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Seagrass digestion by a notorious 'carnivore'

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What an animal consumes and what an animal digests and assimilates for energetic demands are not always synonymous. Sharks, uniformly accepted as carnivores, have guts that are presumed to be well suited for a high-protein diet. However, the bonnethead shark (*Sphyrna tiburo*), which is abundant in critical seagrass habitats, has been previously shown to consume copious amounts of seagrass (up to 62.1% of gut content mass), although it is unknown if they can digest and assimilate seagrass nutrients. To determine if bonnetheads digest seagrass nutrients, captive sharks were fed a ^{13}C-labelled seagrass diet. Digestibility analyses, digestive enzyme assays and stable isotope analyses were used to determine the bonnethead shark's capacity for digesting and assimilating seagrass material. Compound-specific stable isotope analysis showed that sharks assimilated seagrass carbon (13.6 ± 6.77‰ δ^{13}C mean $\pm$ s.d. for all sharks and all amino acid types analysed) with 50 ± 2% digestibility of seagrass organic matter. Additionally, cellulose-component-degrading enzyme activities were detected in shark hindguts. We show that a coastal shark is digesting seagrass with at least moderate efficiency, which has ecological implications due to the stabilizing role of omnivory and nutrient transport within fragile seagrass ecosystems.

1. Background

Understanding what an animal actually digests and assimilates as opposed to what it simply eats allows an understanding of the role of that organism in terms of foraging, nutrient excretion and habitat use [1–6]. Overall, the nutritional ecology of fishes (including sharks) is insufficiently studied outside of a few species used in aquaculture [1,2,7]. Carnivores, such as sharks, appear specialized for digesting high-protein diets, as indicated by elevated digestibility of protein [8,9] and high activity levels of protein-degrading digestive enzymes in their guts [2,10–12]. Omnivores, on the other hand, also digest plant material, and thus face the difficulty of digesting foods (like seagrass) that are low in protein, and are sheathed in fibrous cell walls. As such, omnivores generally have different digestive biochemistry (e.g. greater carbohydrase activities) [13], as well as varying diversities and abundances of enteric microbial communities in comparison to carnivores [14–16]. In an ecological context, the effect of omnivores on ecosystem stability has been debated, but in marine systems, omnivorous predators that feed across trophic levels with strong interactions have been shown to buffer food webs against trophic cascades [17–20].

With population estimates of approximately 4.9 million [21] individuals along the Atlantic and Gulf of Mexico coasts of the USA, the bonnethead shark (*Sphyrna tiburo*) is one of the most abundant and conspicuous members of seagrass meadows and many other soft bottom habitats in US coastal waters and beyond. Although they are frequently listed as carnivorous, consuming mostly crustaceans and mollusks [22], they are also known for consuming copious amounts (up to 62% of gut content mass) of seagrass in

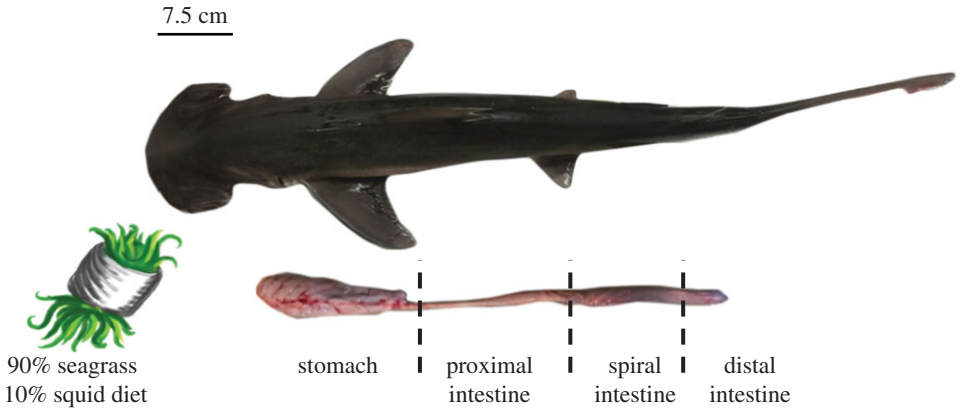

Figure 1. Adult bonnethead shark, *S. tiburo*, with its digestive tract (adapted from [2,10]). Ninety per cent seagrass and 10% squid diet illustration by LLM Pandori. (Online version in colour.)

some populations [23], and for feeding at lower trophic levels than other closely related species [24]. However, what an animal ingests and what they digest and assimilate are not the same thing [7], and hence the scientific community has largely dismissed seagrass ingestion by this shark as incidental intake that does not contribute to the shark's nutritional ecology (with the exception of Bethea *et al*. [23,24]). Sharks are uniformly accepted as carnivorous [1,2,22], so this assumption is not unwarranted. However, the sheer abundance of bonnethead sharks ingesting seagrass in these environments, coupled with the observation that seagrass in the bonnethead distal intestine (DI) appears 'degraded' in comparison to fresh seagrass [23], raises the possibility that these sharks are actually assimilating nutrients from seagrass. If this were the case, it would mean we would need to re-evaluate the roles of bonnethead sharks in seagrass ecology because they could be responsible for significant grazing and nutrient transport within fragile seagrass ecosystems. Seagrass meadows are the most widespread coastal ecosystem on earth [25] and provide a multitude of ecological and economic services [26]. Some of these services include cross-ecosystem nutrient transfer [27], erosion control [28], pollution and pathogen management [25,29,30], providing habitat and protected nursery areas for thousands of fish and invertebrate species, thereby supporting the fishing industry [27], acting as a CO_2 sink [26] and producing large quantities of oxygen [26]. As such, it is imperative that studies of trophic interactions in seagrass habitats correctly identify the diets and digestive strategies of key, abundant taxa.

To determine if bonnethead sharks are capable of digesting and assimilating seagrass nutrients, we fed captive sharks a 90% ^{13}C-labelled seagrass and 10% squid diet (figure 1; totalling 5% of their body weight per day) over a three-week period. Using a combination of captive feeding trials, stable isotope analyses, digestibility analyses and enzymatic biochemistry, we show that bonnetheads are omnivorous and can assimilate plant organic material. Furthermore, they demonstrate positive somatic growth on a plant-based diet, and possess the enzymatic biochemistry needed to digest even some of the fibrous portions of seagrass.

2. Methods

All methods mentioned here are described in detail in the electronic supplementary material.

(a) Seagrass collections and shark capture

Seagrass was collected in Florida Bay and transported in coolers filled with seawater and an aquarium bubbler to the Florida International University (FIU) Biscayne Bay campus outdoor mesocosm facility. Seagrass was re-planted in terracotta pots within a closed, re-circulating, tank system (approx. 454 l) and placed in direct sunlight. We labelled the seagrass by directly adding powdered ^{13}C-labelled sodium bicarbonate (1 g; 99 at. %, Sigma Aldrich Product no. 372382) into the seawater in the tank. A chiller (Aqua Euro USA, model MC-1/2 hp) was used to keep the water in the tank at 30°C. The water in the tank underwent a water change once per week and new ^{13}C-labelled sodium bicarbonate (1 g) was added each time.

Bonnethead sharks were caught off the coast of Layton, FL, on Long Key (24°50′2.6″ N 80°48′32.3″ W) and off the southwestern coast of Key Biscayne (25°41′05.9″ N 80°10′41.0″ W). There were four incidental mortalities and those individuals were immediately dissected for intestinal, liver and muscle tissue samples and henceforth are referred to as the 'wild-caught' sharks. Five additional sharks were transported alive to FIU to undergo feeding trials (henceforth the 'laboratory-fed' sharks).

(b) Feeding events and faecal collections

Once at FIU, bonnethead sharks ($n = 5$) were kept in a 40 337 l circular flow-through tank receiving water pumped directly from Biscayne Bay and acclimated for at least 24 h. After 24 h, the sharks were individually anaesthetized via submersion in a 113 l bin with a 0.2% MS-222 solution buffered with NaOH via recirculating aquarium powerhead. Sharks were quickly weighed, their dorsal fins marked with a unique, non-toxic, water-resistant paint color (ECOS Paints), and then 200 µl of blood (composing less than 1% of the blood volume of each shark) was drawn with a 25 G needle from the haemal arch, just posterior of the anal fin. Blood was centrifuged to separate the plasma and RBC phases, dried at 60°C, and stored in a dry location for later use in stable isotopic measurements. Blood was drawn in this manner once every week for three weeks. Once the blood was drawn, the shark was placed back into the flow-through 40 337 l tank for recovery. Sharks were monitored until normal ventilation resumed.

Each shark was fed a 90% seagrass, 10% squid (*Doryteuthis opalescens*) diet equalling 5% of their initial body weight daily for three weeks. Faecal material was collected daily via siphoning through a 250 µm mesh. Water passed through the mesh while faecal material was collected on top. Faecal material was transferred into 50 ml conical tubes and dried at 60°C for later use in digestibility analyses in order to determine digestive efficiency. Approximately 5 g (dry mass) of faecal material was collected per shark over the course of the three weeks.

(c) Dissections and tissue preparation

At the conclusion of the three-week feeding trial, all laboratory-fed individuals were euthanized in 1% MS-222 solution, measured (standard length (SL), weighed (body mass (BM)) and dissected on a chilled (approx. 4°C) cutting board. Whole gastrointestinal tracts were removed by cutting at the oesophagus and at the cloacal opening. Whole intestines (without the stomach) were weighed and the intestine length (IL) was measured. The intestine was then divided into three sections: proximal intestine (PI), spiral intestine (SI) and DI [31,32]. Each of these sections was then further subdivided into three sections (i.e. PI1, PI2, PI3, etc.) in order to increase the resolution of understanding enzyme activity levels along the digestive tract.

(d) Digestibility analyses

The protein, soluble carbohydrate, lipid and total organic matter contents were determined for the 90% seagrass/10% squid diet, as well as for the faecal material from all of the lab-fed sharks. The following equation was used to determine the percentage digestibility of each macronutrient type by the shark:

$$\%\text{digestibility} = \left(\frac{(\text{ash-adjusted ingested}) - (\text{defecated})}{\text{ash-adjusted ingested}}\right) \times 100.$$

Fibre digestibility was determined using an ANKOM 200/220 Fiber Analyzer, following the ANKOM suggested procedures [33,34] for neutral detergent fibre (NDF; which includes cellulose and hemicellulose) and acid detergent fibre (ADF; which excludes cellulose).

To determine if the laboratory-fed sharks were meeting their daily metabolic demands on the prescribed diet, bonnethead shark metabolic rate was estimated using the equation from Parsons [35]:

$$M = \left(\frac{(68.9 + 177.8W) \cdot 3.25}{W}\right) \times 24,$$

where M is the metabolic rate (kcal kg^{-1} $d^{-1)}$ and W the weight in kilograms. The initial wet weight of the sharks was used here. Coefficients were based on the constants for fish [36]. The amount (g) of the diet consumed by each shark was recorded daily.

(e) Digestive enzyme assays

Intestinal homogenates were produced as described by Leigh *et al*. [32]. In order to determine the activity of enzymes that digest soluble carbohydrate, protein, lipid and fibrous components of seagrass, we assayed α-amylase, maltase, trypsin, aminopeptidase, lipase and β-glucosidase activity for all intestinal regions. All enzyme 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, USA). All assay protocols generally followed methods detailed in Leigh *et al*. [32], unless otherwise noted.

(f) Stable isotope analysis

To measure $\delta^{13}C$ signatures, samples (red blood cells, plasma, liver tissue and seagrass) were thoroughly dried at 60°C. Samples were then individually dipped into liquid nitrogen and ground to a powder using a mortar and pestle. Ground samples (approx. 700 µg for shark blood and tissues samples and approx. 2 mg for seagrass tissues) were then transferred into individual 5 mm × 9 mm tin capsules (Costech Analytical Technologies). Samples were sent to the University of Florida Stable Isotope Facility for processing using a Thermo Delta V Plus isotope ratio mass spectrometer. Lipid was extracted from laboratory-fed shark liver samples and seagrass samples using a soxhlet [37] prior to compound-specific stable isotope analyses (CSSIA). The amino acids measured via CSSIA were aspartate, alanine, glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine and valine because these are commonly measured in studies of nutritional physiology of marine fishes [38,39].

Table 1. Mean (± s.d.) digestibility (%) of protein, lipid, soluble carbohydrates, NDF, ADF and total organic matter of a 90% seagrass, 10% squid diet by the bonnethead shark.

constituent	digestibility (%)
protein	92 ± 3
lipid	51 ± 7
soluble carbohydrate	80 ± 3
neutral detergent fibre	52 ± 3
acid detergent fibre	43 ± 4
total organic matter	50 ± 2

(g) Statistical analysis

Comparisons of enzymatic activities were made among gut regions with analysis of variance followed by a Tukey's honest significant difference with a family error rate of $p = 0.05$. Comparisons of enzymatic activities between laboratory-fed sharks and wild-caught sharks were made using unpaired *t*-tests with a Bonferroni-corrected error rate of $p = 0.006$. Comparisons between laboratory-fed shark liver amino acid $\delta^{13}C$ values and seagrass amino acid $\delta^{13}C$ values were made using unpaired *t*-tests with a Bonferroni-corrected error rate of $p = 0.004$. All statistical tests were performed in R studio (v. 1.0.136).

3. Results and conclusion

We provide conclusive evidence that bonnethead sharks, animals previously thought to be solely carnivorous, can assimilate nutrients from seagrass. This is the first species of shark ever to be shown to have an omnivorous digestive strategy. Laboratory-fed sharks all gained weight on their seagrass-heavy diet (mean: 6.65 ± 3.46% weight gain from initial BM; electronic supplementary material, table S1) and digested the total organic matter (50 ± 2%) and the fibre in seagrass (52 ± 3% for NDF and 43 ± 4% for ADF; table 1) with moderate efficiency. They also more than met their energetic demands on their prescribed laboratory diet (average caloric need: 28 kcal d^{-1} [35], average calories digested in the laboratory feeding trial: 203 kcal d^{-1}; electronic supplementary material, table S1). Remarkably, the bonnethead's digestibility of organic matter is comparable to juvenile green sea turtles (*Chelonia mydas*; mean seagrass organic matter digestibility of 44.7%) [18]. As green sea turtles mature, they become almost entirely herbivorous, and their digestibility of seagrass increases (mean seagrass organic matter digestibility of 64.6%) [18] in parallel with a longer digestive tract and a more diverse microbiome [40]. Therefore, bonnetheads are capable of digesting components of seagrass, with similar effectiveness to omnivores, making them the only shark species known to have the ability to digest plant material [2,10]. For comparison, the carnivorous lizard *Crotaphytus collaris* digested flowers with only 32% efficiency, whereas the herbivorous *Sauromalus obesus* digested these same flowers with 67% efficiency [41], showing

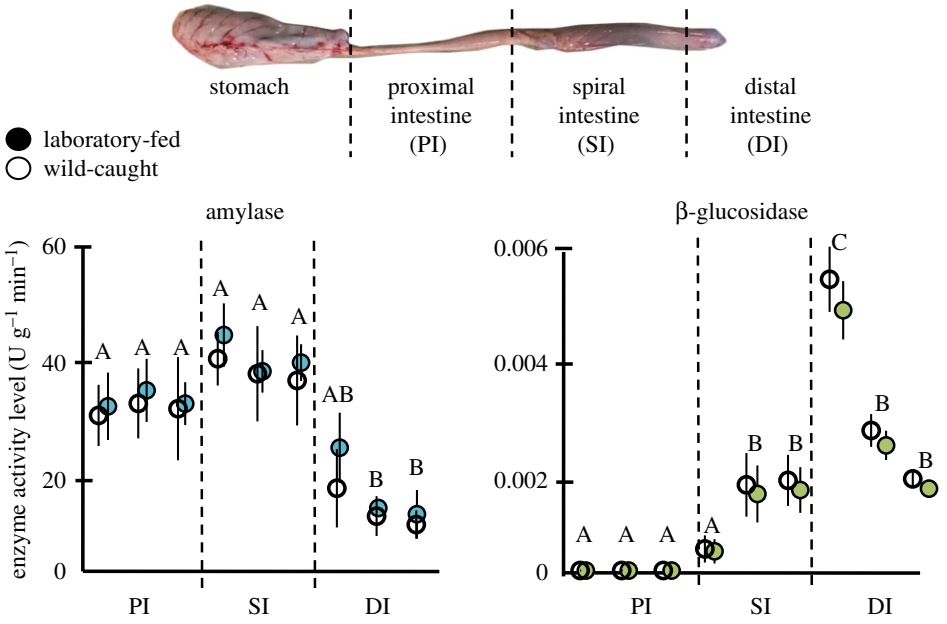

Figure 2. Amylase and β-glucosidase activities in the digestive tracts of bonnethead sharks. Open circles represent wild-caught sharks, while filled circles represent mean ± standard deviation values for laboratory-fed sharks. No significant differences were found between laboratory-fed and wild-caught sharks for any of the enzymes assayed ($p > 0.05$). Differing letters above data points indicate significant difference among gut regions: PI, SI and DI ($p < 0.05$). (Online version in colour.)

that not all carnivores can digest plant material efficiently. Indeed, pandas, which are herbivores with a 'carnivorous' gut [42], have enteric microbiomes that differ from other herbivores [43] and also show about 20% organic matter digestibility of bamboo [42]. Pandas make a living on high intake and digest mostly the soluble portions of bamboo [44]. Thus, bonnethead sharks are considerably better at digesting seagrass than either of these terrestrial examples [41,44].

Enzymatic assays revealed that protein-degrading enzyme (aminopeptidase and trypsin) and lipid-degrading enzyme (lipase) activities peaked in the proximal or SI for both lab-fed and wild-caught sharks, which is congruent with previous work on wild-caught bonnetheads, and other fishes (electronic supplementary material, figure S1) [2,10,13,32,45,46]. The SI is likely the primary site of amino acid and fatty acid absorption in bonnetheads and other shark species [47]. While carbohydrate-degrading enzyme (amylase and maltase) activities were similar between laboratory-fed and wild-caught sharks, maltase activity was relatively low and constant throughout the digestive tract in both groups (figure 2; electronic supplementary material, figure S1) [10]. However, the amylase levels observed in bonnethead sharks are high for a carnivorous fish and comparable with omnivorous fish such as *Xiphister atropurpureus* [13]. Coupled with the bonnethead's high digestibility of soluble carbohydrates (82 ± 5%; table 1), this indicates efficient digestion of the soluble carbohydrates (like starch) [48] found in seagrass material.

The presence of elevated β-glucosidase activity in the hindgut of both the laboratory-fed and wild-caught bonnethead sharks indicate the capacity for the digestion of cellulose breakdown products (e.g. cellobiose), likely with aid from microbial symbionts, as previously suggested for bonnetheads (figure 2) [10]. The fact that β-glucosidase activity was significantly higher in the hindgut compared to other gut regions (PI and SI; figures 1 and 2) indicates likely involvement from the gut microbiome in the digestion of seagrass fibre. Surprisingly, the activity levels of β-glucosidase in the bonnethead hindgut are on a par with activities observed in the hindguts of *Cebidichthys violaceus*, a herbivorous, teleost fish that digests algal material with assistance from their gut microbiome [13]. Evidence of elevated β-glucosidase activities in the hindgut of bonnetheads differentiates them from carnivores and merits further investigation into the role of the microbiome in the digestion of seagrass material. Sharks also have highly acidic stomachs (pH 1–2) [11,49], whereas most herbivorous teleost species have slightly higher average stomach pH values of 2–3 [50,51]. As sharks lack the pharyngeal (secondary) jaws that many herbivorous species use for mastication or trituration of plant material, the highly acidic shark stomach could weaken the cell walls and plasma membranes of seagrass, so that digestive enzymes can enter the cells and digest seagrass cell contents [51]. Bonnethead sharks also have molariform teeth that are presumed to be for crushing hard prey [52], but these teeth may also be capable of seagrass mastication, which could aid in the digestive process.

While digestibility and enzymatic analyses highlight that bonnethead sharks have the capacity to breakdown seagrass, the stable isotope analyses show that they can assimilate plant molecules [53]. We measured a clear increase in the $\delta^{13}C$ signature in the blood and liver tissues of the laboratory-fed sharks over the course of the feeding trial (figures 3 and 4). The ^{13}C-labelled seagrass used in the feeding trials had a mean $\delta^{13}C$ of 104.9‰ (mean atom % of 1.25 ± 0.05) compared with a mean $\delta^{13}C$ of −13.4‰ (mean atom % of 1.08 ± 0.02) for wild, non-labelled seagrass (figure 3). The mean $\delta^{13}C$ signature of the blood plasma from the laboratory-fed sharks increased from −12.1‰ at the beginning of the feeding trial to 2743.9‰ at the end of the feeding trial (figure 3). The red blood cells also exhibited an increase from a mean of −11.5‰ to 19‰ $\delta^{13}C$ over the course of the feeding trial. The liver tissues of wild-caught sharks had a mean $\delta^{13}C$ value of −12.23‰ (mean atom % of 1.09 ± 0.02), while the laboratory-fed sharks had liver tissues with a mean $\delta^{13}C$ value of 357.2‰ (mean atom % of 1.49 ± 0.09) at the conclusion of the three-week feeding trial (figure 4).

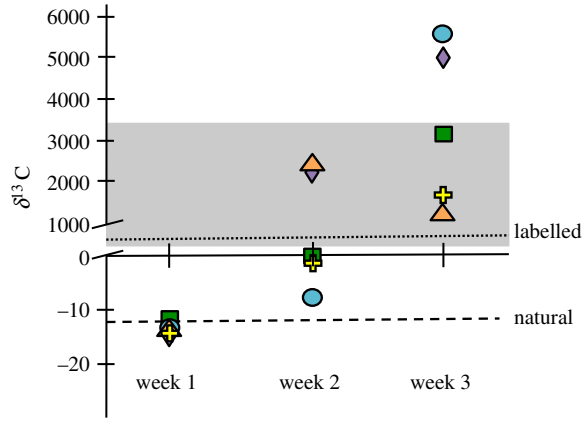

Figure 3. $\delta^{13}C$ values for laboratory-fed bonnethead shark blood plasma for each of the three weeks of the feeding trial. Differently shaped (and coloured) data points represent different individual laboratory-fed sharks. The mean values for ^{13}C-labelled seagrass and natural seagrass are shown as differently patterned horizontal lines. The total $\delta^{13}C$ range for the ^{13}C-labelled seagrass is denoted by a light grey box. (Online version in colour.)

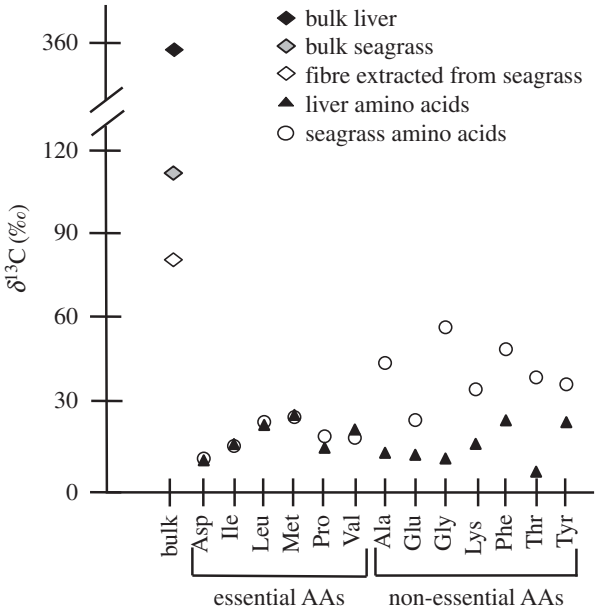

Figure 4. Bulk bonnethead shark liver tissue, bulk seagrass tissue, fibre extracted from labelled seagrass and individual amino acid (AA) $\delta^{13}C$ values (means). Asp, aspartic acid; Ile, isoleucine; Leu, leucine; Met, methionine; Pro, proline; Val, valine; Ala, alanine; Glu, glutamic acid; Gly, glycine; Lys, lysine; Phe, phenylalanine; Thr, threonine; Tyr, tyrosine.

The combination of these data shows that bonnethead sharks are not only consuming copious amount of seagrass (8.8–62.1% of gut content mass) [23], but they are actually capable of digesting and assimilating seagrass nutrients, making them clear omnivores. As the bonnethead shark digestive tract is morphologically similar to other closely related strict carnivores, it shows that a 'carnivorous' gut can digest at least parts of ingested plant material. These results in the bonnethead shark are also consistent with observations that many herbivorous fishes lack what would be called a 'specialized' gut morphology for housing enteric symbionts that aid in the digestion of plants [7,20], unlike the myriad specializations seen in mammals [54].

We do recognize that the $\delta^{13}C$ values for both blood plasma and liver tissues are exceptionally high compared to the bulk $\delta^{13}C$ values for the seagrass used in the feeding trial. The most likely explanation for this elevated signal has to do with urea, which in sharks, is synthesized via the ornithine urea cycle in the liver, making urea a sink for bicarbonate carbon [55–60]. Sharks are unique from most teleost fishes in that their total blood osmolarity (1118 mOsm l^{-1} for dogfish sharks) [58] is similar to that of seawater (1050 mOsm l^{-1}) [59] and that nearly half (441 mM l^{-1}) of this is accounted for by urea [58]. As urea synthesis occurs in the liver and uses CO_2 [55,61], if ^{13}C-labelled bicarbonate in the seagrass was absorbed in the digestive tract and then equilibrated with the blood bicarbonate, this would explain the exceptionally high $\delta^{13}C$ values in the blood plasma and liver tissues [57,60,62–64]. This also explains the discrepancy between the high $\delta^{13}C$ values in the plasma versus the red blood cells, where the red blood cells have a much slower isotopic turnover rate (more than four months versus approximately one to two months for plasma proteins) [60,62], and the red blood cells do not contain bicarbonate or urea. The red blood cell isotopic signature, therefore, represents labelled proteins, which are similar to the labeled amino acids in the liver (figure 4).

Furthermore, the CSSIA shows that amino acids in the laboratory-fed sharks livers were also labelled, making it unlikely that ^{13}C-labelled sodium bicarbonate in the sharks livers caused this result (figure 4; electronic supplementary material, table S2). Moreover, the CSSIA analysis allowed us to identify those amino acids that shared the same $\delta^{13}C$ signature among the sharks livers and the seagrass as some of the essential amino acids for bonnetheads: aspartate, isoleucine, leucine, methionine, valine and proline (figure 4; electronic supplementary material, table S3) [38,39]. The other amino acids (alanine, glutamate, glycine, lysine, phenylalanine, threonine, tyrosine) were more enriched in ^{13}C in the grass than in the sharks (electronic supplementary material, table S3), but this could reflect the fact that a three-week feeding trial was not sufficient to allow complete turnover of all amino acids in the liver protein [53]. Previous analyses of wild seagrass amino acids using CSSIA showed that all of the amino acid $\delta^{13}C$ values were negative, similar to the bulk signatures ($\delta^{13}C$ values −11.1 to −15.9‰) of the wild seagrass [53]. Hence, each of our analyses (including bulk seagrass, seagrass fibre and CSSIA) show that all of the components of the seagrass in the current study were indeed labelled with ^{13}C (positive $\delta^{13}C$ values), and therefore the assimilation of the labelled carbon into the bonnethead sharks must have come from the labelled seagrass and cannot represent some components of wild seagrass (or any marine resource) still persisting in the sharks' tissues. The CSSIA and enriched seagrass fibre isotopic signatures also argue against the assimilated labelled carbon only coming from the labelled bicarbonate, and in fact, some of the bulk liver isotopic signature could be contributed by liver glycogen synthesized from ^{13}C-labelled glucose assimilated from seagrass tissues, including the fibre, which was heavily labeled (figure 4). Finally, the red blood cells $\delta^{13}C$ values were similar to those found in the liver amino acids, showing that the actual proteins are enriched at the level of amino acids in the red blood cells.

The sheer abundance of bonnethead sharks in coastal communities (approx. 4.9 million individuals in the Atlantic and Gulf of Mexico coastal waters of the USA) [21] coupled with consumption and digestion of seagrass by these animals suggests that we need to re-evaluate the role that bonnetheads play in seagrass meadows, critical ecosystems that provide habitat for thousands of fish species, filter the surrounding

water, act as a sink for atmospheric CO_2 and produce large quantities of oxygen [25,26]. Understanding how the consumption and digestion habits of bonnethead sharks impacts seagrass ecosystems is important as these omnivores may stabilize food web dynamics and even play a role in nutrient redistribution and transport. Bonnethead sharks often display short-term residency to core areas within seagrass meadows, but shift the location of these areas within a large home range, suggesting that individuals may be able to transport nutrients between and within habitat patches [65]. Considering bonnethead sharks as omnivores, rather than carnivores, in models of seagrass meadow function, and then testing the predictions of those models for management purposes, changes our understanding of the fluxes of nutrients and energy among trophic levels within each part of these ecosystems. To better understand the ecological influence of sharks and other marine predators, or any mobile consumers for that matter, and how they may act as nutrient vectors, we need to move beyond observations of just consumption or bite rates and strive to understand, not only what consumers are eating, but also what they are digesting and excreting back into their environments (i.e. their nutritional physiology). This is critical to effectively formulating conservation efforts including trophic models [5,66].

Ethics. Seagrass was collected with a special activity license issued to James Fourqurean (SAL-15-1754-SR). Sharks were collected with a special activity license issued to Y.P.P. (SAL-16-1825A-SRP). All experiments were approved by FIU IACUC (15-026-CR01).

Data accessibility. All data are presented within the manuscript, figures or electronic supplemental material associated with this manuscript. Raw data can be accessed at http://german.bio.uci.edu/images/pdf/PRSB_Leigh_data.pdf.

Authors' contributions. Conceptualization, S.C.L., Y.P.P. and D.P.G.; methodology, S.C.L., Y.P.P. and D.P.G.; investigation, S.C.L.; writing original draft, S.C.L.; writing, reviewing and editing, S.C.L., Y.P.P. and D.P.G.; funding acquisition, S.C.L., Y.P.P. and D.P.G.

Competing interests. The authors declare no competing interests.

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