape variables and body length (Figure 3.4 and Table 3.2), with slopes that deviated to some degree from expected isometric values (Table 1). Specifically, gapes became proportionally shorter and wider (-12% and +24% from an expected slope of one (Figure 3.4A & 3.4B). The product of these two metrics, gape area, showed an allometric increase of 9% (Figure 3.4C). Although the Gape-to-Gut Index did not scale isometrically (p=0.234), the average GGI at the 25% position was over the critical value of 1 for small (1.40±0.52), medium (1.51±0.11), and large (1.75±0.08) size classes (Figure 3.5).

Analysis of the gut histological sections indicated that the outer circumference and wall thickness exhibited a positive and significant correlation with body length (p<0.01; Figure 3.6A & 3.6B), while the relative percent wall thickness of the gut did not (p=0.256; Figure 3.6C). Both outer circumference and wall thickness showed positive allometry, with slopes that were 17% and 23%

percent higher, respectively, from expected isometric values ($p<0.01$; Table 3.1). The slope for relative percent thickness was not significantly different from the expected slope of zero ($p=0.256$; Table 3.3). Overall, the thickness of the load-bearing gut wall was approximately 7% of the structural radius.

3.4.2 Mechanical Properties

The alimentary tract mechanical properties (strength, extension ratio, and toughness) depended strongly on size class as well as position (Figure 3.7 and Table 3.4). There were no significant interactions between these two effects for any of the mechanical properties measured. A post-hoc pairwise comparison test showed that mechanical strength was significantly greater at the 25% and 50% positions compared to the 75% position. The toughness of the 25% position was significantly higher than the 75% position, while the 50% position was intermediate. The extension ratio at the 25% position was significantly higher than both the 50% and 75% position. Overall, the 25% position was 5% stronger, 34% more extensible and 29% tougher than the 75% position.

Tissue mechanical properties did not scale linearly with fish size using Eqn 3.2, so we instead used an ANOVA to compare the three size classes. Overall the mechanical properties of the smaller fish were substantially lower than larger size classes, which were modestly different. For example, the mean difference in strength between the large and medium size classes along the length of the alimentary tract at the three positions was 1.1%, 3.5%, and 1.9%, respectively, whereas the mean difference between the large and small size classes were 18.0%, 18.2%, and 17.7% (Figure 3.7A). A similar positional trend was found in mean toughness among size classes, with a small difference found between large and medium size class of 1.2%, 3.6%, and 2.3%, and large difference between large and small size classes of 20.0%, 20.5%, and 20.7%, respectively (Figure 3.7C). Interestingly, the extension ratio was slightly higher in the medium verses large size class, with a mean difference of 2.8%, 4.4%, and 1.1%, respectively. As with the other mechanical properties, there was a considerable difference in the extension ratio between large and small size classes of 49.0%, 38.6%, 17.7%, respectively. Furthermore, as with overall trends, the 25% position was more extensible than both 50% and 75% positions (Figure 3.7B).

3.5 DISCUSSION

Our results show strong scaling relationships for the morphological and histological parameters with respect to body length. Fish mass showed positive allometry with respect to body length, which led to deeper and wider bodies (Figure 3.1, Figure 3.3 & Table 3.1). Previously, fish mass has been shown to be affected by numerous trophic, geographic, and seasonal influences. Árnason et al. (2009) has shown that increased food availability in cod (*Gadus morhua*) leads to positive allometric growth in their wild counterpart, whereas increasing temperatures in larval and juvenile fishes led to disproportionate weight increases (Otterlei et al. 1999). Interestingly, Jisr et al. (2018) has shown that some fishes species only show positive allometric growth during either the winter or summer months. Growth models have also shown to be influenced by changes in diet over ontogeny, as diets of fishes change continuously with body size (Werner and Gilliam 1984). As fishes increase in size, there is a shift towards choosing larger prey items and becoming more size selective (Newman and Waters 1984; Grant and Noakes 1986; Ingram et al. 2011); potentially due to energetic advantages as well (Werner and Hall 1974). Surfperches also have the ability to stimulate growth by 20 mm in length during the summer months (Deleon 2005). This growth acceleration seems to have a positive effect on ontogenesis, especially gape and alimentary systems.

Gape morphology plays an important role in prey acquisition and may therefore have consequences for growth. Contrary to our hypothesis of isometry, our data shows that gape area becomes disproportionately larger as fish increased in size largely because the width increase outpacing the decrease in height, so gape height and width showed both negative and positive allometry, respectively (Figure 3.4 & Table 3.2). This scaling pattern increases the intake area of the mouth thus allowing for larger prey items to be consumed. Although a smaller gape can generate higher suction forces (Wainwright et al. 2007), larger gapes allow fishes to not only consume prey of an increased size but also increase the diversity of prey types that can be captured (Schmitt and Holbrook 1984; Busch 1996; Russo et al. 2009). In fact, Wainwright and Richard (1995) stressed gape width and height as underlying factors that promote changes in diet over ontogeny. Haldorson and Moser (1979) did find that populations of surfperch restricted to smaller prey items had smaller mouths. Holbrook et al. (1985) also found that the youngest fish were gape limited and shifted to larger prey when body length was roughly 120 mm (slightly older than 1 year). Moreover, striped surfperches from kelp beds consumed larger more diverse prey then shoreline individuals (Alevizon 1975). While habitat selection and prey availability influence feeding morphology, diet can also affect alimentary tract morphology (Kramer and Bryant 1995; Elliott and Bellwood 2003; Wagner et al. 2009).

As hypothesized, intestinal tissue length and mass both scaled isometrically with respect to body length (Figure 3.3 & Table 3.1). These results also compare well with other studies on ontogenetic changes in gut length in fishes (Benavides et. al. 1994, Ward-Campbell and Beamish 2005). However, our histological results show both outer circumference and wall thickness of the alimentary tissues exhibit positive allometry (Figure 3.6). The disproportionate increases of outer circumference and wall thickness is likely due to both environmental and dietary influences, as previous studies have shown an increase in these traits are associated with diet and energy demands (Elliott and Bellwood 2003). Moreover, as with the related *Cymatogaster aggregata*, we found no distinguishable difference in the percent wall thickness (of approximately 7%) along the alimentary tract at the three relative positions (25%, 50%, 75%), suggesting this structural parameter is constrained; *i.e.* there is a relative percent thickness associated with the basic structural integrity of the intestinal wall.

Additionally, results support our hypothesis that the mechanical properties (ultimate strength, extension ratio, and toughness) increase significantly over ontogeny (Figure 3.7 & Table 3.4), but these changes do not follow allometric scaling relationships. Overall, the proximal region (25% position) was found to be significantly stronger, more extensible and tougher than the distal region (75%). This finding is similar to observation for phylogenetically related *C. aggregata* from Horton et al. (2020). Of our three size groupings, the alimentary tissues of the smaller size class were significantly weaker, less extensible and less tough than the middle and larger size classes (Figure 3.7). Larval surfperches are larger and more developed than most larval fishes (given nurturing within females before a live birth) and the diet of smaller fishes are similar to their larger counterparts, though younger fish ate smaller prey items and few complex prey types (Holbrook et al. 1985). Therefore, the similarities in alimentary tissue architecture are not surprising given digestive capabilities and maturity of this fish at birth. There is an exploratory period during the larval stage to identify and develop search image and preference patterns for prey items, which becomes established as fish increase in size (Gisbert 1999). Therefore, the shift of both middle and larger size classes to gain the ability to exploit larger prey may possibly coincide not only with an increase in body and gape size but also the increase in the mechanical properties of intestinal tissues. This increase in alimentary mechanical capabilities would be highly adaptive when diets shift in order to be able to accommodate larger prey and resist potential damage associated with the structural complexity of hard prey processing.

Although our data did not investigate tissue ultrastructure, given our tissue mechanics results and similarities in histological images across size class (Figure 3.8), alimentary tissue fiber composition and orientation are potentially the main influences for the observed differences in the mechanical properties along the length of the alimentary tract. Fiber orientation can affect load responses and thus directly affect the mechanical properties of these tissues (Clark and Cowey 1958; Gosline 1971; Wainwright et al. 1978). Moreover, elastin and collagen are the primary components that provide the majority of the structural integrity of the intestinal wall (Krafka 1939; Fung 1981; Wainwright et al. 1982; Storkholm 1998). Given the importance of fiber orientation, it is plausible that a system of cross-linkages traverses the length of the intestinal tract. It is worth noting that segmenting the area of the "intestinal bulb" could disrupt or severe connecting collagen and elastin fibers from adjacent positions, particularly in the smaller segments. A more detailed study on the alimentary ultrastructure could resolve these concerns and provide insight into the effect of fiber orientation on mechanical properties.

In addition to ultrastructure, a detailed phylogenetic perspective would shed light on the complex role of mechanical properties on evolutionary success. There have been several cases where a single genus adapted to new niches through new modes of feeding and digestion utilization to exploit novel food resources in relatively short evolutionary time (Campbell et al. 2005; Olsson et al. 2007). This is likely due to the plastic nature of intestinal tissues, which adapt to geographic and dietary availability. The extent to which alterations in the mechanical properties of alimentary tissues contributed to the exploitation of these niches remains unknown. It would be interesting to test if the mechanical properties in phylogenetically related fish species remain similarly constrained or would change based on the dietary specificity of a given species. Overall, this study showed strong relationships between morphological and histological parameters with respect to body length and with alimentary mechanical properties.

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3.7 TABLES AND FIGURES

Figure 3.1 Photographs of the striped perch, *Embiotoca lateralis*, at various lengths. These fish are stomachless, demersal, and durophagous; scale bar = 5 cm.

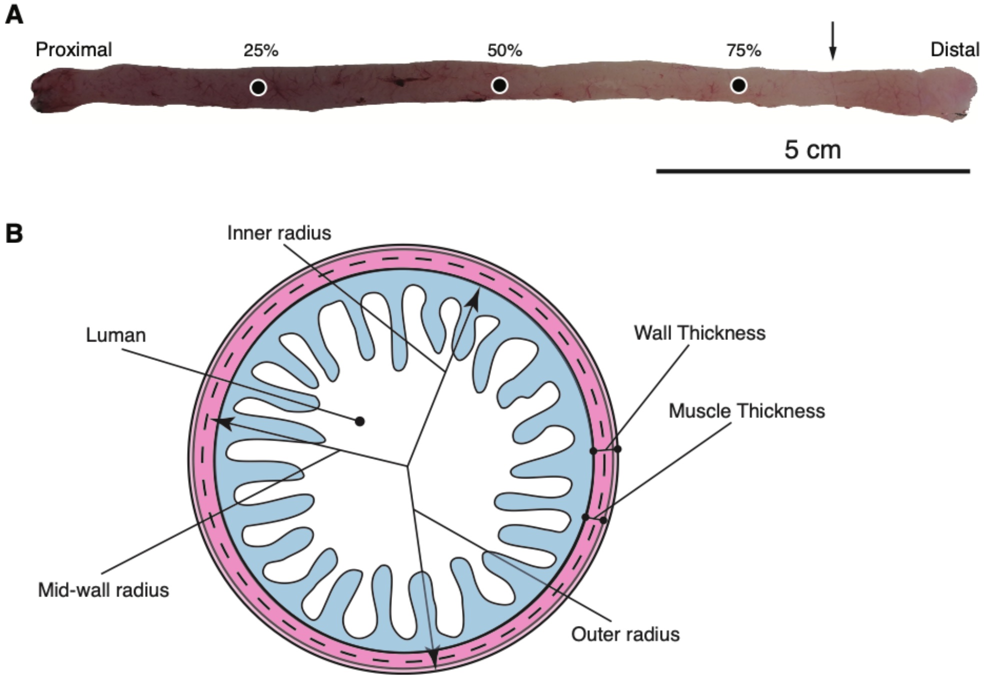


Figure 3.2 **A**. Uncoiled alimentary tract of *Embiotoca lateralis* from proximal to distal end. Start of rectal tissue is indicated by vertical arrow. Note: No stomach is present, and there is a normal darker coloration of the tissue in the proximal region. **B**. Illustration of the morphological and dimensional measurements taken from an intestinal cross-section at each of the three relative positions (25%, 50%, 75%) along the length. Wall thickness is the sum of the muscle thickness and elements of the serous coat (dark + light pink); blue area contains the villi and intestinal glands, which do not contain parietal cells (HCL secretion).

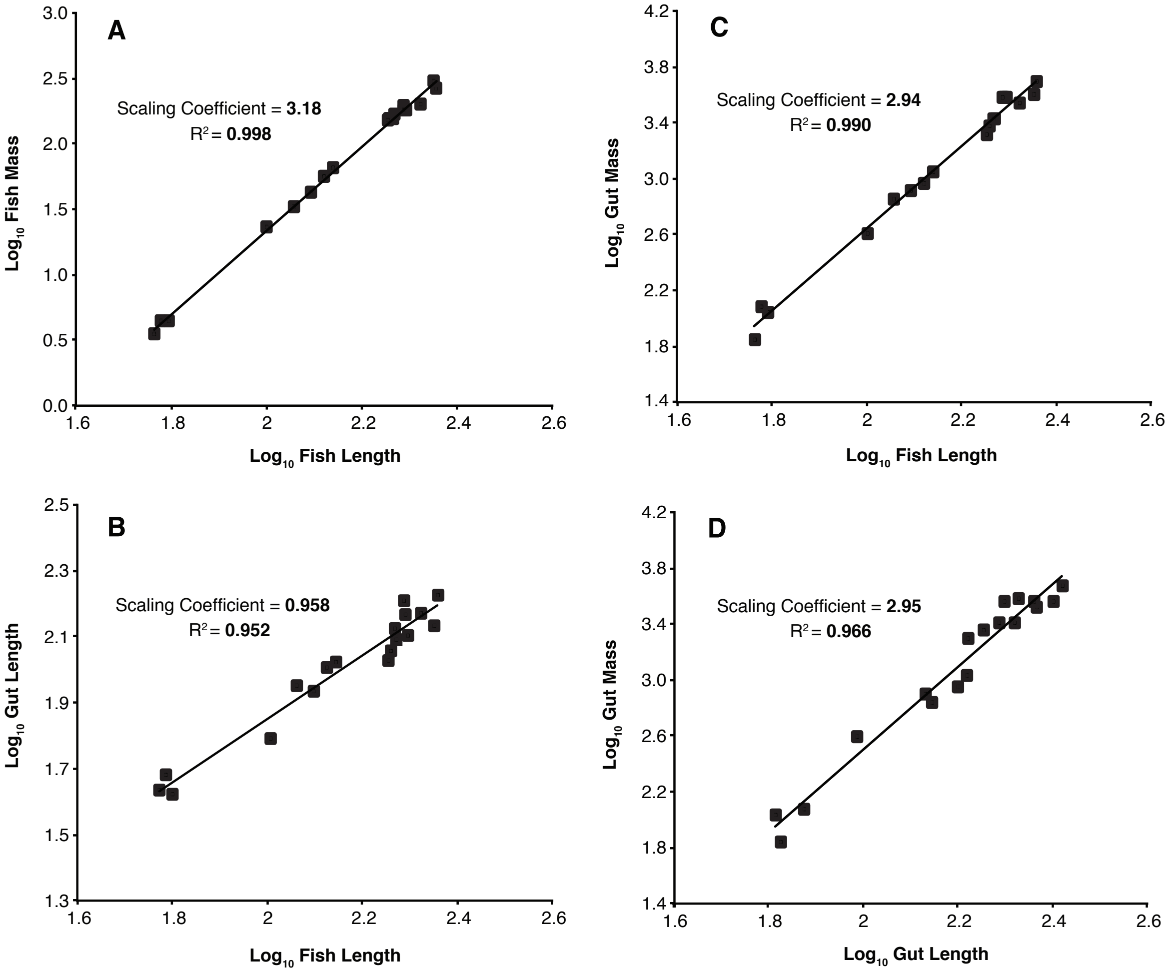
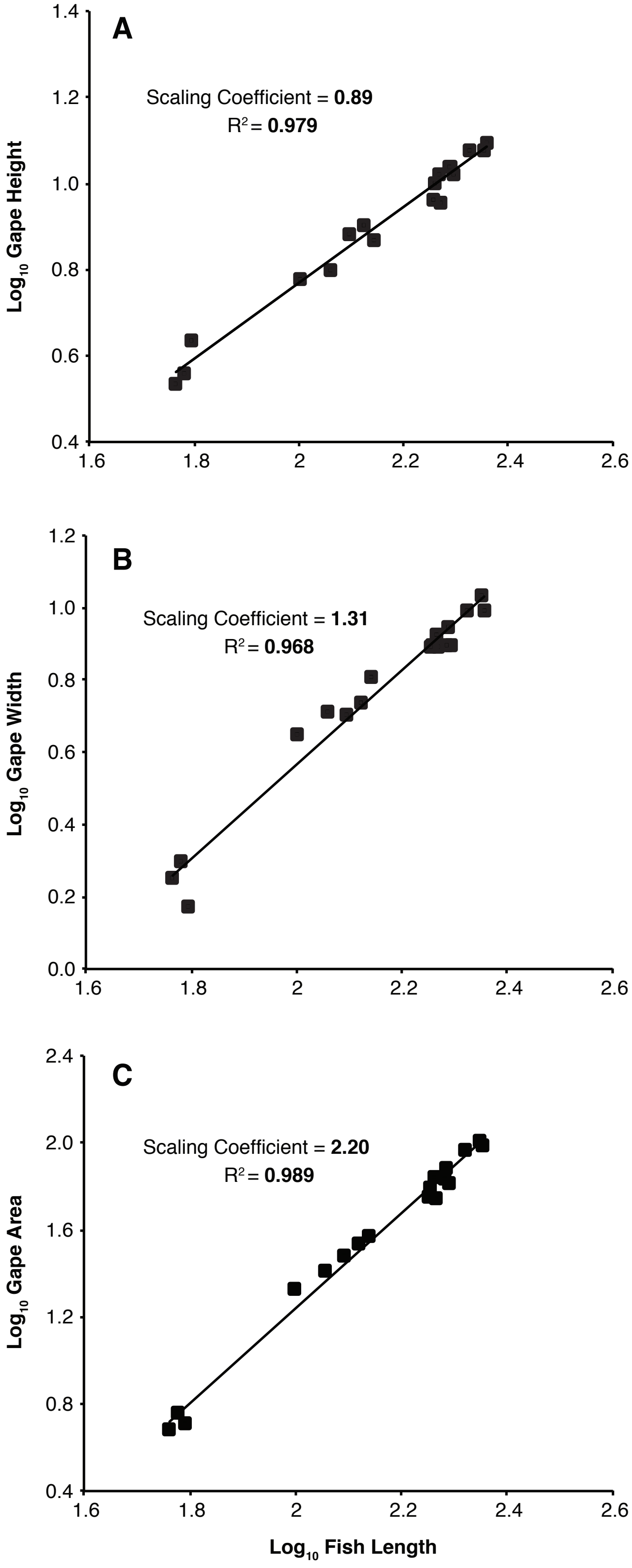


Figure 3.3 Log-transformed scaling relationships of fish and gut morphology. **A.** Fish mass, **B.** Gut length, **C.** Gut mass vs. Body length, and **D.** Gut mass vs. Gut length using ordinary least squares regression analyses for all individuals (n=18). The scaling and correlation coefficients (slope and R^2, respectively) are listed for each. See Table 1 for statistical summary.

Figure 3.4 Scaling relationships of log-transformed gape morphology vs body length. **A.** Gape height. **B.** Gape Width. **C.** Gape area verse body length using ordinary least squares regression analyses for all individuals (n=18). The scaling and correlation coefficients (slope and *R^2*, respectively) are listed for each. See Table 2 for statistical summary.



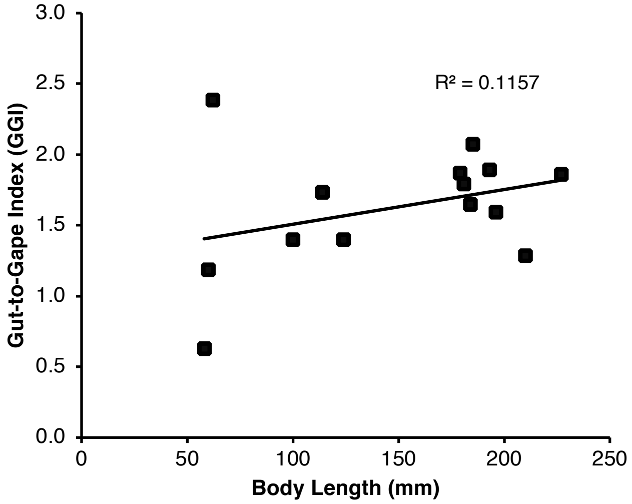


Figure 3.5 Gut-to-Gape Index (GGI) at the 25% position. Although the Gape-to-Gut Index did not scale isometrically, the average GGI was over the critical value of 1 for small (1.40±0.52), medium (1.51±0.11), and large (1.75±0.08) size classes (n=14).

Figure 3.6 Scaling relationships of mean histological parameters from each of the three positions (25%, 50%, 75%) along the alimentary tract. Used ordinary least squares regression analyses for all individuals (n=18). **A.** Outer circumference. **B.** Wall thickness. **C.** Percent wall thickness. The scaling and correlation coefficients (slope and R^2, respectively) are listed for each. Note: relative wall thickness is relatively constant across all body lengths (~7%). See Table 3 for statistical summary.

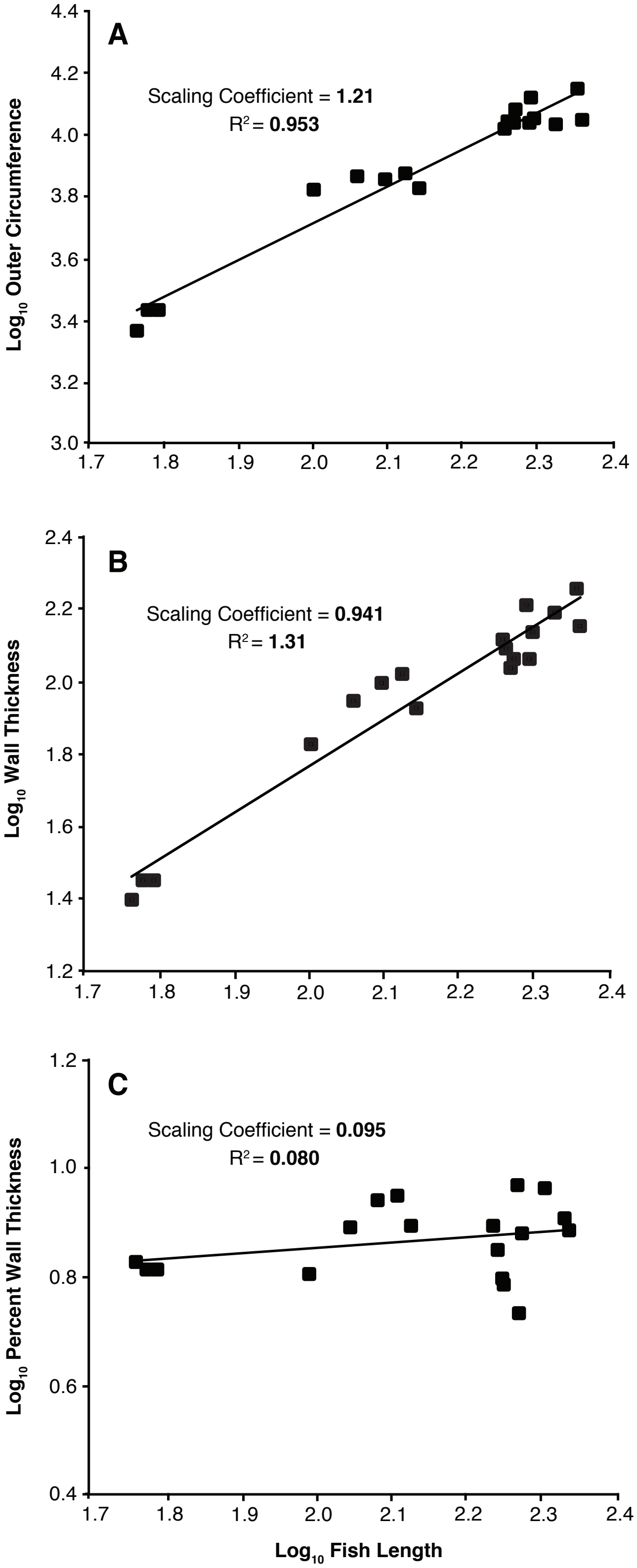
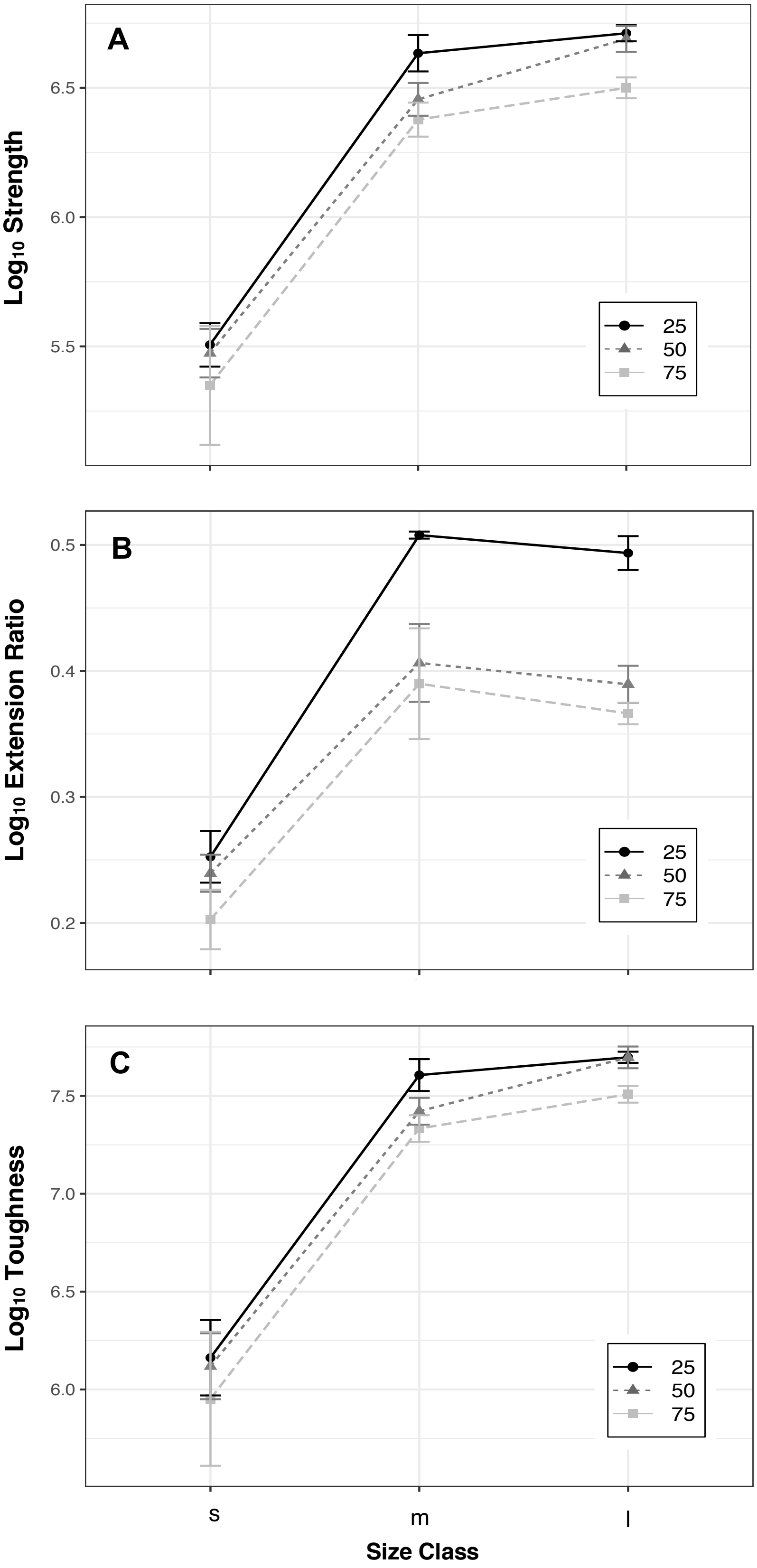


Figure 3.7 Alimentary mechanical properties at each position (25%, 50%, 75%) within a given size class. Small = s, Medium = m, and Large = l using $\log_{10}$ transformed data. Symbols are means of n = 4 - 8 samples; bars are standard error.



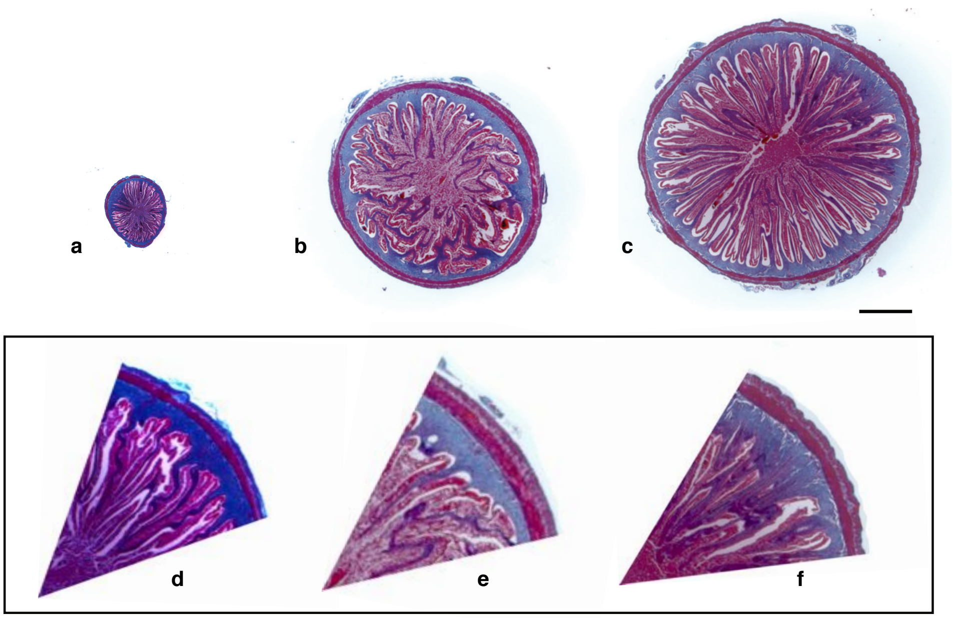


Figure 3.8 Representative radial cross-section in the striped perch, *Embiotoca lateralis*. Showing general wall structure of alimentary tissues in small (a), medium (b), and large (c) sizes classes; scale bar = 500 microns. Bottom insert box contains an enlarged wedge segments (d-f) highlighting radial wall of corresponding size classes above.

Table 3.1 Summary of ordinary least-squared regression analyses of body and gut morphometric data for all individuals. For each combination of independent and dependent variable, regression statistics (r^2, *p-value*) are listed, followed by coefficient estimates (±SE); n=18. Each regression slope was compared to the expected value using a one sample t-test; *p*-values in bold indicate significance for alpha = 0.05.

Independent variable (x)	***Dependent variable (y)***	***Regression***		***Intercept***	***Slope***			
		r^2	***p-value***	***Estimated ± SE***	***Estimated ± SE***	***Expected***	***t-stat***	***p-value***
Log$_{10}$ Body length	Log$_{10}$ Fish Mass	0.998	**<0.001**	-5.039±*0.08*	*3.18±0.04*	3	-4.828	**<0.001**
Log$_{10}$ Body length	Log$_{10}$ Gut Length	0.952	**<0.001**	-0.068±*0.08*	*0.96±0.05*	1	0.773	0.451
Log$_{10}$ Body length	Log$_{10}$ Gut Mass	0.990	**<0.001**	-3.235±*0.08*	*2.94±0.07*	3	0.867	0.399
Log$_{10}$ Gut Length	Log$_{10}$ Gut Mass	0.966	**<0.001**	-2.806±*0.08*	*2.95±0.14*	3	0.341	0.736

Table 3.2 Summary of ordinary least-squared regression analyses of log-transformed gape morphology for all individuals. For each combination of independent and dependent variable, regression statistics (r^2, *p-value*) are listed, followed by coefficient estimates (±SE); n=18. Each regression slope was compared to the expected value using a one sample t-test; *p*-values in bold indicate significance for alpha = 0.05.

Independent variable (x)	***Dependent variable (y)***	***Regression***		***Intercept***	***Slope***			
		r^2	***p-value***	***Estimated ± SE***	***Estimated ± SE***	***Expected***	***t-stat***	***p-value***
Log_{10} Body length	Log_{10} Gape Height	0.980	**<0.001**	-1.007±*0.07*	*0.89±0.03*	1	3.427	**0.003**
Log_{10} Body length	Log_{10} Gape Width	0.968	**<0.001**	-2.046±*0.13*	*1.31±0.06*	1	-5.202	**<0.001**
Log_{10} Body length	Log_{10} Gape Area	0.990	**<0.001**	-3.159±*0.12*	*2.20±0.06*	2	-3.418	**0.004**

Table 3.3 Summary of ordinary least-squared regression analyses on log-transformed average histological data for all individuals. For each combination of independent and dependent variable, regression statistics (*r^2*, *p-value*) are listed, followed by coefficient estimates (±SE); n=18. Each regression slope was compared to the expected value using a one sample t-test; *p*-values in bold indicate significance for alpha = 0.05. Note: percent wall thickness is relatively constant across all body lengths.

Independent variable (x)	***Dependent variable (y)***	***Regression***		***Intercept***	***Slope***			
		r^2	***p-value***	***Estimated ± SE***	***Estimated ± SE***	***Expected***	***t-stat***	***p-value***
Log$_{10}$ Body length	Log$_{10}$ Outer Circumference	0.953	**<0.001**	1.306±*0.14*	*1.206±0.07*	1	32.864	**0.007**
Log$_{10}$ Body length	Log$_{10}$ Wall Thickness	0.941	**<0.001**	-0.084±*0.18*	*1.305±0.08*	1	-3.739	**0.002**
Log$_{10}$ Body length	Log$_{10}$ Percent Thickness	0.080	*0.282*	0.661±*0.17*	*0.095±0.08*	0	-1.178	0.256

Table 3.4 Summary of two-way ANOVA of alimentary material properties (strength, extension ratio and toughness). Using $\log_{10}$ transformed data, and size class (large, medium, small) as a fixed factor. Post-hoc pairwise comparisons are also included for both size class and position along alimentary tract. Significant effects ($p < 0.05$) are indicated in bold.

Material Property	**Factor**	***df***	***Sum of squares***	***Mean sums of squares***	***F-value***	***p-value***
Strength	Position	*2*	0.135	0.068	3.805	**0.033**
	Size class	*2*	8.580	4.290	241.335	**<0.001**
	Position x Size class	*4*	0.038	0.010	0.540	0.707
	Residuals	*33*	0.587	0.018		
	Pairwise comparison					
	size class	*size class*	***p-value***	*position*	*position*	***p-value***
	large	medium	**0.013**	25	50	0.533
	large	small	**<0.001**	25	75	**0.007**
	medium	small	**<0.001**	50	75	**0.028**
Extension Ratio	Position	*2*	0.075	0.037	21.092	**<0.001**
	Size class	*2*	0.241	0.121	67.371	**<0.001**
	Position x Size class	*4*	0.011	0.003	1.524	0.218
	Residuals	*33*	0.059	0.002		
	Pairwise comparison					
	size class	*size class*	***p-value***	*position*	*position*	***p-value***
	large	medium	0.61	25	50	**<0.001**
	large	small	**<0.001**	25	75	**<0.001**
	medium	small	**<0.001**	50	75	0.118
Toughness	Position	*2*	0.098	0.049	1.513	0.235
	Size class	*2*	14.627	7.313	226.477	**<0.001**
	Position x Size class	*4*	0.044	0.011	0.338	0.850
	Residuals	*33*	1.066	0.032		
	Pairwise comparison					
	size class	*size class*	***p-value***	*position*	*position*	***p-value***
	large	medium	**0.013**	25	50	0.741
	large	small	**<0.001**	25	75	**0.033**
	medium	small	**<0.001**	50	75	0.063

3.8 ACKNOWLEDGMENTS

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Vita

Jaquan Horton came to this planet and quickly became a Red Sox fan. He received his Bachelors from University of Massachusetts Amherst and Masters from the University of California Irvine. His research interests align with the field of Biomechanics and Functional Morphology, with focus on the mechanical and material properties of biological tissues. However, he has conducted research on swimming fish, aquatic walking in salamanders, and antlion pit construction. He has a passion for learning and teaching others about the amazing elements of the natural world, and is adamant about creating, fortifying, and developing outreach programs for under-representative groups in STEM. Jaquan is an avid sailor, enjoys photography, and hopes science will dominate the minds of those on Earth so that greatness can be achieved for this species. Now, back to the home planet.