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Digestive enzyme activities in the guts of bonnethead sharks (*Sphyrna tiburo*) provide insight into their digestive strategy and evidence for microbial digestion in their hindguts

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ABSTRACT

Few investigations have studied digestive enzyme activities in the alimentary tracts of sharks to gain insight into how these organisms digest their meals. In this study, we examined the activity levels of proteases, carbohydrases, and lipase in the pancreas, and along the anterior intestine, spiral intestine, and colon of the bonnethead shark, *Sphyrna tiburo*. We then interpreted our data in the context of a rate-yield continuum to discern this shark's digestive strategy. Our data show anticipated decreasing patterns in the activities of pancreatic enzymes moving posteriorly along the gut, but also show mid spiral intestine peaks in aminopeptidase and lipase activities, which support the spiral intestine as the main site of absorption in bonnetheads. Interestingly, we observed spikes in the activity levels of *N*-acetyl-β-D-glucosaminidase and β-glucosidase in the bonnethead colon, and these chitin- and cellulose-degrading enzymes, respectively, are likely of microbial origin in this distal gut region. Taken in the context of intake and relatively long transit times of food through the gut, the colonic spikes in *N*-acetyl-β-D-glucosaminidase and β-glucosidase activities suggest that bonnetheads take a yield-maximizing strategy to the digestive process, with some reliance on microbial digestion in their hindguts. This is one of the first studies to examine digestive enzyme activities along the gut of any shark, and importantly, the data match with previous observations that sharks take an extended time to digest their meals (consistent with a yield-maximizing digestive strategy) and that the spiral intestine is the primary site of absorption in sharks.

1. Introduction

Generally, the digestive strategies of animals fit within a spectrum of physiological parameters called a rate-yield continuum (Fig. 1). On one end, yield maximizers consume relatively large meals less frequently, hold the digesta in their digestive tract for long periods of time, and have relatively high digestive efficiency of total organic matter. Rate maximizers, on the other hand, tend to consume large amounts of low-quality food at a high frequency, pass food through the gut quickly, and have relatively low digestibility of total organic matter. Rate maximizers tend to readily digest the more soluble fractions of their diet (German, 2009; German and Bittong, 2009; Karasov and Douglas, 2013; German et al., 2015), whereas yield maximizers can also digest the more structural elements (e.g., chitin) of their food, either endogenously or with the aid of an enteric microbial community (Crossman et al., 2005; Skea et al., 2005; Karasov and Martínez del Rio, 2007; Karasov and Douglas, 2013). Hence, in addition to diet itself, an animal's digestive strategy affects its ecological role, and thus, it is important to move beyond diet analysis in studies of trophic ecology and also investigate an animal's nutritional physiology (Crossman et al., 2005; Skea et al., 2005, 2007; Karasov and Martínez del Rio, 2007; Karasov and Douglas, 2013; German et al., 2015).

Most fishes, including sharks, do not masticate their food before ingesting it into the digestive tract. Thus, the chemical means of digestion (i.e., hydrochloric acid, digestive enzymes) are crucial in nutrient acquisition in fishes (Papastamatiou and Lowe, 2004, 2005; Clements and Raubenheimer, 2006; German, 2011). In fact, the activity levels of digestive enzymes are often used to infer digestive function in fishes. Generally, carbohydrase activities (e.g., amylase) are elevated in herbivores, whereas some proteases (e.g., aminopeptidase) can be elevated in carnivores (Hidalgo et al., 1999; German et al., 2004; German et al., 2015). Moreover, the patterns of enzymatic activity along a fish's gut can provide insight into the digestive strategy taken by a fish to digest a given diet (Fig. 2A; Skea et al., 2005, 2007; Day et al., 2011; German, 2009; German and Bittong, 2009; German et al., 2015). The key to these studies is a spike in the activities of microbially derived digestive enzymes—especially enzymes that degrade insoluble, structural compounds like cellulose, carrageenan, and chitin—in the hindguts of fishes adopting a yield-maximizing strategy, and a lack

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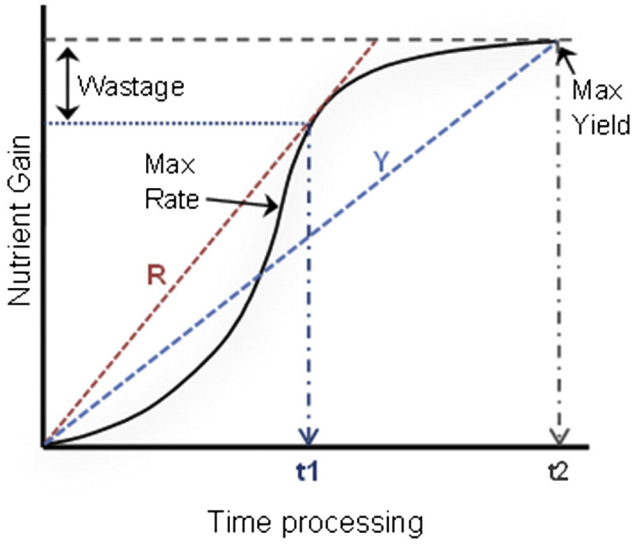

Fig. 1. Cumulative nutrient gained (solid black line) by a fish as a function of time spent processing a meal (modified from German et al., 2015). The slope of the black line labeled "Max Rate" is the maximum rate at which the nutrient can be absorbed from the meal. A rate-maximizing strategy is characterized by a line tangential to the curve (red line "R"), with defecation of gut contents occurring at time 1 (t1). A portion of the nutrient consumed is lost in the feces ("Wastage"), but at t1, the animal can take a new meal. This is the rate-maximizing strategy with high intake. Maximum yield (blue line "Y") is attained by extending processing time to time 2 (t2); however, this is done at the cost of reduced digestive rate. In animals with lower intake, this strategy tends to involve longer retention times of food in the gut and can include microbial fermentation in the hindgut (especially in herbivorous vertebrates).

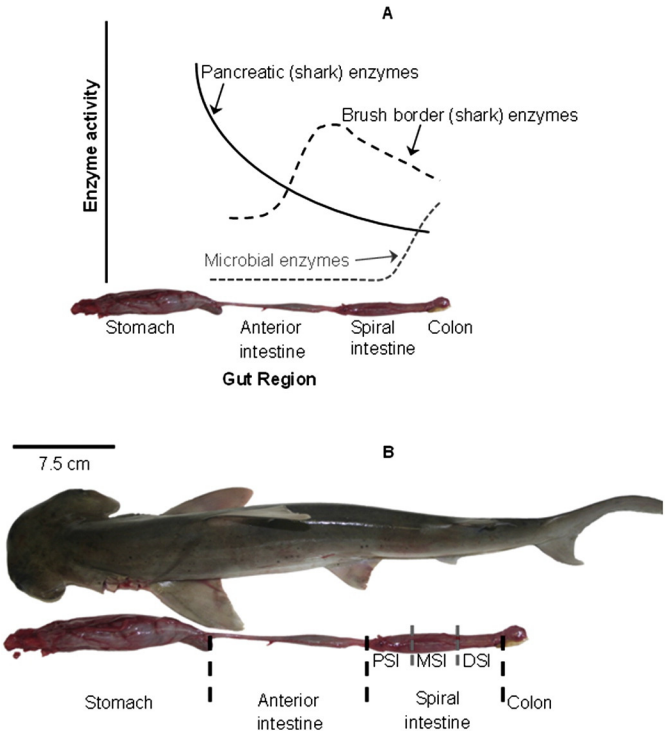

Fig. 2. (A) Potential patterns of digestive enzyme activities along a shark gut. Pancreatic enzymes are made in the acinar cells of the pancreas (not shown). Thus, other than the pancreas itself, activities of pancreatic digestive enzymes would be expected to be highest in the anterior intestine (where they are carried after traveling down the ductus choledochus). Brush border enzymes tend to peak in the mid-intestine of many fishes, which would be the spiral intestine in sharks. However, microbially produced enzymes peak in the distal intestines of fish utilizing a yield-maximizing strategy because microbes tend to be more concentrated in the distal intestines of fishes (see Skea et al., 2005; German et al., 2015). Fish adopting a rate-maximizing strategy would not show a spike in microbial digestive enzymes in their distal intestines (see German, 2009; German and Bittong, 2009; German et al., 2015). (B) *Sphyrna tiburo* with its digestive tract. For this study, the stomach was excised, and the remaining digestive tract was divided into the anterior intestine, proximal-, mid-, and distal-spiral intestine (PSI, MSI, and DSI, respectively), and colon.

of such an activity spike in rate-maximizing fishes. The reason for the distal intestine spike in microbial enzymatic activity in fishes taking a yield-maximizing strategy is that yield maximizers tend to have rich microbial communities in their hindguts (i.e., foregut microbial digestion, as in ruminant mammals, is unknown in fishes; Moran et al., 2005; Clements and Raubenheimer, 2006; Clements et al., 2014). Thus, patterns of digestive enzymatic activity along a fish's gut are useful in understanding their digestive strategy and trophic ecology.

As of late, there has been an increase in the interest in shark trophic ecology, and yet, investigations of digestive strategies in sharks are limited and have primarily focused on the stomach (e.g., Papastamatiou and Lowe, 2004, 2005; Papastamatiou, 2007; Newton et al., 2015). Thus, in this study, we investigated the bonnethead shark, *Sphyrna tiburo*, which is a small, coastal hammerhead species and is one of the most abundant elasmobranch taxa in coastal Florida waters. Bonnethead sharks generally consume crustaceans, cephalopods, and mollusks (Cortés et al., 1996), although some young-of-the-year bonnetheads consume copious amounts of sea grass (Bethea et al., 2007). Hence, while having a typical carnivorous diet, bonnetheads may also need to digest chitin (in crustacean exoskeletons) and plant structural polysaccharides (e.g., cellulose in sea grass).

We measured digestive enzyme activities in the guts of bonnethead sharks to examine what compounds these fish could digest and to infer their digestive strategy based on the digestive enzyme activity patterns along their guts (Table 1; Fig. 2A). We, therefore, tested the hypothesis that bonnethead sharks are "yield maximizers" and consume relatively large meals relatively infrequently (Fig. 1; German et al., 2015). Thus, sharks, including bonnetheads, would be expected to have enzymatic patterns consistent with their yield-maximizing strategy that would include some amount of microbial digestion in the hindgut (Fig. 2A; German et al., 2015). Sharks are known to have long transit times (i.e., >20 h) of food in the gut (Wetherbee et al., 1987), which is another indication of a yield-maximizing strategy towards digestion. However, elasmobranchs (including sharks) have a relatively short intestine coupled to a "spiral valve" (Fig. 2B), which is a convoluted region of the intestine that resembles a spiral staircase or a rolled scroll of paper in cross section (Holmgren and Nilsson, 1999; Chatchavalvanich et al., 2006; Theodosiou et al., 2007). Although it is accepted that the spiral valve (heretofore called the spiral intestine) increases the absorptive surface area in the elasmobranch gut (Holmgren and Nilsson, 1999; Chatchavalvanich et al., 2006; Wilson and Castro, 2011), patterns of digestive enzyme activities in the anterior intestine versus the spiral intestine, or how enzyme activities change along the spiral intestine, are largely unknown (Kuz'mina, 1990; Holmgren and Nilsson, 1999). Activities of the intestinal enzymes maltase, sucrase, trehalase, and alkaline phosphatase have been measured in membrane vesicle preparations of dogfish spiral intestine tissue (Crane et al., 1979), but it was not clear from where in the spiral intestine these vesicle preparations were taken, or how activities change moving along the spiral intestine. Therefore, our investigation also provides insight into the role of the spiral intestine as a site of digestion and/or absorption in sharks.

We measured the activity levels of six digestive enzymes in the guts of bonnethead sharks that reflected this species ability to digest carbohydrates (maltase, *N*-acetyl-β-D-glucosaminidase, and β-glucosidase), protein (trypsin and aminopeptidase), and lipids (lipase; Table 1). We measured the activities of these enzymes in the pancreas, anterior intestine, along the spiral intestine, and in the colon (Fig. 2B) and used the activity patterns to infer whether these sharks are rate or yield maximizers, predicting the latter. We did not measure digestive enzyme activities (e.g., pepsin, chitinase) in

Table 1
Digestive enzymes assayed in tissues and contents of the digestive tract of *Sphyrna tiburo*.

Enzyme	Synthesis[1]	Substrate	Dietary source	Expected pattern[2]	>Fraction[3]
Maltase	Brush border	Maltose	Animals, sea grass	Middle spike	Tissue
β-Glucosidase	Microbial	β-Glucosides	Sea grass	Colonic spike	Contents
N-acetyl-β-D-glucos[4]	Brush border, microbial	*N*-acetyl-β-D-glucoam	Crustaceans	Middle spike	Tissue
Trypsin	Pancreatic	Protein	Animals	Decrease	Tissue
Aminopeptidase	Brush border	Dipeptides	Animals	Middle spike	Tissue
Lipase	Pancreatic	Lipid	Animals	Decrease	Tissue

[1] Indicates where the enzyme is synthesized, either from fish (pancreatic or brush border), or from microbial sources.
[2] This column shows the expected patterns of activity along the GI tracts of *S. tiburo*. For example, "decrease" means that the activity of this enzyme should decrease toward the distal-most regions of the digestive tract, whereas a "colonic spike" means that the enzyme is expected to increase in activity in the colon (see Fig. 2), which may indicate microbial production of this enzyme.
[3] Predictions of which assayed fractions will have higher activity of a particular enzyme. For example, "Tissue" means that the activity of that enzyme is expected to be greater in the fishes' gut tissues than in the contents of a given gut region.
[4] Complete name of the enzyme is *N*-acetyl-β-D-glucosaminidase, and the substrate is *N*-acetyl-β-D-glucosaminides.

the stomachs of the sharks, as that is the focus of a different study, although a cursory examination of the stomach contents of the sharks used in this study confirmed their carnivorous diet (Cortés et al., 1996; Bethea et al., 2007).

2. Materials and methods

2.1. Shark capture and tissue preparation

Six bonnethead sharks were collected in gill nets off the coast of Cedar Key (29.115° N, 83.034° W) and Cumberland Sound (30.795° N, 81.492° W), Florida, USA. Sharks were incidental mortalities from monthly surveys of shark nursery habitat within Florida coastal waters (e.g., Bethea et al., 2011). Immediately following collection, freshly dead sharks were measured (stretch total length ± 0.5 cm) and then dissected on a cutting board kept on ice (4 °C). The sharks were 98 ± 9.4 cm (mean ± SEM) in length and were small adults or large juveniles. Each digestive system was removed by cutting just anterior to the stomach and at the anus. The pancreas was excised and frozen individually on dry ice in a 50 mL centrifuge vial. The guts were gently uncoiled, measured, and the stomachs excised. The stomachs were placed in individual bags and frozen on dry ice for later use in gut content analyses. The remaining digestive tract was divided into the following sections: anterior intestine, spiral intestine (SI), and colon (Fig. 2B). The SI was further subdivided into three sections of equal length: the proximal, mid, and distal SI (Fig. 2B). Each section was emptied of their contents by pushing with the blunt side of a razorblade, and the tissue was rinsed with shark Ringer's solution; the contents and intestinal tissues were then placed in separate 50 mL centrifuge vials and frozen on dry ice (German and Bittong, 2009). Contents were recovered from each gut region of each shark, and total gut content masses ranged from 0.05% to 0.7% of body mass (mean ± SEM: 0.31 ± 0.10%). Because we did not measure body masses of our sharks, we estimated their body masses from the length–weight relationship for scalloped hammerheads (*Sphyrna lewini*), which are similarly sized and closely related to bonnetheads (Cavalcanti, 2007), using the equations described in Kohler et al. (1996). Frozen samples were then shipped on dry ice to UC Irvine where they were stored at −80 °C until analyzed (within 6 months).

Gut tissues or contents from each gut region from individual sharks were weighed (regional gut or content mass ± 0.001 g) and homogenized following German and Bittong (2009). Intestinal contents were homogenized in 25 mM Tris–HCl, pH 7.5, whereas intestinal tissues were homogenized in 350 mM mannitol with 1 mM HEPES, pH 7.5. Colon tissue and contents were homogenized in sodium acetate pH 5.5, based on the acidic conditions documented in this gut region of the bamboo shark (*Chiloscyllium plagiosum*; Anderson et al., 2010). The supernatants of homogenates were collected and stored in small aliquots (100–200 μl) at −80 °C until just before use in spectrophotometric or fluorometric assays of digestive enzyme activities. The protein content of the homogenates was measured using bicinchoninic acid (German and Bittong, 2009; Smith et al., 1985). Stomach contents were cursorily examined in all specimens confirming the carnivorous diet of the bonnetheads. Gut content masses were used to determine the percent of total gut content mass found in each gut region following German et al. (2015).

2.2. Assays of digestive enzyme activity

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

Maltase and Sucrase activities were measured following Dahlqvist (1968), as described by German and Bittong (2009). We used 112 mM maltose (or 100 mM sucrose) dissolved in 200 mM phosphate buffer, pH 7.5 (sodium acetate pH 5.5 for the colon tissue and contents). The maltase and sucrase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

β-Glucosidase and *N*-acetyl-β-D-glucosaminidase activities were measured following German et al. (2015), using 200 μM solutions of the substrates 4-methylumbelliferyl-β-D-glucoside and 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide, respectively, dissolved in 25 mM Tris–HCl (pH 7.5; sodium acetate pH 5.5 for the colon tissue and contents). Briefly, 90 μL of substrate was combined with 10 μL of homogenate in a black microplate and incubated for 30 min. Following incubation, 2.5 μL of 1 M NaOH was added to each microplate well, and the fluorescence read immediately at 365 nm excitation and 450 nm emission. Each plate included a standard curve of the product (4-methylumbelliferone), substrate controls, and homogenate controls, and enzymatic activity (μmol product released per minute per gram wet weight tissue) was calculated from the MUB standard curve (German et al., 2011).

Trypsin activity was assayed using a modified version of the method designed by Erlanger et al. (1961). The substrate, 2 mM Nα-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA), was dissolved in 100 mM Tris–HCl buffer (pH 7.5; sodium acetate pH 5.5 for the colon tissue and contents). Trypsin activity was determined with a *p*-nitroaniline standard curve, and expressed in U

(μmol *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase activity was measured using 2.04 mM L-alanine-*p*-nitroanilide HCl dissolved in 200 mM sodium phosphate buffer (pH 7.5; sodium acetate pH 5.5 for the colon tissue and contents). Aminopeptidase activity was determined with a *p*-nitroaniline standard curve, and activity was expressed in U (μmol *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Lipase (nonspecific bile-salt activated) activity was assayed using 0.55 mM *p*-nitrophenyl myristate (in ethanol) in the presence of 5.2 mM sodium cholate dissolved in 25 mM Tris–HCl (pH 7.5; sodium acetate pH 5.5 for the colon tissue and contents). Lipase activity was determined with a *p*-nitrophenol standard curve and expressed in U (μmol *p*-nitrophenol liberated per minute) per gram wet weight of gut tissue.

The activity of each enzyme was regressed against the protein content of the homogenates to confirm that there were no significant correlations between the two variables. Because no significant correlations were observed, the data are not reported as U per mg protein.

2.3. Statistical analyses

Prior to all significance tests, a Levene's test for equal variance was performed and residual versus fits plots were examined to ensure the appropriateness of the data for parametric analyses. Where necessary, data were log-transformed prior to analysis. All tests were run using SPSS statistical software (version 20). Comparisons of mass-specific enzymatic activities were made among gut regions with ANOVA followed by a Tukey's HSD with a family error rate of $P = 0.05$.

3. Results

The gut contents of the sharks were concentrated in the spiral intestine, with the distal-spiral intestine having more contents than the anterior intestine or colon (Fig. 3). Collectively, the spiral intestine contained approximately 86% of the total gut content mass of the sharks (not accounting for stomach content mass). The digestive enzyme activities of the bonnetheads generally followed the patterns predicted in Table 1 and Fig. 2A. Trypsin showed a strong decreasing pattern moving distally along the gut, with significantly ($P < 0.001$) higher activities in the pancreas than other gut regions, and activities in the tissues tended to be of the same magnitude as those in the contents (Fig. 4). Lipase activities showed a similar pattern moving along the guts as trypsin did ($P = 0.003$), but there were elevated activities of this enzyme in the mid SI samples (Fig. 4). Aminopeptidase activities showed a significant spike ($P < 0.001$) in the mid SI samples, and activities were generally elevated in the tissues in comparison to the contents (Fig. 5). *N*-acetyl-β-D-glucosaminidase activities were more elevated in the SI tissues than the anterior intestine ($P < 0.001$), but there were spikes in the activity of this enzyme in the tissue and contents ($P = 0.003$) of the colon (Fig. 5), suggesting potential microbial production of this enzyme in the distal-most regions of the sharks' digestive tract. Maltase activities generally decreased ($P = 0.057$) moving distally along the digestive tract, whereas β-glucosidase activities clearly spiked ($P = 0.062$) in the colon of the sharks (Fig. 6). This latter result again suggests a microbial source for this enzyme, although β-glucosidase activity was not reliably detectable in the gut contents of any gut region.

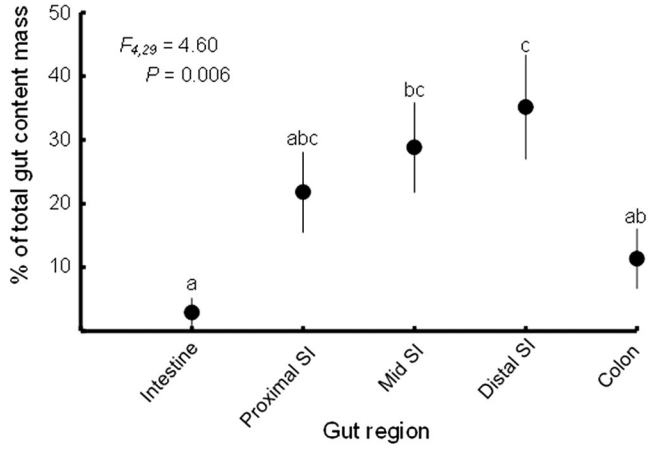

Fig. 3. Regional gut content mass as a percent of total gut content mass in different gut regions of *Sphyrna tiburo*. See Fig. 2 for gut region definitions. Activities are mean ± SEM. Values were compared among gut regions with ANOVA followed by Tukey's HSD multiple comparisons test. Regional gut content mass percentages that share a letter are not significantly different from one another ($P > 0.05$). SI = spiral intestine.

4. Discussion

Two key observations support the contention that bonnethead sharks adopt a yield-maximizing strategy to digestion: the activity levels of *N*-acetyl-β-D-glucosaminidase and β-glucosidase were elevated in the sharks' colons. These distal intestine enzyme activity spikes are consistent with other fish species known to have a yield-maximizing strategy in the digestive process (Skea et al., 2005; German et al., 2015) and suggest that bonnetheads have an active microbial population in their hindguts that may aid in digestion. Overall, the patterns of digestive enzyme activities in the bonnethead guts largely matched our predictions (Table 1; Fig. 2A), with pancreatic enzyme activities largely decreasing moving down the gut (Fig. 4) and some brush border enzyme activities (e.g., aminopeptidase) peaking in the mid spiral intestine (Fig. 5). This latter result, along with contents being concentrated in the spiral intestine (Fig. 3), suggests that the spiral intestine is likely the most active, absorptive section of the shark intestine.

Sharks are known for consuming large meals, and holding those meals for extended periods of time in the stomach (Wetherbee et al., 1987; Holmgren and Nilsson, 1999; Papastamatiou, 2007) before releasing chyme into the anterior intestine (Meyer and Holland, 2012). What happens to digesta after passage into the anterior intestine is largely unstudied in many elasmobranchs, but flow likely follows the typical "plug-flow" model (Penry and Jumars, 1987) through the anterior intestine until the digesta reaches the spiral intestine, where transit may be slowed (Holmgren and Nilsson, 1999); indeed, about 86% of the intestinal content mass of the bonnetheads was concentrated in the spiral intestine (Fig. 3). Slowed flow anywhere in the intestine is also consistent with a yield-maximizing strategy. In one of the most detailed analyses of the intestinal epithelium in any elasmobranch, Chatchavalvanich et al. (2006) showed that the spiral intestine of the white-edge freshwater ray (*Himantura signifer*) had more complex folding patterns (i.e., more absorptive surface area) than the anterior intestine in that species. Observations in other elasmobranchs support this contention (Holmgren and Nilsson, 1999; Wilson and Castro, 2011). Given that we observed that aminopeptidase and lipase activities peaked in the mid spiral intestine, similar to mid-intestine spikes in activities of these enzymes in other fish species that lack a spiral intestine (Chakrabarti et al., 1995; Harpaz and Uni, 1999; Smoot and Findlay, 2000; German, 2009; German et al., 2015), this portion of the intestine seems to be the primary site of amino acid and fatty acid absorption in bonnetheads. Thus, the spiral intestine essentially encompasses what is called the "intestine" in most other fishes, with regionality of function changing from proximal to distal ends (German, 2009; German et al., 2015), unlike some earlier cursory analyses that claimed little regionality in digestive enzyme activity in the elasmobranch gut (Kuz'mina, 1990). Measurements of nutrient transport rates along the elasmobranch epithelium are lacking, but we hypothesize that these rates would be highest in the spiral intestine. Our data, along with others (e.g., Holmgren and Nilsson, 1999; Chatchavalvanich et al., 2006), suggest differing roles for the

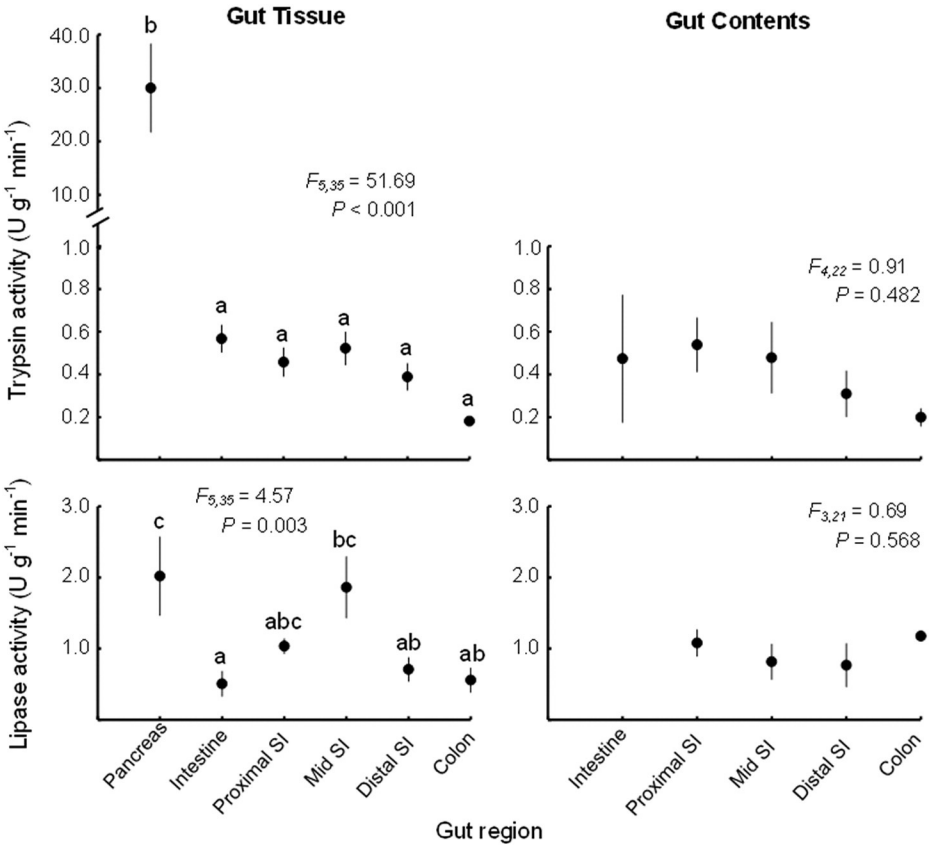

Fig. 4. Trypsin (top) and lipase (bottom) activities in gut tissue (left column) or gut contents (right column) in different gut regions of *Sphyrna tiburo*. See Fig. 2 for gut region definitions. Activities are mean ± SEM. Trypsin or lipase activities were compared among gut regions independently for tissue or contents with ANOVA followed by Tukey's HSD multiple comparisons test. Regional enzymatic activity values for an enzyme and gut fraction (tissue or contents) that share a letter are not significantly different from one another ($P > 0.05$). There were not enough intestinal contents in which to perform the lipase assay, and hence these values are missing from the lipase gut content graph (right bottom). SI = spiral intestine.

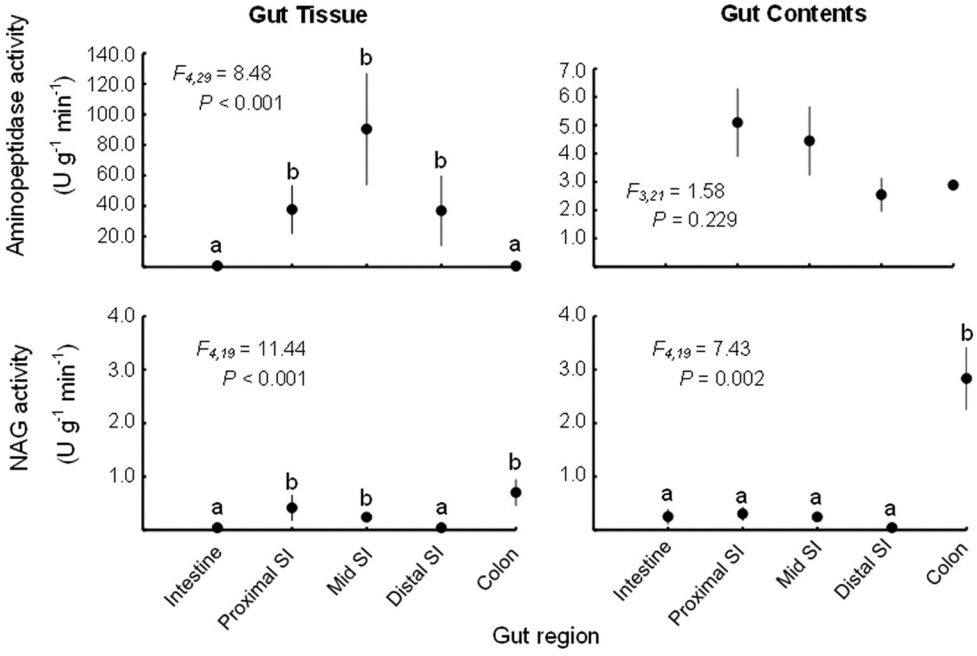

Fig. 5. Aminopeptidase (top) and *N*-acetyl-β-D-glucosaminidase (NAG; bottom) activities in gut tissue (left column) or gut contents (right column) in different gut regions of *Sphyrna tiburo*. See Fig. 2 for gut region definitions. Note the different scales for the *y*-axis of the aminopeptidase graphs. Activities are mean ± SEM. Aminopeptidase or NAG activities were compared among gut regions independently for tissue or contents with ANOVA followed by Tukey's HSD multiple comparisons test. Regional enzymatic activity values for an enzyme and gut fraction (tissue or contents) that share a letter are not significantly different from one another ($P > 0.05$). There were not enough intestinal contents in which to perform the aminopeptidase assay, and hence these values are missing from the aminopeptidase gut content graph (right top). SI = spiral intestine.

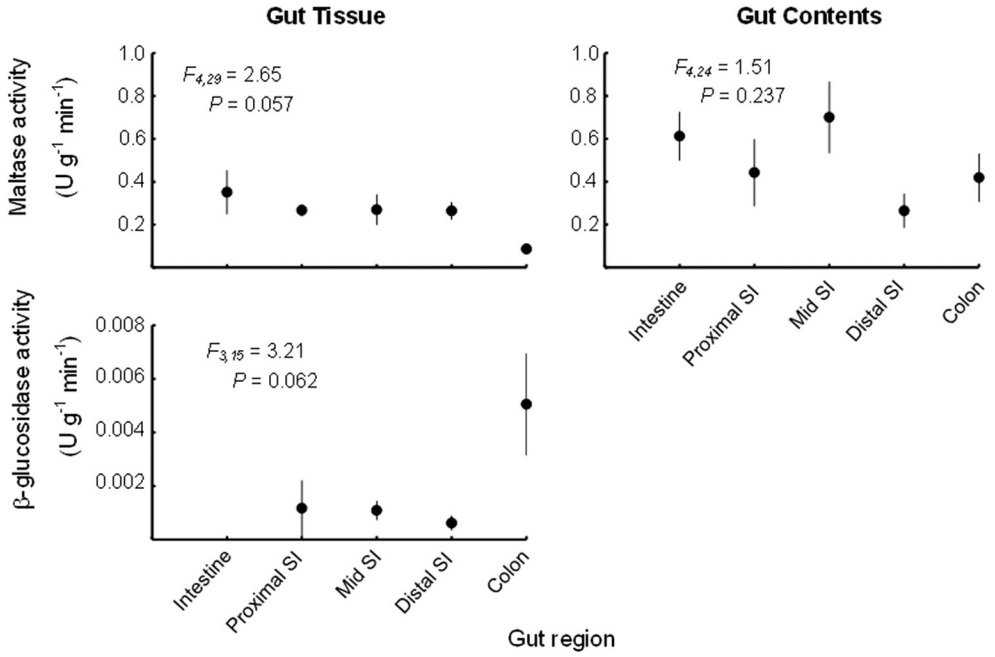

Fig. 6. Maltase (top) and β-glucosidase (bottom) activities in gut tissue (left column) or gut contents (right column) in different gut regions of *Sphyrna tiburo*. See Fig. 2 for gut region definitions. Activities are mean ± SEM. Maltase or β-glucosidase activities were compared among gut regions independently for tissue or contents with ANOVA followed by Tukey's HSD multiple comparisons test. Regional enzymatic activity values for an enzyme and gut fraction (tissue or contents) that share a letter are not significantly different from one another ($P > 0.05$). There was no detectable β-glucosidase activity in the intestine, and hence these values are missing from the β-glucosidase gut tissue graph (left bottom). β-Glucosidase was not repeatedly detectable in gut contents, and hence, this graph is not shared. SI = spiral intestine.

anterior and spiral intestines, and this should be the focus of future work on shark digestive tracts.

The elevated trypsin and aminopeptidase activities in the bonnethead pancreatic and spiral intestinal tissues, respectively, make sense, as the pancreas is the site of synthesis for trypsin and enterocytes the sites of synthesis for aminopeptidase (Karasov and Martínez del Rio, 2007). However, the bonnethead trypsin and aminopeptidase activity levels are about 10× higher than activities in the pancreatic or intestinal tissues of carnivorous teleost fishes measured using the same methods and equations for calculations as this study (German, 2009; German et al., 2015). Bonnethead trypsin activity levels are also qualitatively similar to trypsin activities measured in Mako sharks at a similar temperature (25 °C; Newton et al., 2015). Thus, bonnetheads appear to be efficient at digesting protein, which is probably an important nutrient for these carnivorous animals. Lipase activity in the bonnetheads is not exceptionally elevated in comparison to other fishes, or sharks (Newton et al., 2015), but the broad distribution of lipolytic activity along the gut (Fig. 4) suggests that bonnetheads likely readily digest lipids with great efficiency. To our knowledge, there are not any studies of protein and/or lipid digestibility in sharks consuming their natural prey items, but Wetherbee and Gruber (1993) measured apparent digestive efficiencies of 62–83% and 76–88% for energy and organic matter, respectively, in lemon sharks (*Negaprion brevirostris*) consuming different-sized meals of a fish diet. Given that fish would be primarily protein and lipid (Horn, 1989), it follows that the high organic matter digestibility by lemon sharks would primarily be of protein and lipid. We attempted to measure amylolytic activity in the bonnetheads, but we were not able to reliably detect enzymatic activity against starch, suggesting that bonnetheads may be poor at digesting soluble carbohydrates, like starch. This is corroborated by the relatively low maltase activities in the bonnethead intestine (Fig. 5), although using membrane vesicle preparations may improve detection of maltase (Crane et al., 1979).

What is intriguing is the presence of elevated *N*-acetyl-β-D-glucosaminidase activity in the colons of the bonnetheads. Bonnetheads clearly consume chitin with their diet rich in crustaceans (Bethea et al., 2007), and like other fishes that consume chitin (Goodrich and Morita, 1977; Gutowska et al., 2004; German et al., 2010; German et al., 2015), this may be an important source of carbon and nitrogen for these sharks. Indeed, *N*-acetyl-β-D-glucosaminidase activities in the bonnethead intestine are also at least 5× higher than carnivorous, omnivorous, and detritivorous teleost fishes measured using the same methods and equations for calculations as this study (German and Bittong, 2009; German et al., 2015). The source of the *N*-acetyl-β-D-glucosaminidase is likely endogenous along the gut walls of the anterior and spiral intestine, but the spike in *N*-acetyl-β-D-glucosaminidase activity in the colon (including the colon contents) strongly suggests a microbial origin of these activities in the hindgut (German and Bittong, 2009; German et al., 2015).

Interestingly, the colons of sharks and skates are known to be acidic (pH 5.5–6.4, depending on species), which is more acidic than their intestines (which tend to be pH 7.0–7.5; Anderson et al., 2010; but see Wood et al., 2007) and is more similar to the pH of a typical vertebrate colon, which is a site of microbial fermentation (Karasov and Martínez del Rio, 2007; Karasov and Douglas, 2013). The colonic environment is anaerobic, which allows enteric microbes to use fermentative pathways to produce short chain fatty acids (SCFA); these SCFA (e.g., acetate, propionate) are the reason for the lower pH of the colon in most vertebrates and the SCFA can be absorbed by the host and used for ATP production (Bergman, 1990; Stevens and Hume, 1998; Karasov and Martínez del Rio, 2007; Karasov and Douglas, 2013). Although we did not measure SCFA production in the bonnetheads, SCFA production is well known in the hindguts of fishes and is usually higher in herbivores than in carnivores (Clements and Choat, 1995; Clements et al., 2014; German et al., 2015), although some carnivores (e.g., largemouth bass, *Micropterus salmoides*) do show seasonally high levels of SCFA production in their hindguts (Smith et al., 1996). The omnivorous *Phytichthys chirus*, which also consumes a crustacean-rich diet, has elevated levels of *N*-acetyl-β-D-glucosaminidase activities in its hindgut (German et al., 2015), further suggesting that hindgut microbial digestion of chitin may be wide-spread in carnivorous fishes with chitin-rich diets, as we see in the bonnetheads. The slower transit time of food through a

carnivorous gut is amenable to a yield-maximizing strategy, and the activities of microbially produced enzymes being elevated in the hindgut also support that carnivores can be yield maximizers with some reliance on microbial symbionts in the digestive process.

Along these lines, we were surprised to observe a spike in β-glucosidase activity in the bonnethead colon, as this enzyme digests the breakdown products of cellulose and other β-glucosides, like laminarin (German and Bittong, 2009). Up to 62% of young-of-the-year bonnethead diet (by mass) can be composed of sea grass, which appears degraded by the time it reaches the hindguts of the sharks (Bethea et al., 2007). Certainly, if bonnetheads have a microbial community in their hindguts that are capable of degrading chitin, they may also be able to degrade other β-glucosides. The activity levels of β-glucosidase in the bonnethead colon are about 2× higher than those observed in the hindguts of *Cebidichthys violaceus*, an herbivorous, teleost fish that digests algal material in its hindgut with the aid of an enteric microbial community (German et al., 2015). Clearly, feeding trials to examine the digestibility of sea grass by bonnetheads are necessary to confirm this supposition, but it does appear possible that bonnetheads have the enzymatic machinery to degrade components of sea grass. Indeed, the main carbohydrate in sea grass is cellulose, and sucrose is the photosynthate (Kuiper-Linley et al., 2007). We did not readily detect sucrase activities in the bonnethead intestine, but attempting sucrase assays on membrane vesicle preparations (as we suggest for maltase) may produce more consistent results for this enzyme (Crane et al., 1979). Using stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$ signatures), Bethea et al. (2011) showed that the bonnetheads occupied a different trophic space (in particular, a lower trophic level from the perspective of $\delta^{15}N$) than their congener, the scalloped hammerhead shark (*Sphyrna lewini*), which is more piscivorous. Although invertebrate versus fish diets would be enough to result in niche segregation from the perspective of stable isotope analysis, it is possible that the digestion of sea grass and its epibionts may contribute to the lower $\delta^{15}N$ and enriched $\delta^{13}C$ signatures observed in bonnethead tissues relative to scalloped hammerhead tissues (Bethea et al., 2011), but this needs to be explored in more detail.

In conclusion, we measured digestive enzyme activities along the guts of bonnethead sharks in an effort to understand their digestive strategy and discern what compounds they might be able to digest. The patterns of enzymatic activity along their guts suggest that bonnetheads take a yield-maximizing strategy to the digestive process, and that these sharks likely harbor an enteric microbial community in their colons that may aid in digestion of complex carbohydrates (e.g., chitin, cellulose). We also elucidated that the spiral intestine is likely the primary site of digestion and absorption in the bonnethead gut, and future studies should focus on the spiral intestine to discern the digestive capabilities of elasmobranchs, but also determine the role of the anterior intestine, which is currently unclear. Indeed, there is broad interest in sharks, and to better understand their ecological roles, we need to move beyond feeding observations and truly grasp what they are eating, digesting, and excreting back into their environments in order to make better predictions of how sharks will thrive in a changing world.

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References

Anderson, W.G., Dasiewicz, P.J., Liban, S., Ryan, C., Taylor, J.R., Grosell, M., Weihrauch, D., 2010. Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea* and the clear nose skate *Raja eglanteria*. Comp. Biochem. Physiol. A 155, 493–502.

Bergman, E., 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70, 567–590.

Bethea, D.M., Hale, L., Carlson, J.K., Cortés, E., Manire, C.A., Gelsleichter, J., 2007. Geographic and ontogenetic variation in the diet and daily ration of the bonnethead shark, *Sphyrna tiburo*, from the eastern Gulf of Mexico. Mar. Biol. 152, 1009–1020.

Bethea, D.M., Carlson, J.K., Hollensead, L.D., Papastamatiou, Y.P., Graham, B.S., 2011. A comparison of the foraging ecology and bioenergetics of the early life-stages of two sympatric hammerhead sharks. Bull. Mar. Sci. 87, 873–899.

Cavalcanti, M.J., 2007. A phylogenetic supertree of the hammerhead sharks (Carcharhiniformes: Sphyrnidae). Zool. Stud. 46, 6–11.

Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R., Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. Comp. Biochem. Physiol. 112A, 167–177.

Chatchavalvanich, K., Marcos, R., Poonpirom, J., Thongpan, A., Rocha, E., 2006. Histology of the digestive tract of the freshwater stingray *Himantura signifer* Compagno and Roberts, 1982 (Elasmobranchii, Dasyatidae). Anat. Embryol. 211, 507–518.

Clements, K.D., Choat, J.H., 1995. Fermentation in tropical marine herbivorous fishes. Physiol. Biochem. Zool. 68, 355–378.

Clements, K.D., Raubenheimer, D., 2006. Feeding and nutrition. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press, Boca Raton, FL, pp. 47–82.

Clements, K.D., Angert, E.R., Montgomery, W.L., Choat, J.H., 2014. Intestinal microbiota in fishes: what's known and what's not. Mol. Ecol. 23, 1891–1898.

Cortés, E., Manire, C.A., Hueter, R.E., 1996. Diet, feeding habits, and diel feeding chronology of the bonnethead shark, *Sphyrna tiburo*, in southwest Florida. Bull. Mar. Sci. 58, 353–367.

Crane, R.K., Boge, G., Rigal, A., 1979. Isolation of brushborder membranes in vesivular form from the intestinal spiral valve of the small dogfish (*Scyliorhinus canicula*). Biochim. Biophys. Acta 554, 264–267.

Crossman, D.J., Choat, J.H., Clements, K.D., 2005. Nutritional ecology of nominally herbivorous fishes on coral reefs. Mar. Ecol. Prog. Ser. 296, 129–142.

Dahlqvist, A., 1968. Assay of intestinal disacharidases. Anal. Biochem. 22, 99–107.

Day, R.D., German, D.P., Manjakasy, J.M., Farr, I., Hansen, J., Tibbetts, I.R., 2011. Enzymatic digestion in stomachless fishes: how a simple gut accommodates both herbivory and carnivory. J. Comp. Physiol. B. 181, 603–613.

Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

German, D.P., 2009. Do herbivorous minnows have "plug-flow reactor" guts? Evidence from digestive enzyme activities, gastrointestinal fermentation, and luminal nutrient concentrations. J. Comp. Physiol. B. 179, 759–771.

German, D.P., 2011. Digestive efficiency. In: Farrel, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, pp. 1596–1607.

German, D.P., Bittong, R.A., 2009. Digestive enzyme activities and gastrointestinal fermentation in wood-eating catfishes. J. Comp. Physiol. B. 179, 1025–1042.

German, D.P., Horn, M.H., Gawlicka, A., 2004. Digestive enzyme activities in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Physiol. Biochem. Zool. 77, 789–804.

German, D.P., Nagle, B.C., Villeda, J.M., Ruiz, A.M., Thomson, A.W., Contreras-Balderas, S., Evans, D.H., 2010. Evolution of herbivory in a carnivorous clade of minnows (Teleostei: Cyprinidae): effects on gut size and digestive physiology. Physiol. Biochem. Zool. 83, 1–18.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387–1397.

German, D.P., Sung, A., Jhaveri, P.K., Agnihotri, A., 2015. More than one way to be an herbivore: convergent evolution of herbivory using different digestive strategies in prickleback fishes (family Stichaeidae). Zoology 118, 161–170.

Goodrich, T.D., Morita, R.Y., 1977. Incidence and estimation of chitinase activity associated with marine fish and other estuarine samples. Mar. Biol. 41, 349–353.

Gutowska, M., Drazen, J., Robison, B., 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. Comp. Biochem. Physiol. A 139, 351–358.

Harpaz, S., Uni, Z., 1999. Activity of intestinal mucosal brush border membrane enzymes in relation to the feeding habits of three aquaculture fish species. Comp. Biochem. Physiol. Part A 124, 155–160.

Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267–283.

Holmgren, S., Nilsson, S., 1999. Digestive system. In: Hamlett, W.C. (Ed.), Sharks, skates, and rays: the biology of elasmobranch fishes. The Johns Hopkins University Press, Baltimore, MD, pp. 144–173.

Horn, M.H., 1989. Biology of marine herbivorous fishes. Oceanogr. Mar. Biol. Annu. Rev. 27, 167–272.

Karasov, W.H., Douglas, A.E., 2013. Comparative digestive physiology. Compr. Physiol. 3, 741–783.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological ecology: how animals process energy, nutrients, and toxins. Princeton University Press, Princeton, NJ USA.

Kohler, N.E., Casey, J.G., Turner, P.A., 1996. Length–length and length–weight relationships for 13 shark species from the western north Atlantic. NOAA Technical Memorandum NMFS-NE-110pp. 1–29.
Kuiper-Linley, M., Johnson, C.R., Lanyon, J.M., 2007. Effects of simulated green turtle regrazing on seagrass abundance, growth and nutritional status in Moreton Bay, south-east Queensland, Australia. Mar. Freshw. Res. 58, 492–503.
Kuz'mina, V.V., 1990. Characteristics of enzymes involved in membrane digestion in elasmobranch fishes. Zh. Evol. Biokhim. Fiziol. 26, 161–166.
Meyer, C.G., Holland, K.N., 2012. Autonomous measurement of ingestion and digestion processes in free-swimming sharks. J. Exp. Biol. 215, 3681–3684.
Moran, D., Turner, S., Clements, K.D., 2005. Ontogenetic development of the gastrointestinal microbiota in the marine herbivorous fish *Kyphosus sydneyanus*. Microb. Ecol. 49, 590–597.
Newton, K.C., Wraith, J., Dickson, K.A., 2015. Digestive enzyme activities are higher in the shortfin mako shark, *Isurus oxyrinchus*, than in ectothermic sharks as a result of visceral endothermy. Fish Physiol. Biochem. 41, 887–898.
Papastamatiou, Y.P., 2007. The potential influence of gastric acid secretion during fasting on digestion time in leopard sharks (*Triakis semifasciata*). Comp. Biochem. Physiol. A 147, 37–42.
Papastamatiou, Y.P., Lowe, C.G., 2004. Postprandial response of gastric pH in leopard sharks (*Triakis semifasciata*) and its use to study foraging ecology. J. Exp. Biol. 207, 225–232.
Papastamatiou, Y.P., Lowe, C.G., 2005. Variations in gastric acid secretion during periods of fasting between two species of shark. Comp. Biochem. Physiol. A 141, 201–214.
Penry, D.L., Jumars, P.A., 1987. Modeling animal guts as chemical reactors. Am. Nat. 129, 69–96.
Skea, G., Mountfort, D., Clements, K.D., 2005. Gut carbohydrases from the New Zealand marine herbivorous fishes *Kyphosus sydneyanus* (Kyphosidae), *Aplodactylus arctidens* (Aplodactylidae), and *Odax pullus* (Labridae). Comp. Biochem. Physiol. B 140, 259–269.
Skea, G., Mountfort, D., Clements, K.D., 2007. Contrasting digestive strategies in four New Zealand herbivorous fishes as reflected by carbohydrase activity profiles. Comp. Biochem. Physiol. Part B 146, 63–70.
Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., Klenk, D., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Smith, T., Wahl, D., Mackie, R., 1996. Volatile fatty acids and anaerobic fermentation in temperate piscivorous and omnivorous freshwater fish. J. Fish Biol. 48, 829–841.
Smoot, J.C., Findlay, R.H., 2000. Digestive enzyme and gut surfactant activity of detrivorous gizzard shad (*Dorosoma cepedianum*). Can. J. Fish. Aquat. Sci. 57, 1113–1119.
Stevens, C.E., Hume, I.D., 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol. Rev. 78, 393–427.
Theodosiou, N., Hall, D.A., Jowdry, A.L., 2007. Comparison of acid mucin goblet cell distribution and Hox13 expression patterns in the developing vertebrate digestive tract. J. Exp. Zool. 308B, 442–453.
Wetherbee, B.M., Gruber, S.H., 1993. Absorption efficiency of the lemon shark *Negaprion brevirostris* at varying rates of energy intake. Copeia 1993, 416–425.
Wetherbee, B.M., Gruber, S.H., Ramsey, A.L., 1987. X-radiographic observations of food passage through digestive tracts of lemon sharks. Trans. Am. Fish. Soc. 116, 763–767.
Wilson, J.M., Castro, L.F.C., 2011. Morphological diversity of the gastrointestinal tract in fishes. In: Grosell, M., Farrell, A.P., Brauner, C.J. (Eds.), The Multifunctional Gut of Fish. Elsevier, San Diego, pp. 1–55.
Wood, C.M., Kajimura, M., Bucking, C., Walsh, P.J., 2007. Osmoregulation, ionoregulation and acid–base regulation by the gastrointestinal tract after feeding in the elasmobranch (*Squalus acanthias*). J. Exp. Biol. 210, 1335–1349.