# Digestive enzyme activities in the guts of bonnethead sharks (*Shyrna 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, respectively, degrading enzymes 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 support the contention 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.

Key words: elasmobranch, trypsin, lipase, β-glucosidase, maltase, chitin, spiral intestine, pancreas

## 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 Martinez 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 Martinez 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). 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 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 el 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). 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 seagrass (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 seagrass).

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 hours) 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 vs. 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 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 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. Frozen samples were then shipped on dry ice to UC Irvine where they were stored at -80°C until analyzed (within six 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.

*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

(22ºC), 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 were combined with 10 μL of homogenate in a black microplate and incubated for 30 minutes. 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

186 (BAPNA), was dissolved in 100 mM Tris-HCl buffer (pH 7.5; sodium acetate pH 5.5 for the
187 colon tissue and contents). Trypsin activity was determined with a p-nitroaniline standard curve,
188 and expressed in U (μmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.
189 Aminopeptidase activity was measured using 2.04 mM L-alanine-p-nitroanilide HCl
190 dissolved in 200 mM sodium phosphate buffer (pH 7.5; sodium acetate pH 5.5 for the colon
191 tissue and contents). Aminopeptidase activity was determined with a p-nitroaniline standard
192 curve, and activity was expressed in U (μmol p-nitroaniline liberated per minute) per gram wet
193 weight of gut tissue.
194 Lipase (nonspecific bile-salt activated) activity was assayed using 0.55 mM p-nitrophenyl
195 myristate (in ethanol) in the presence of 5.2 mM sodium cholate dissolved in 25 mM Tris-HCl
196 (pH 7.5; sodium acetate pH 5.5 for the colon tissue and contents). Lipase activity was
197 determined with a p-nitrophenol standard curve, and expressed in U (μmol p-nitrophenol
198 liberated per minute) per gram wet weight of gut tissue.
199 The activity of each enzyme was regressed against the protein content of the
200 homogenates to confirm that there were no significant correlations between the two variables.
201 Because no significant correlations were observed, the data are not reported as U per mg protein.
202
203 *2.3 Statistical analyses*
204 Prior to all significance tests, a Levene's test for equal variance was performed and
205 residual versus fits plots were examined to ensure the appropriateness of the data for parametric
206 analyses. Where necessary, data were log-transformed prior to analysis. All tests were run using
207 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 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. 3). 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. 3). 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. 4). N-acetyl-β-D-glucosaminidase activities were more elevated in the SI tissues than the 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. 4), 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. 5). 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.

## 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. 3), and some brushborder enzyme activities (e.g., aminopeptidase) peaking in the mid spiral intestine (Fig. 4). This latter result suggests that the spiral intestine is likely the most active, absorptive section of the shark intestine, consistent with increased epithelial surface area in this gut region (Holmgren and Nilsson, 1999; Chatchavalvanich et al., 2006; Wilson and Castro, 2011). Measurements of nutrient transport rates along the elasmobranch epithelium are lacking, but we hypothesize that these rates would be highest in the spiral 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). 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).

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 site of synthesis for aminopeptidase (Karasov and Martinez del Rio, 2007). However, the bonnethead trypsin and aminopeptidase activity levels are about 10X 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). 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, but the broad distribution of lipolytic activity along the gut (Fig. 3) suggests that bonnetheads likely readily digest lipids with great efficiency. To our knowledge, there aren't 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

277 lemon sharks (*Negaprion brevirostris*) consuming different sized meals of a fish diet. Given that
278 fish would be primarily protein and lipid (Horn, 1989) it follows that the high organic matter
279 digestibility by lemon sharks would primarily be of protein and lipid. We attempted to measure
280 amylolytic activity in the bonnetheads, but we were not able to reliably detect enzymatic activity
281 against starch, suggesting that bonnetheads may be poor at digesting soluble carbohydrates, like
282 starch. This is corroborated by the relatively low maltase activities in the bonnethead intestine
283 (Fig. 4), although using membrane vesicle preparations may improve detection of maltase (Crane
284 et al., 1979).

285 What is intriguing is the presence of elevated N-acetyl-β-D-glucosaminidase activity in
286 the colons of the bonnetheads. Bonnetheads clearly consume chitin with their diet rich in
287 crustaceans (Bethea et al., 2007), and like other fishes that consume chitin (Goodrich and Morita,
288 1977; Gutowska et al. 2004; German et al., 2010; German et al., 2015), this may be an important
289 source of carbon and nitrogen for these sharks. Indeed, N-acetyl-β-D-glucosaminidase activities
290 in the bonnethead intestine are also at least 5X higher than carnivorous, omnivorous, and
291 detritivorous teleost fishes measured using the same methods and equations for calculations as
292 this study (German and Bittong, 2009; German et al., 2015). The source of the N-acetyl-β-D-
293 glucosaminidase is likely endogenous along the gut walls of the anterior and spiral intestine, but
294 the spike in N-acetyl-β-D-glucosaminidase activity in the colon (including the colon contents)
295 strongly suggests a microbial origin of these activities in the hindgut (German and Bittong, 2009;
296 German et al., 2015).

297 Interestingly, the colons of sharks and skates are known to be acidic (pH 5.5-6.4,
298 depending on species), which is more acidic than their intestines (which tend to be pH 7.0-7.5;
299 Anderson et al., 2010), and is more similar to the pH of a typical vertebrate colon, which is a site

300 of microbial fermentation (Karasov and Martinez del Rio, 2007; Karasov and Douglas, 2013).
301 The colonic environment is anaerobic, which allows enteric microbes to use fermentative
302 pathways to produce short chain fatty acids (SCFA); these SCFA (e.g., acetate, propionate) are
303 the reason for the lower pH of the colon in most vertebrates and the SCFA can be absorbed by
304 the host and used for ATP production (Bergman, 1990; Stevens and Hume, 1998; Karasov and
305 Martinez del Rio, 2007; Karasov and Douglas, 2013). Although we didn't measure SCFA
306 production in the bonnetheads, SCFA production is well known in the hindguts of fishes, and is
307 usually higher in herbivores than in carnivores (Clements et al. 1995; Clements et al. 2014;
308 German et al. 2015), although some carnivores (e.g., largemouth bass, *Micropterus salmoides*)
309 do show seasonally high levels of SCFA production in their hindguts (Smith et al., 1996). The
310 omnivorous *Phytichthys chirus*, which also consumes a crustacean-rich diet, has elevated levels
311 of N-acetyl-β-D-glucosaminidase activities in its hindgut (German et al., 2015), further
312 suggesting that hindgut microbial digestion of chitin may be wide-spread in carnivorous fishes
313 with chitin-rich diets, as we see in the bonnetheads. The slower transit time of food through a
314 carnivorous gut is amenable to a yield-maximizing strategy, and the activities of microbially-
315 produced enzymes being elevated in the hindgut also support that carnivores can be yield-
316 maximizers with some reliance on microbial symbionts in the digestive process.
317 Along these lines, we were surprised to observe a spike in β-glucosidase activity in the
318 bonnethead colon, as this enzyme digests the breakdown products of cellulose and other β-
319 glucosides, like laminarin (German and Bittong, 2009). Up to 62% of young-of-the-year
320 bonnethead diet (by mass) can be composed of seagrass, which appears degraded by the time it
321 reaches the hindguts of the sharks (Bethea et al., 2007). Certainly, if bonnetheads have a
322 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 2X 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 seagrass by bonnetheads are necessary to confirm this supposition, but it does appear possible that bonnetheads have the enzymatic machinery to degrade components of seagrass. Indeed, the main carbohydrate in seagrass 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 *vs* fish diets would be enough to result in niche segregation from the perspective of stable isotope analysis, it is possible that the digestion of seagrass, 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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**Figure 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).

**Figure 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.

**Figure 3.** 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. 1 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.

**Figure 4.** 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. 1 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.

**Figure 5.** 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. 1 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.