UNIVERSITY OF CALIFORNIA,
IRVINE

The Resource Acquisition Strategies and Digestive Physiology of Sharks

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

Samantha Christine Leigh

Dissertation Committee:
Professor Donovan P. German, Chair
Professor Timothy Bradley
Professor Matthew Bracken

2019

DEDICATION

To my daughter Reighlyn, for giving me the most precious title in the world: Mom, and for providing the inspiration behind everything that I do.

To my husband, for enduring months of being apart for field work, for constantly encouraging and believing in me, and for never telling me "good luck," but instead, "be confident."

To my parents, for supporting my dreams since day one, and for instilling in me the confidence that I can accomplish anything that I put my mind to.

To my sisters, for inspiring me by following your own dreams and for being my best friends, always and forever.

To all of my teachers over the years for pushing me to do my best and challenging me to reach my full potential.

To all of the women who came before me and paved the way for me to pursue science. And to all of the women who will come after me: you can do anything.

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ACKNOWLEDGMENTS

Foremost, I thank my advisor, Dr. Donovan P. German for his support in every aspect of my journey over the last five years in pursuit of my PhD. Donovan's enthusiasm for science is an inspiration and it has helped to shape me into the researcher that I am today. Not only has he taught me so much about being a great scientist, but he has modeled what it means to be an incredible teacher, mentor, and leader. I will be forever grateful that he took a chance on pursuing a project about a "seagrass-eating shark" that most people thought would not pan out. He believed in the idea and he believed in me to carry it out. Thank you, Donovan, for encouraging me to follow my passions, for writing countless letters of recommendation, for reading more manuscript and grant proposal drafts than I can count, for sitting through practice presentations, and for pushing me to reach my full potential.

Thank you to my lab mates who have been instrumental in my growth as a researcher. I am especially thankful to all the undergraduates who helped with data collection and analysis: Quang Nguyen-Phuc, Cam Vandenakker, Chloe Richards, Nicole Dwyer, Caitlyn Catabay, Siyan Chen, Emily Urena, Eleazar Paniagua, Maria Sabando, Sarah Sisco.

I am indebted to my collaborator Dr. Yannis P. Papastamatiou. He opened up his lab to me and provided so much guidance, support, and hands-on help in making this project a reality. He taught me an immense amount about handling sharks in the field and read countless drafts of manuscripts and grant proposals. Thank you, Yannis, for acting as my "advisor-away-from-home."

Thank you to all of those who helped in the field: Steven Kajiura, Bill Chamberlain, Joel Trexler, Sarah Hoffmann, Beth Bowers, Megan Kelley, Mo Bergmann, Claire Burgett, Jim Fourqueran, Kara Carpenter, and especially Katie Sobczak, not only for helping in the field, but for being one of my best friends since kindergarten.

Many thanks to my committee members Dr. Timothy Bradley, Dr. Matthew Bracken, and to my advancement committee members Dr. Katerine Whiteson and Dr. James Hicks. All of you have provided suggestions and support throughout the duration of my project and I am forever grateful for the advice that made this dissertation stronger.

Sharks were collected under SAL-16-1825A-SRP issued to Yannis Papastamatiou. All experiments were approved by Florida International University (FIU) IACUC (15-026-CR01). This study was funded by a National Science Foundation Graduate Research Fellowship, a National Geographic Society Young Explorers Grant, the UCI OCEANS Graduate Research Fellowship, the UCI Newkirk Center Graduate Research Fellowship, a UCI Public Impact Distinguished Fellowship, two Sigma XI GIARs, a Society for Integrative and Comparative Biology GIAR, the Raney Award from the American Society of Ichthyologists and Herpetologists, a GAANN award from the US Department of Education, the UCI Graduate Division, and the UCI Microbiome Initiative Pilot Project Award.

Finally, thank you to my friends and family. I am incredibly fortunate that there are far too many of you to list, but every single person in my life has contributed to the person that I am today. I love you all.

Samantha Christine (Wright) Leigh
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EDUCATION

2019 ***PhD in Ecology and Evolutionary Biology*, University of California, Irvine**
Advisor: Dr. Donovan German
NSF GRFP Fellow

2017 ***MS in Ecology and Evolutionary Biology*, University of California, Irvine**
Advisor: Dr. Donovan German

2013 ***BS in Marine and Environmental Science*, Coastal Carolina University**
Wall Fellows Leadership Development Program Fellow

SCIENTIFIC PUBLICATIONS

4) Hoffman SL, Donatelli CD, **Leigh SC**, Brainerd EL, and Porter ME (2018) Three-dimensional movements of the pectoral fin during yaw turns in the Pacific spiny dogfish, *Squalus suckleyi*. *Biology Open*. DOI:10.1242/bio.037291.
3) **Leigh SC,** Papastamatiou YP, and German DP (2018) Seagrass digestion by a notorious "carnivore." *Proceedings of the Royal Society B*. DOI: 10.1098/rspb.2018.1583.
2) **Leigh SC,** Nguyen Q, and German DP (2018) The effects of protein and fiber content on gut structure and function in zebrafish (*Danio rerio*). *Journal of Comparative Physiology B.* 188(2): 237-253.
1) **Leigh SC**, Papastamatiou YP, and German DP (2017) The nutritional physiology of sharks. *Reviews in Fish Biology and Fisheries,* 27(3): 561-585.

TEACHING PUBLICATIONS

2) **Leigh SC** (2019) Plants and animals have requirements for survival. *STEM Taught Journal*. In Press.
1) **Wright SC** (2014) Tutor training techniques and topics. *Southern Discourse.* 18(1). Publication of the Southeastern Writing Center Association.

MANUSCRIPTS UNDER REVIEW OR IN ADVANCED PREP (Available upon request)

4) **Leigh SC** et al. (In Prep) Optimization of digestive enzyme assay methodology.
3) **Leigh SC**, Brodie S, Hazen E, Muhling B, Garfield T, Dewar H (In Prep) Analysis and forecast of opah (*Lampris spp*.) distribution along the pacific coast of the US.
2) **Leigh SC**, Hoffmann S, Summers A, German DP (In Prep) An investigation of the spiral intestine in elasmobranchs.
1) **Leigh SC** and German DP (In Prep) The role of microbial symbionts in bonnethead shark seagrass digestion.

SELECTED GRANTS

2018 ***Sigma Xi Grant-in-Aid of Research*: $1,000**
Rewards scientific excellence by proving funds for research travel and equipment. Funding the acquisition, preparation, and CT scanning of elasmobranch spiral intestines.

2018 ***Microbiome Initiative Pilot Project Award*** (UC Irvine)
Provides in-kind support for exceptional applicants to analyze microbiome samples.

2017 ***Public Impact Distinguished Fellowship Award:* $12,000** (UC Irvine)
Only four distinguished fellows are chosen from university-wide nominations. Supports graduate students whose current research has the potential for substantial impact in the local, national, or global public sphere.

2017 ***Newkirk Center for Science and Society Graduate Research Fellowship*: $10,000** (UC Irvine)
Support to engage the community as a partner in scientific knowledge production. Funds used for *16s* and metagenomics sequencing of the gut microbiome in bonnethead sharks and to set up an outreach project incorporating art, science, and community engagement at Crystal Cove

State Park.

2017 ***OCEANS Graduate Student Research Fellowship***: **$7,000** (UC Irvine)
Encourages marine research crossing traditional disciplinary boundaries. Funds used for stable isotope analysis to determine bonnethead shark seagrass assimilation and for the creation of an interactive art exhibit (walk-through shark digestive tract) at Crystal Cove State Park's "Art in the Park" event.

2017 ***Friday Harbor Laboratories Research Fellowship***: **$1,300** (U. of Washington)
Supports exceptional marine research to be conducted at Friday Harbor Laboratories. Funded investigation of the function of the spiral intestine in elasmobranchs.

2016 ***National Geographic Society, Young Explorers Research Grant***: **$5,000**
Covers field costs for hard-working, creative individuals with a passion for improving our understanding of the world. Funded field work for investigation of the resource acquisition of seagrass-eating bonnethead sharks and their ecological role in coastal habitats.

2016 ***Sigma Xi Grant-in-Aid of Research***: **$1,000**
Rewards scientific excellence by proving funds for research travel and equipment. Funded enzymatic and histological analysis of bonnethead shark digestive tract.

2016 ***American Society of Ichthyologists and Herpetologists, Raney Fund Award***: **$800**
Supports outstanding research conducted by a pre-doctoral ichthyologist or herpetologist. Funds contributed to physiological analysis of bonnethead shark digestion.

2016 ***Society for Integrative and Comparative Biology Grant-in-Aid of Research***: **$1,000**
Funds excellent research proposals in comparative biology fields. Contributed to physiological analysis of bonnethead shark digestion.

SELECTED FELLOWSHIPS, AWARDS, & HONORS

2019 ***Teaching Excellence and Service to the University Award*** (UC Irvine)

2018 ***Abstract Award*** (American Physiological Society)

2018 ***Dr. William F. Holcomb Scholarship:*** **$1,000** (UC Irvine)
Recognizes excellence in marine science research by a single student in the Biology Dept.

2018 ***Best Overall Presentation*** (Ecology & Evolutionary Bio. Grad Student Symposium)

2018 ***Best Oral Presentation Award*** (SICB Div. of Comparative Physio. & Biochemistry)

2018 ***Pedagogical Fellowship Program:*** (UCI Division of Teaching Excellence)
A highly regarded "preparing future faculty" program; selection is competitive, based on a record of excellent teaching, promising scholarship, and service to the University, department, and professional community.

2018 ***Broadening Participation Award*****: $500** (SICB)
Defers travel costs to the annual meeting for exceptional applicants who demonstrate a commitment to increasing diversity in STEM fields.

2017 ***Best Graduate Student Presentation (2nd place):*** (Southwestern Organismal Bio.)

2017 ***Samuel H. Gruber Outstanding Presentation Award***: (American Elasmo. Society)
Prestigious award given for the best oral presentation at annual meeting.

2017 ***Grover C. Stephens Excellence in Physiology Research Award***: **$1, 000** (UC Irvine)
Recognizes excellent physiological research by a student in the Biology Dept.

2017 ***Climate Action Training Program Fellow*** (UC Irvine)
Students from interdisciplinary backgrounds are selected to take part in problem-based climate data science workshops, science communication seminars, and a 3-month internship experience with the goal of solving climate-related problems.

2016 ***NSF GRFP***: **$138,000**
Recognizes and supports three years of tuition and fees for outstanding graduate students in diverse STEM fields (~2,000 awardees are chosen out of over 13,000 applicants/year).

2016 ***Friday Harbor Laboratories Fish Functional Morphology Course Fellow***
Selected students take part in a 5-week intensive course on fish biomechanics and are expected to complete a self-designed research project. Results are written in manuscript form and presented in an oral presentation at the conclusion

of the program.

2015&16 ***Ford Foundation Fellowship Honorable Mention***
Recognizes outstanding graduate students seeking to increase diversity in STEM.

2015 ***GAANN Fellowship Award***: **$7,500** (U.S. Department of Education)
Awarded to students who exhibit an outstanding capacity for teaching & research.

2014 ***Provost PhD Fellowship Award***: **$20,000** (UC Irvine)
Awarded to the top 5% of students admitted to UCI graduate programs school-wide.

2014 ***Diversity Recruitment Fellowship Award***: **$10,000** (UC Irvine)
Awarded to exceptional incoming graduate students with the goal of increasing diversity.

2014 ***Competitive Edge Summer Research Fellowship Award***: **$5,000** (UC Irvine)
Provides funding for motivated incoming graduate students to begin their research during the summer prior to their first official quarter. Students present at a symposium at the conclusion of the program.

INVITED SEMINARS

7) **S. Leigh**. (2019). Physiology Impacts Ecology: Sharks in their Habitats. ***University of Southern California***.
6) **S. Leigh**. (2019). Predator vs Prey: How do sharks make a living? ***Claremont McKenna Colleges***.
5) **S. Leigh.** (2018). The Resource Acquisition Strategies of Sharks. ***University of California – San Diego.***
4) **S. Leigh**. (2018). JAWS on a Diet: An Omnivorous Predator. ***Aquarium of the Pacific.***
3) **S. Leigh.** (2018). The Nutritional Physiology of Sharks. ***The University of Utah***.
2) **S. Leigh.** (2017). The Resource Acquisition Strategies of Sharks. ***McDaniel College***.
1) **S. Leigh.** (2016). Pursuing a PhD: Research, Teaching, Mentoring, & Learning. ***University of Southern California****: Wrigley Institute for Environmental Studies Career Symposium.*

PRESENTATIONS & PUBLISHED ABSTRACTS

21) **S. Leigh** and D. German (2019) The role of microbial symbionts in bonnethead shark seagrass digestion. *Society for Integrative and Comparative Biology*. Oral Presentation.
20) **S. Leigh** and D. German (2018) The role of microbial symbionts in bonnethead shark seagrass digestion. *American Physiological Society Conference*. Oral Presentation.
19) **S. Leigh**. (2018). Omnivorous predators: seagrass digestion in the bonnethead shark. *Newkirk Center for Science and Society.* Oral Presentation.
18) **S. Leigh**. (2018). Omnivorous predators: seagrass digestion in the bonnethead shark. *Ecology & Evolutionary Biology Graduate Student Symposium*. Oral Presentation.
17) **S. Leigh**. (2018). An Omnivorous Shark? *UCI Grad Slam Symposium.* Oral Presentation.
16) **S. Leigh**, Y. Papastamatiou, & D. German. (2018). Omnivorous sharks? Analysis of bonnethead shark digestive physiology provides evidence for seagrass digestion and assimilation. *Society for Integrative and Comparative Biology.* Oral Presentation.
15) **S. Leigh,** S. Brodie, E. Hazen, B. Muhling, T. Garfield, & H. Dewar. (2017). Analysis of Opah (*Lamris sp.*) Distribution Along the Pacific US Coast. *Environmental Research Symposium*. Poster.
14) **S. Leigh,** Y. Papastamatiou & D. German. (2017). The Resource Acquisition Strategies of Seagrass-eating Bonnethead Sharks and Their Role in the Environment. *Ecological Society of America*. Oral Presentation.
13) S. Hoffmann, C. Donatelli**. S. Leigh**, E. Brainerd, & M. Porter. (2017). Functional ecomorphology of shark pectoral fins. *Florida SeaGrant Coastal Science Symposium*. Poster.
12) **S. Leigh**, S. Hoffman, A. Summers, & D. German. (2017). Spiraling into Control: The function of the spiral intestine in Elasmobranchs. *Joint Meeting of Ichthyologists and Herpetologists*. Oral Presentation.
11) S. Hoffmann, **S. Leigh**, C. Donatelli, E. Brainerd, & M. Porter. (2017). Three-dimensional movements of the pectoral fin during routine turns in the Pacific Spiny Dogfish (*Squalus suckleyi*). *Joint Meeting of Ichthyologists and Herpetologists.* Oral Presentation.
10) **S. Leigh,** Y. Papastamatiou & D. German. (2017). The Resource Acquisition Strategies of Seagrass-eating Bonnethead Sharks. *Joing Meeting of Ichthyologists and Herpetologists.* Poster.

9) **S. Leigh**, S. Hoffman, A. Summers, & D. German. (2017). The function of the spiral intestine in Elasmobranchs. *Society for Integrative and Comparative Biology Conference.* Oral Presentation.
8) S. Hoffmann, **S. Leigh**, C. Donatelli, E. Brainerd, & M. Porter. (2017). Three-dimensional movements of the pectoral fin during routine turns in the Pacific Spiny Dogfish (*Squalus suckleyi*). *Society for Integrative and Comparative Biology Conference.* Oral Presentation.
7) **S. Leigh**, S. Hoffman, A. Summers, & D. German. (2016). Spiraling into Control: the function of the spiral intestine in Elasmobranchs. *Friday Harbor Laboratories Research Symposium.* Oral Presentation.
6) **S. Leigh** & D. German. (2016). The Resource Acquisition Strategies of Seagrass-eating Bonnethead Sharks. *Society for Integrative and Comparative Biology Conference*. Poster.
5) **S. Leigh** & D. German. (2016). The Resource Acquisition Strategies of Seagrass-eating Bonnethead Sharks. *Ecology & Evolutionary Biology Graduate Student Symposium*. Oral Presentation.
4) **S. Leigh** & D. German. (2015). The Role of Diet Type on the Gut Size and Function of Zebrafish. *Society for Integrative and Comparative Biology Conference.* Oral Presentation.
3) **S. Wright** & D. German. (2014). The Role of Diet Type on the Gut Size and Function of Zebrafish. *Southwestern Organismal Biology Conference.* Oral Presentation.
2) **S. Wright** & D. German. (2014). The Role of Diet Type on the Gut Size and Function of Zebrafish. *Summer Research Symposium, UC Irvine*. Oral Presentation.
1) **S. Wright**, N. McNabb, & K. Heidelberg. (2012). Diel Patterns of Zooplankton Diversity and Abundance in Big Fisherman's Cove. *Summer Undergraduate Research Experience (SURE) Symposium*. Poster.

TEACHING & MENTORING EXPERIENCE

***1) Instructor of Record (Lecturer),* California State University Fullerton**

a) I am currently teaching Human Anatomy and Physiology (Spring 2019). I was responsible for creating the syllabus and student learning outcomes, making the lectures, as well as designing the active learning activities, assignments, and assessments.

***2) Pedagogical Fellowship Program,* UCI Division of Teaching Excellence and Innovation (2018)**

a) A highly regarded "preparing future faculty" program; selection is competitive, based on a record of excellent teaching, promising scholarship, and service to the University**.** It consists of three courses focusing on both general and discipline-specific pedagogical theory, observing and implementing evidence-based teaching practices, and developing and leading the UCI TA Training Workshops.

b) Culminates in the Certificate of Teaching Excellence.

***3) Teaching Assistant,* UCI Ecology and Evolutionary Biology Department**

a) DNA to Organisms (Fall 2014)

b) Human Physiology Laboratory (Winter 2015, Fall 2015, Winter 2016, Spring 2016)

***4) Guest Lecturer,* UCI**

a) Processes in Ecology and Evolution, Topic: Predation (Spring 2018)

b) Organisms to Ecosystems, Topic: Intro. to Ecology (Winter 2018)

5) Tutoring

a) **The College Trail** (2014-2015): Weekly, I tutored middle and high school students in various subjects, including biology, earth system science, algebra, statistics, and calculus.

b) **Coastal Carolina University Writing Center** (2010-2013): I tutored undergraduate students in every aspect of the writing process, from brainstorming to proof-reading. I also lead monthly writing workshops and specialized in ESL students and student athletes. Work resulted in publication in *Southern Discourse* (see teaching publications list above).

6) Mentoring (* = published during mentorship, ^ = went on to graduate/medical school)

a) **University of California, Irvine:** Quang Nguyen*^, Caitlyn Catabay^, Cam Vandenakker^, Siyan Chen^, Emily Urena^, Chloe Richards, Nicole Dwyer, Eleazar Paniagua^

b) **Florida International University:** Maria Sabando, Sarah Sisco

c) **California State University Fullerton:** Newton Hood^ (TA), Robert Courville (TA)

PROFESSIONAL & LEADERSHIP SERVICE

9) Loh Down on Science (2018-2019)

Invited by Sandra Tsing Loh, Executive Producer and Host of the "Loh Down on Science" radio show to research, write, and edit scripts for the show. The show is broadcast 5x per week to over 4 million listeners on 150 public radio stations across the country. Our goal is to make science fun and accessible to all.

8) UC Graduate Research Advocacy (2018)

I was one of two students selected by UCI Vice Provost Dr. Frances Leslie to represent UCI graduate students at UC Graduate Research Advocacy Day in Sacramento. I met with California senators and assembly members to discuss my research and advocate for UC graduate program funding.

7) UCI Climate Solutions Summit (2018)

Co-organizer of summit with the goal of facilitating interactions among UC Irvine researchers, community leaders, stakeholders, and policy makers in order to identify socially meaningful research priorities, important climate problems and solutions, as well as ways to improve public communication with the local community and government officials.

6) UCI Evolutionary Genetics Hiring Committee (2018)

Represented graduate student interests on the hiring committee for an assistant professor position. My role was to contribute to reading, discussing, and voting on applications, as well as organizing interview schedules and selecting the final hire.

5) Graduate Student Representative (2017-2018)

Responsible for representing the graduate students in the UCI Ecology and Evolutionary Biology Department during monthly faculty meetings, organizing new student orientation and quarterly graduate student meetings, and assigning and communicating with student-run committees.

4) UCI Policy Prep (P3) Program (2017-2018)

Three-tiered program which fuses learning workshops/seminars focused on public policy processes with practical experience related to advocacy and science policy. I am co-organizing the UCI Climate Solutions Summit (see below) as my science policy project.

3) NOAA Southwest Fisheries Science Center (2017)

As a Climate Action Training Program Fellow, I created the first model of preferred opah (*Lampris sp.*) habitat distribution along the Pacific coast of the U.S. and projected changes of this habitat preference due to both seasonal and longer term environmental changes.

2) Ecological Society of America (2017)

Co-organizer of the organized oral session "Nutritional Ecology in a Changing World: The Transduction of Energy Between the Environment, Individuals and Communities."

1) Winter Ecology and Evolutionary Biology Graduate Student Symposium (2017)

Co-organizer of departmental symposium event featuring oral presentations from graduate students.

<u>OUTREACH PROJECTS</u>

10) UCI Homecoming Festival (2019)

I set up a booth at the event to discuss my research and the importance of shark and marine habitat conservation with alumni and other visitors. I used a multi-media approach with photos and videos as well as shark artifacts (shark skin, jaws, egg cases, etc.) that visitors could touch and explore.

9) Targeted Instruction Generating Excitement about Research and Science (TIGERS: 2014-2019)

Through this program, I aim to increase science literacy in our local community and inspire students to pursue STEM careers. Every month, I visit regular and AP biology classes at Valencia HS, which has a high number of students in underrepresented groups in the STEM disciplines, and carryout a lesson & lab activity related to their current curriculum.

8) Art in the Park: Crystal Cove State Park Community Outreach (2018)

In collaboration with UCI art graduate student Lauryn Moles, I created a walk-through art installation of a shark digestive system. Community members who visited the exhibit were given an ipad containing a video that would guide them through the digestive processes occurring in each stage of the digestive system that they walked through. This ipad video can be found here: https://youtu.be/TGQSR9G1R5g

7) Downey High School Career Mentoring (2018)

I met one-on-one with high school students interested in pursuing STEM career paths and provided guidance for accomplishing their goals.

6) Shark Camp: Back Bay Science Center (2017)

Once a month during the summer (June-September), the Back Bay Science Center exposes local

youths (ages 7-15) to shark science through an interactive natural history and biology lesson followed by a fishing and identification session in Upper Newport Bay. I lead the biology lesson and assist campers with the fishing and elasmobranch identification process.

5) Gills Club Science Mentor (2017-2018)

Using social media, I answer questions about my research experiences and interact with the younger club members in order to empower girls and young women to pursue leadership positions in science and to expose them to ocean-related conservation issues.

4) California State Science Fair (2016 & 2017)

I volunteered as a judge for the Junior Zoology Category (6^{th}-8^{th} grade).

3) "SciGirls" PBS (2014-2015)

I was chosen by PBS producer Marie Domingo to appear as a science mentor to three culturally diverse middle school girls on the television program *SciGirls*. I aided in developing the science project that the girls completed and helped them analyze their data. The full episode, titled "Terrific Pacific" is available online here: https://goo.gl/STvx2C.

2) Equitable Science Curriculum Integrating Arts in Public Education (ESCAPE: 2014 & 2015)

Served as a research science specialist to teach elementary school teachers about science misconceptions while incorporating artistic lessons into their curriculums.

1) Crystal Cove Alliance Citizen Science Cruises (2014 & 2015)

Aided in developing the cruise curriculum (geared towards middle school and high school students) and regularly serve as a science expert aboard the cruises.

SELECTED SCIENCE COMMUNICATION AND MEDIA ATTENTION

19) The New York Times (2018): "The Omnivorous Sharks That Eat Grass," by Veronique Greenwood
https://www.nytimes.com/2018/09/06/science/omnivorous-sharks-seagrass.html

18) Newsweek (2018): "Vegetarian Sharks? World's First Omnivorous Sea Beasts…," by Hannah Osborne
https://www.newsweek.com/vegetarian-omnivorous-shark-bonnethead-discovered-seagrass-1103831

17) The Guardian (2018): "First known omnivorous shark species identified," by Ian Sample
https://www.theguardian.com/environment/2018/sep/05/bonnethead-omnivorous-shark-species-identified

16) USA Today (2018): "The bonnethead is the first known plant-eating shark, scientists say," by Brett Molina
https://www.usatoday.com/story/news/nation-now/2018/09/06/bonnethead-shark-scientists-reveal-first-known-plant-eating-shark/1213376002/

15) ABC News (2018): "Side of seagrass please: Scientists find omnivorous shark," The Associated Press
https://abcnews.go.com/Technology/wireStory/side-sea-grass-scientists-find-omnivorous-shark-57652873

14) Fox News (2018): "Vegetarian shark discovery," by James Rogers
http://www.foxnews.com/science/2018/09/05/vegetarian-shark-discovery-first-omnivorous-species-sea-predator-stuns-scientists.html

13) NBC (2018): "Researchers Find First Known Plant-Eating Shark," by Andrew Johnson
https://www.nbcsandiego.com/news/local/First-Omnivorous-Shark-Discovered-UCI-492560841.html

12) Scientific American (2018): "Bonnethead Sharks Are Underwater Lawnmowers," by Christopher Intagliata
https://www.scientificamerican.com/podcast/episode/bonnethead-sharks-are-underwater-lawnmowers/

11) Nature (2018): "The world's first flexitarian shark grazes like a cow"
https://www.nature.com/articles/d41586-018-06173-y

10) The OC Register (2018): "Omnivore sharks?", by Laylan Connelly
https://www.ocregister.com/2018/09/05/omnivore-sharks-uci-scientists-find-bonnetheads-digest-greens-as-well-as-meat/

9) UCI News (2018): "Shaking Up the Shark's Image," by Roy Rivenburg
https://news.uci.edu/2018/10/29/shaking-up-the-sharks-image/

8) KUCI: Ask a Leader (2018): "Climate Solutions Summit: Live at the Beckman," hosted by Claudia Shambaugh

http://askaleader.com/?p=1336

7) Popular Science (2018): "This tiny shark eats seagrass and is doing just fine," by Kate Baggaley
https://goo.gl/RieXqA

6) Science Magazine (2018): "Meet the world's first salad eating shark," by Elizabeth Pennisi
https://goo.gl/yrtkus

5) National Geographic Society (2017): "This shark eats grass and no one knows why," by Hannah Lang
https://goo.gl/9ARDDB

4) SciComm Mondays (2017): "A shark that eats plants?" hosted by Nicole Wood
https://goo.gl/YycRSN

3) Brews and Brains (2017): "Beyond the jaws: shark digestive physiology," hosted by Sarah Cross
https://goo.gl/8kSm1E

2) Earth Touch News (2017): "This shark has an appetite for…grass?" by David Moscato
https://goo.gl/7LPi15

1) Keys News (2017): "Study: Bonnethead sharks dine on seagrass," by Theresa Java
https://goo.gl/dPwcWz

RECENT RESEARCH EXPERIENCE

University of California, Irvine: Dr. Donovan German Laboratory

Title: PhD Student/ Teaching Assistant and Pedagogical Fellow (June 2014 present)

Projects: 1) Investigating *Sphyrna tiburo* (bonnethead sharks) to understand how they capitalize on vegetation by using a combination of molecular methods, biochemical assays, stable isotope analysis, and histology. 2) Exploring the ability of *Danio rerio* (zebrafish) to exhibit plasticity of gut structure and function when exposed to various diets (herbivorous, omnivorous, & carnivorous) over the course of multiple generations. 3) Teaching Assistant for undergraduate courses and research mentor to students.

Friday Harbor Laboratories: Fish Functional Morphology Course Fellow

Title: Student Fellow (July-August 2016)

Projects: 1) Investigated the functional morphology of the spiral intestine in elasmobranchs using CT scanning, 3D modeling techniques, fluid dynamics, and chemically induced intestinal muscle contractions. 2) Used Video Reconstruction of Moving Morphology (VROMM) combined with post mortem electrical stimulation (EMG) to investigate 3D movement of the pectoral fin in *Squalus suckleyi.*

University of Georgia: Dr. Richard Steet Laboratory

Title: Research Technician/Lab Manager (September 2013-June 2014)

Projects: Used zebrafish as a model to study pathogenic mechanisms of lysosomal disease. Responsible for carrying out experiments using RT-PCR, cloning, transformation, western blots, RNA preparation, cDNA synthesis, husbandry, dissections, etc. Also responsible for ordering supplies, editing manuscripts, and maintaining the department Stock Center which entailed handling over $20,000 worth of lab supplies.

Southern California Coastal Water Research Project: Microbiology Laboratory

Title: Research Assistant (May 2013-September 2013)

Projects: Worked on developing a faster, more effective way to monitor water quality. Responsible for collecting water samples in field, water filtering, qPCR, culturing samples, microbial source tracking, data entry and analysis, and inventory of lab freezers and chemicals.

University of Southern California: Dr. Karla Heidelberg Laboratory

Title: Research Intern (August 2012-November 2012)

Projects: Investigated how the stress of temperature increase impacts the symbiotic relationship between the California Golden Gorgonian, *Muricea californica*, and their microbiomes. Responsible for PCR, DNA gel electrophoresis, creating clone libraries, and epifluorescent/dissecting microscopy.

PEER REVIEWS

Journal of Experimental Biology
Public Library of Science (PLoS ONE)
Oecologia
Croatian Journal of Fisheries (CJF)

Ecology and Evolution
Integrative and Comparative Biology

AFFILIATIONS
American Physiological Society (2018-present)
American Elasmobranch Society (2016-present)
American Society of Ichthyologists and Herpetologists (2016-present)
Society for Integrative and Comparative Biology (2014-present)
Southwest Organismal Biology Division (2014-present)
Ecological Society of America (2017)

ABSTRACT OF THE DISSERTATION

The Resource Acquisition Strategies and Digestive Physiology of Sharks
By

Samantha Christine Leigh

Doctor of Philosophy in Ecology and Evolutionary Biology

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Professor Donovan P. German, Chair

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 13C-labeled 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. Additionally, cellulose-component-degrading enzyme activities were detected in shark hindguts. I 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. Furthermore, the intestinal microbiome of vertebrates has been shown to play a crucial role in their digestive capabilities. This is particularly true for omnivores and herbivores that rely on enteric microbes to digest components of plant material that are indigestible

endogenously. The bonnethead shark represents an interesting opportunity to explore how intestinal microbes provide a mechanism for omnivory in a marine vertebrate. I use digestive enzyme assays, histological imaging, measurements of microbial fermentation, and 16S rDNA sequencing to identify processes by which the bonnethead shark can digest and assimilate plant material. Finally, I delve into the functional morphology of the spiral intestine in sharks since this unique structure appears to be important in terms of housing enteric microbes and slowing the transit rate of digesta. I use CT scanning technology to provide a new way of investigating the spiral intestine. In addition to being one of the most conclusive investigations of shark nutritional physiology to date, my results highlight the importance combining studies of structure and function in order to better understand the nutritional physiology of organisms.

INTRODUCTION

Sharks make up one of the most abundant and diverse groups of consumers in the ocean (Compagno 2008). They may play an important ecological role in energy fluxes in marine environments and in impacting the biodiversity of lower trophic levels that we depend on as a food and economic resource (e.g., Wetherbee et al. 1990; Cortés et al. 2008). However, beyond prey capture methods and dietary analyses, the nutritional physiology of sharks is woefully understudied. They consume a broad range of diet types (smaller sharks, marine mammals, teleosts, crustaceans, zooplankton, etc.) and are generally known to be largely carnivorous, consuming prey items high in protein and lipids (Wetherbee et al. 1990; Cortés et al. 2008; Bucking 2016). Indeed, matching physiological concepts with genetic underpinnings and evolutionary background is crucial to understanding the patterns and processes involved in the evolution of the digestive strategies that sharks possess.

The broader field of nutritional physiology has a foundation based largely on economic theory: the digestive tract is energetically expensive to maintain (Cant et al. 1996), and thus, from basic economic principles, the Adaptive Modulation Hypothesis (AMH; Karasov 1992; Karasov and Martinez del Rio 2007) suggests that gut function should match with what is consumed in terms of quantity and biochemical composition (Martine and Fuhrman1995; Karasov and Douglas 2013). Shark evolution presumably follows AMH and sharks should, therefore, have guts well-suited for their diet types. Sharks are also generally known for eating large meals on an infrequent basis, potentially going days, or even weeks, without a meal (Wetherbee et al. 1987; Cortés et al. 2008; Armstrong and Schindler 2011). Hence, in order to acquire ample nutrients from their

infrequent meals, sharks must have mechanisms of slowing the rate of digesta transit to allow sufficient time for digestion and nutrient absorption, yet there has been minimal investigation into shark nutritional physiology. Although the field of comparative nutritional physiology is relatively young (e.g., Karasov and Diamond 1983; Diamond and Karasov 1987; Karasov and Martinez del Rio 2007), much has been learned about gut function in ecological and evolutionary contexts, albeit mostly about terrestrial organisms because of research in biomedical and livestock fields (Choat and Clements 1998; Clements et al. 2009). Within marine biology, far more advances have been made concerning the nutritional physiology of teleost fishes (e.g., German 2011) than for sharks. New methods of investigation have been developed, as well as new theories and models that could be applied to sharks (e.g., German et al. 2015; Clements et al. 2017). The most recent reviews of elasmobranch digestive physiology (Cortés et al. 2008; Bucking 2016; Ballantyne 2016; Leigh et al. 2017) lament the dearth of data available on shark digestion, and thus, make logical connections to the recent advances in the understanding of teleost nutritional physiology, where there have been efforts to integrate diet with digestive function and metabolism. In the following chapters, I delve into the digestive physiology of sharks, first by focusing on bonnethead sharks, *Sphyrna tiburo*.

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. In my first chapter, I determine if bonnetheads digest seagrass nutrients. Captive sharks were fed a 13C-labeled 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 with 50±2% digestibility of seagrass organic matter. Additionally, cellulose-component-degrading enzyme activities were detected in shark hindguts. I 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.

My second chapter investigates the role that the intestine microbiome plays in seagrass digestion of the bonnethead shark. Intestinal microbiomes of vertebrates has been shown to play a crucial role in their digestive capabilities. This is particularly true for omnivores and herbivores that rely on enteric microbes to digest components of plant material that are indigestible endogenously. While studies of microbe-host interactions are becoming more frequent in terrestrial systems, studies of this type are still largely lacking in marine systems, particularly for higher trophic level organisms. The bonnethead shark represents an interesting opportunity to explore how intestinal microbes provide a mechanism for omnivory in a marine vertebrate. I use digestive enzyme assays, histological imaging, measurements of microbial fermentation, and 16S rDNA sequencing to identify processes by which the bonnethead shark can digest and assimilate plant material. I found evidence of cellobiose and chitin degrading enzymes (β-glucosidase and N-acetyl-β-D-glucosaminidase) in their distal intestine, microbial

fermentation in their spiral and distal intestines (as evident by high short-chain-fatty-acid concentrations), increased epithelial surface area in their spiral intestine, and we have identified the specific taxa of microbes that make up the majority of the bonnethead shark gut microbiome (Vibrionales, Closdridiales, Pseudomondales, Mycoplasmatales, Rhizobiales, and others). In addition to being one of the most conclusive investigations of a shark gut microbiome to date, our results highlight the importance combining studies of microbial community composition with an informed context of host ecology and physiology.

Since the spiral intestine of the bonnethead shark seems to be the location with the most microbial activity, as well as the region of the digestive tract where digest spends the majority of its time, my final chapter focuses on the functional morphology of the spiral intestine in sharks. It has long been stated, despite very little quantifiable evidence, that the spiral intestine present in all known sharks, skates, and rays is used to slow the rate of transit of digesta through the gut and provides increased surface area for the absorption of nutrients. In this investigation, we use a novel technique - creating 3D reconstructions from CT scans of spiral intestines - in order to identify the morphology of spiral intestines from at least one representative species of each shark family. Using this information, we start to provide an evolutionary as well as dietary context to the different structures. We also provide the first quantification of the flow rate of material through the spiral intestine. This project opens the door to using 3D morphometrics to examine the function of the gastrointestinal tract of sharks and fishes in general. The sum of these three chapters results in one of the most conclusive investigations in the digestive physiology of sharks to date.

Chapter 1

Seagrass Digestion by a Notorious 'Carnivore'

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 (Bucking 2016; Leigh et al. 2017; Taylor et al. 2006; Karasov and Martinez del Rio 2007; Tracy et al. 2006; Olin et al. 2013). Overall, the nutritional ecology of fishes (including sharks) is insufficiently studied outside of a few species used in aquaculture (Bucking 2016; Leigh et al. 2017; German 2011). Carnivores, such as sharks, appear specialized for digesting high-protein diets, as indicated by elevated digestibility of protein (Wetherbee and Gruber 1993; Di Santo and Bennett 2011) and high activity levels of protein-degrading digestive enzymes in their guts (Leigh et al. 2017; Jhaveri et al. 2015; Papastamatiou 2007; Newton et al. 2015). 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; German et al. 2015), as well as varying diversities and abundances of enteric microbial communities in comparison to carnivores (Nayak 2010; Clements et al. 2014; Givens et al. 2015). 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 (Ward et al. 2017; Bjorndal 1980; Clements and Raubenheimer 2006).

With population estimates of approximately 4.9 million (NOAA/NMFS Highly Migratory Species Management Division 2007) individuals along the Atlantic and Gulf of Mexico coasts of the United States of America (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 USA coastal waters and beyond. Although they are frequently listed as carnivorous, consuming mostly crustaceans and mollusks (Cortés et al. 2008), they are also known for consuming copious amounts (up to 62% of gut content mass) of seagrass in some populations (Bethea et al. 2007), and for feeding at lower trophic levels than other closely-related species (Bethea et al. 2011). However, what an animal ingests and what they digest and assimilate are not the same thing (German 2011), 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. 2007 & 2011). Sharks are uniformly accepted as carnivorous (Bucking 2016; Leigh et al. 2017; Cortés et al. 2008), 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 appears "degraded" in comparison to fresh seagrass (Bethea et al. 2007), 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 since they could be responsible for significant grazing and nutrient transport within fragile seagrass ecosystems. Seagrass meadows are the most widespread coastal ecosystem on earth (Lamb et al. 2017) and provide a multitude of ecological and economic services (Nordlund et al. 2016). Some of these services include

cross-ecosystem nutrient transfer (Barbier et al. 2011), erosion control (Grech et al. 2012), pollution and pathogen management (Lamb et al. 2017; Cullen-Unsworth et al. 2014; Waycott et al. 2009), providing habitat and protected nursery areas for thousands of fish and invertebrate species thereby supporting the fishing industry (Barbier et al. 2011), acting as a CO_2 sink (Nordlund et al. 2016), and producing large quantities of oxygen (Nordlund et al. 2016). 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-labeled seagrass and 10% squid diet (Fig. 1.1; totaling 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.

Methods:

All methods mentioned here are described in detail in the Supplemental Methods.

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 terra-cotta pots within a closed, re-circulating, tank system (~454 L) and placed in direct sunlight. We

labeled the seagrass by directly adding powdered ^{13}C-labeled sodium bicarbonate (1g; 99-atom-%, Sigma Aldrich Product #372382) into the seawater in the tank. A chiller (Aqua Euro USA, Model: MC-1/2hp) was used to keep the water in the tank from reaching above 30°C. The water in the tank underwent a water change once per week and new ^{13}C-labeled 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 Florida International University to undergo feeding trials (henceforth the "lab-fed" sharks).

Feeding Events and Fecal 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 hrs. After 24 hrs, the sharks were individually anesthetized 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-gauge 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 equaling 5% of their initial body weight daily for three weeks. Fecal material was collected daily via siphoning through a 250 μm mesh. Water passed through the mesh while fecal material was collected on top. Fecal material was transferred into 50mL 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 fecal material was collected per shark over the course of the 3 weeks.

Dissections and Tissue Preparation

At the conclusion of the three-week feeding trial, all lab fed individuals were euthanized in 1% MS-222 solution, measured [standard length (SL)], weighed [body mass (BM)], and dissected on a chilled (~4°C) cutting board. Whole GI tracts were removed by cutting at the esophagus 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 distal intestine (DI; German 2009; Leigh et al. 2018). 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.

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 fecal material from all

of the lab-fed sharks. The following equation was used to determine the percent digestibility of each macronutrient type by the shark:

% Digestibility = [((Ash-adjusted Ingested) – (Defecated)) / (Ash-adjusted Ingested)] x 100

Fiber digestibility was determined using an ANKOM 200/220 Fiber Analyzer, following the ANKOM suggested procedures (Goering and Van Soest 1970; Vogel et al. 1997) for neutral detergent fiber (NDF; which includes cellulose and hemicellulose) and acid detergent (ADF; which excludes cellulose).

To determine if the lab-fed sharks were meeting their daily metabolic demands on the prescribed diet, bonnethead shark metabolic rate was estimated using the equation from Parsons 1990:

$$M= ((68.9 + 177.8W) 3.25/W) \times 24$$

where M is metabolic rate (kcal kg^{-1} d^{-1}) and W is weight in kilograms. Initial wet weight of the sharks was used here. Coefficients were based on the constants for fish (Solomon and Brafield 1972). The amount (g) of the diet consumed by each shark was recorded daily.

Digestive Enzyme Assays

Intestinal homogenates were produced as described by Leigh et al. 2018. 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). All assay protocols generally followed methods detailed in Leigh et al. 2018, unless otherwise noted.

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 (~700 μg for shark blood and tissues samples and ~2 mg for seagrass tissues) were then transferred into individual 5 mm x 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 lab-fed shark liver samples and seagrass samples using a soxhlet (Bligh and Dyer 1959) 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 since these are commonly measured in studies of nutritional physiology of marine fishes (Wilson 1985; McMahon et al. 2015).

Statistical Analysis

Comparisons of enzymatic activities were made among gut regions with ANOVA followed by a Tukey's HSD with a family error rate of $P = 0.05$. Comparisons of enzymatic activities between lab-fed sharks and wild caught sharks were made using unpaired t-tests with a Bonferonni corrected error rate of $P = 0.006$. Comparison of lab-fed shark liver amino acid $\delta^{13}C$ values to 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 (version 1.0.136).

Results and Conclusions:

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. Lab-fed sharks all gained weight on their seagrass-heavy diet (mean: 6.65 ± 3.46% weight gain from initial body mass; Table 1.1) and digested the total organic matter (50 ± 2%) and the fiber in seagrass (52 ± 3% for neutral detergent fiber and 43±4% for acid detergent fiber; Table 1.2) with moderate efficiency. They also more than met their energetic demands on their prescribed lab diet (average caloric need: 28 kcal per day, average calories digested in the laboratory feeding trial: 203 kcal per day; Parsons 1990; Table 1.1). 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%; Bjorndal 1980). As green sea turtles mature, they become almost entirely herbivorous, and their digestibility of seagrass increases (mean seagrass organic matter digestibility of 64.6%; Bjorndal 1980) in parallel with a longer digestive tract and a more diverse microbiome (Price et al. 2017). 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 (Leigh et al. 2017; Jhaveri et al. 2015). 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 (Ruppert 1980), showing that not all carnivores can digest plant

material efficiently. Indeed, Pandas, which are herbivores with a "carnivorous" gut (Stevens and Hume 1988), have enteric microbiomes that differ from other herbivores (Ley et al. 2008) and also show about 20% organic matter digestibility of bamboo (Stevens and Hume 1988). Pandas make a living on high-intake and digest mostly the soluble portions of bamboo (Dierenfield et al. 1982). Thus, bonnethead sharks are considerably better at digesting seagrass than either of these terrestrial examples (Ruppert 1980; Dierenfield 1982).

Enzymatic assays revealed that protein-degrading enzyme (aminopeptidase and trypsin) and lipid-degrading enzyme (lipase) activities peaked in the proximal or spiral intestine for both lab-fed and wild-caught sharks, which is congruent with previous work on wild-caught bonnetheads, and other fishes (Fig. 1.2; Leigh et al. 2017; Jhaveri et al. 2015; German et al. 2015; Leigh et al. 2018; Buddington et al. 1997; Harpaz and Uni 1999). The spiral intestine is likely the primary site of amino acid and fatty acid absorption in bonnetheads and other shark species (Hart et al. 2016). While carbohydrate-degrading enzyme (amylase and maltase) activities were similar between lab-fed and wild-caught sharks, maltase activity was relatively low and constant throughout the digestive tract in both groups (Fig. 1.2 & Fig. 1.3; Jhaveri et al. 2015). However, the amylase levels observed in bonnethead sharks are high for a carnivorous fish and comparable to omnivorous fish such as *Xiphister atropurpureus* (German et al. 2015). Coupled with the bonnethead's high digestibility of soluble carbohydrates (82 ± 5%, Table 1.2), this indicates efficient digestion of the soluble carbohydrates (like starch; Govers et al. 2015) found in seagrass material.

The presence of elevated β-glucosidase activity in the hindgut of both the lab-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 (Fig. 1.3; Jhaveri et al. 2015). The fact that β-glucosidase activity was significantly higher in the hindgut compared to other gut regions (proximal intestine and spiral intestine, Figs. 1.2 & 1.3) indicates likely involvement from the gut microbiome in the digestion of seagrass fiber. Surprisingly, the activity levels of β-glucosidase in the bonnethead hindgut are on par with activities observed in the hindguts of *Cebidichthys violaceus*, an herbivorous, teleost fish that digests algal material with assistance from their gut microbiome (German et al. 2015). 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; Papastamatiou 2007; Papastamatiou and Lowe 2004), whereas most herbivorous teleost species have slightly higher average stomach pH values of 2-3 (Horn 1989; Zemke-White et al. 1999). Since 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 material (Zemke-White et al. 1999). Bonnethead sharks also have molariform teeth that are presumed to be for crushing hard prey (Mara et al. 2010), 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 (Larsen et al. 2013). We measured a clear increase in the $\delta^{13}C$ signature in the blood and liver tissues of the lab-fed sharks over the course of the feeding trial (Figs. 1.4 & 1.5). The ^{13}C-labeled seagrass used in the feeding trials had a mean $\delta^{13}C$ of 104.9‰ (mean atom % of 1.25 ± 0.05) compared to a mean $\delta^{13}C$ of -13.4‰ (mean atom % of 1.08 ± 0.02) for wild, non-labeled seagrass (Fig. 1.4). The mean $\delta^{13}C$ signature of the blood plasma from the lab-fed sharks increased from -12.1‰ at the beginning of the feeding trial to 2,743.9‰ at the end of the feeding trial (Fig. 1.4). 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 lab-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 (Fig. 1.5).

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; Bethea et al. 2007), but they are actually capable of digesting and assimilating seagrass nutrients, making them clear omnivores. Since 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 (German 2011; Karasov and Douglas 2013), unlike the myriad specializations seen in mammals (Choat and Clements 1998).

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 (Watford 2003; Yancey 2015; Shipley et al. 2017; Forster et al. 1972; Evans 2009). Sharks are unique from most teleost fishes in that their total blood osmolarity (1118 mOsm/L for dogfish sharks; Forster et al. 1972) is similar to that of seawater (1050 mOsm/L; Evans 2009) and that nearly half (441 mM/L) of this is accounted for by urea. Since urea synthesis occurs in the liver and uses CO_2 (Watford 2003; Ballantyne 1997), if ^{13}C-labeled 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 (Shipley et al. 2017; Malpica-Cruz et al. 2012; Kim et al. 2011; Kim et al. 2012; MacNeil et al. 2005). 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 (>4 months vs ~1-2 months for plasma proteins; Malpica-Cruz et al. 2012; Kim et al. 2011), and the red blood cells don't contain bicarbonate or urea. The red blood cell isotopic signature, therefore, represents labeled proteins, which are similar to the labeled amino acids in the liver (Fig. 1.5).

Furthermore, the compound specific stable isotope analyses (CSSIA) shows that amino acids in the lab-fed sharks livers were also labeled, making it unlikely that ^{13}C-

labeled sodium bicarbonate in the sharks livers caused this result (Fig. 1.5; Table 1.3). 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 (Fig. 1.5; Table 1.4; Wilson 1985; McMahon et al. 2015). The other amino acids (alanine, glutamate, glycine, lysine, phenylalanine, threonine, tyrosine) were more enriched in ^{13}C in the grass than in the sharks (Table 1.4), 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 (Larsen et al. 2013). 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 (Larsen et al. 2013). Hence, each of our analyses (including bulk seagrass, seagrass fiber, and CSSIA) show that all of the components of the seagrass in the current study were indeed labeled with ^{13}C (positive $\delta^{13}C$ values), and therefore, the assimilation of the labeled carbon into the bonnethead sharks must have come from the labeled seagrass and cannot represent some components of wild seagrass (or any marine resource) still persisting in the sharks' tissues. The CSSIA and enriched seagrass fiber isotopic signatures also argue against the assimilated labeled carbon only coming from the labeled bicarbonate, and in fact, some of the bulk liver isotopic signature could be contributed by liver glycogen synthesized from ^{13}C-labeled glucose assimilated from seagrass tissues, including the fiber, which was heavily labeled (Fig. 1.4). 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 (~4.9 million individuals in the Atlantic and Gulf of Mexico coastal waters of the USA; NOAA/NMFS 2007) 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 (Lamb et al. 2017; Nordlund et al. 2016). 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 (Heupel et al. 2006). 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 (Tracy et al. 2006; Clements et al. 2017).

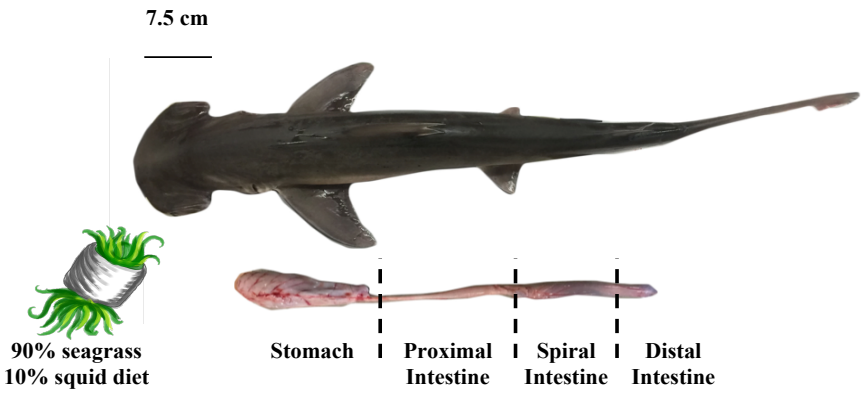

Figure 1.1: Adult bonnethead shark, Sphyrna tiburo, with it's digestive tract. 90% seagrass and 10% squid diet illustration by LLM Pandori.

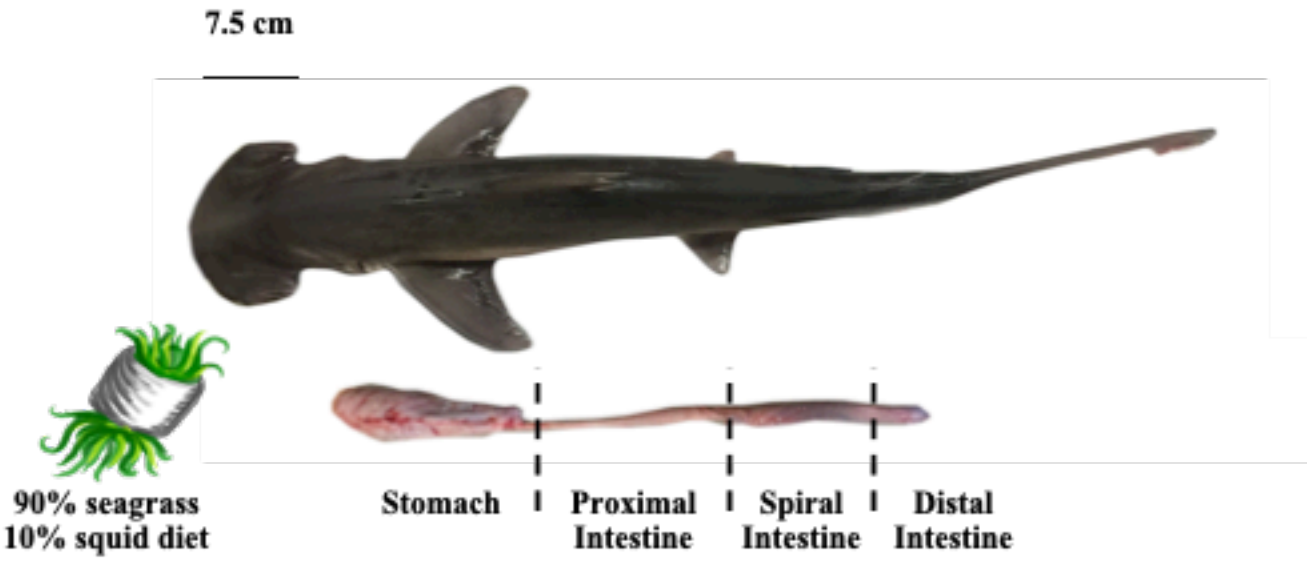

Figure 1: Adult bonnethead shark, *Sphyrna tiburo*, with it's digestive tract (Adapted[2, 10]). 90% seagrass and 10% squid diet illustration by LLM Pandori.

Figure 1.2: Trypsin, aminopeptidase, maltase, and lipase activities in the digestive tracts of bonnethead sharks. Open circles represent wild-caught sharks, while filled circles represent mean ± standard deviation values for lab-fed sharks. Protein- degrading enzymes (trypsin and aminopeptidase) are in red, soluble carbohydrate- degrading enzymes (maltase) are in blue, and lipid-degrading enzymes (lipase) are in purple. No significant differences were found between lab-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 ($P<0.05$).

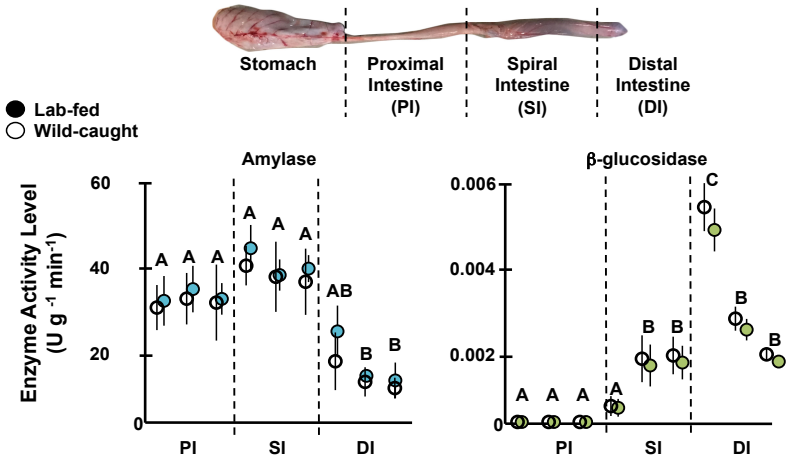

Figure. 1.3: Amylase and β-glucosidase activities in the digestive tracts of bonnethead sharks. Open circles represent wild-caught sharks, while filled circles represent mean ± standard deviation values for lab-fed sharks. The carbohydrate-degrading enzyme (amylase) is in blue and the cellulobiose-degrading enzyme (β-glucosidase) is in green. No significant differences were found between lab-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 ($P<0.05$).

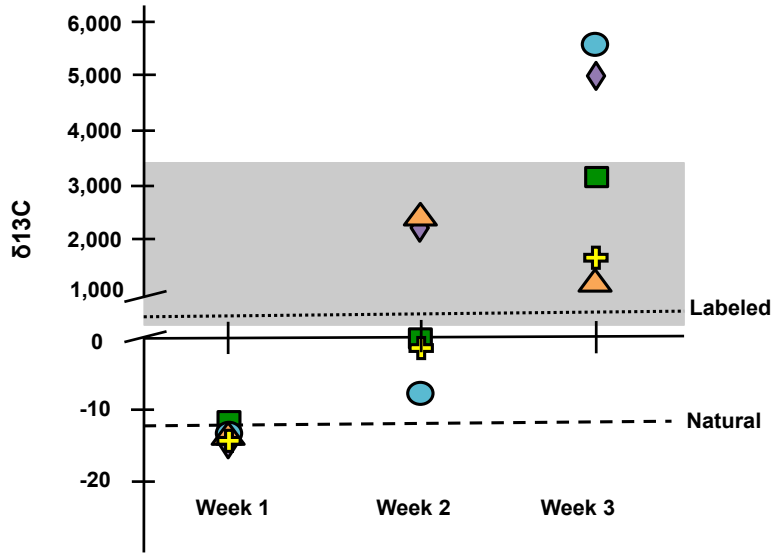

Figure 1.4: $\delta^{13}C$ values for lab-fed bonnethead shark blood plasma for each of the three weeks of the feeding trial. Different shaped (and colored) data points represent different individual lab-fed sharks. Mean values for ^{13}C-labled seagrass and natural seagrass are shown as different-patterned horizontal lines. The total $\delta^{13}C$ range for the ^{13}C-labeled seagrass is denoted by a light grey box.

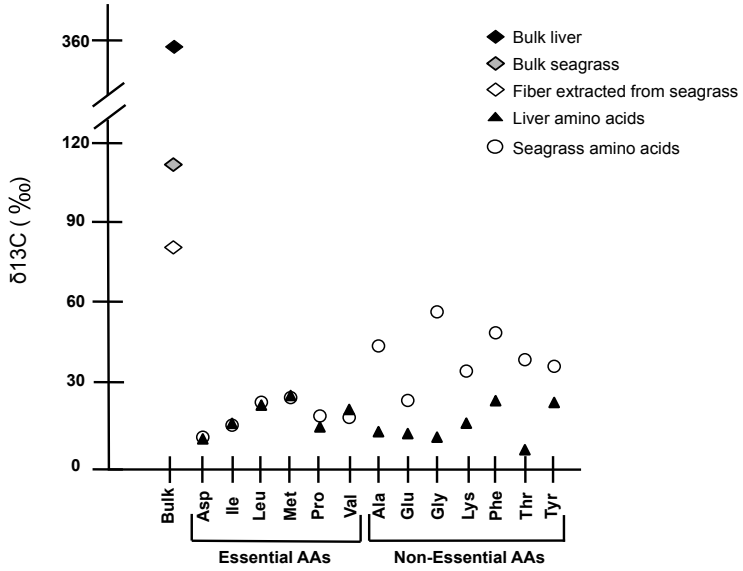

Figure 1.5: Bulk bonnethead shark liver tissue, bulk seagrass tissue, fiber extracted from seagrass, and individual amino acid (AA) $\delta^{13}C$ values (means). Abbreviations: Asp (aspartic acid), Ile (isoleucine), Leu (leucine), Met (methionine), Pro (proline), Val (valine), Ala (alanine), Glu (glutamic acid), Gly (glycine), Lys (lysine), Phe (phenylalanine), Thr (threonine), and Tyr (tyrosine).

Shark ID	Initial mass (g)	Terminal Mass (g)	Weight Increase (%)	Caloric Demands (kcal/day)	Calories Consumed (kcal/day)	Calories Digested (kcal/day)
1	1376	1414	2.687411598	25.414038	278.885	179.45455
2	1445	1518	4.808959157	24.4571184	265.568	170.88544
3	1313	1492	11.99731903	23.5834092	253.409	163.06147
4	1942	2080	6.634615385	32.3066328	374.806	241.17698
5	2116	2274	6.948109059	34.7197344	408.388	262.78604
Mean:	1638.4	1755.6	6.6152828	28.0961865	316.2112	203.472896
Standard Deviation:	364.8	392.6204	3.4557357	5.0596724	70.413081	45.308811

Table 1.1: The initial and terminal masses (g) of each individual lab-fed bonnethead shark. The weight increase (%) for each individual shark, as well as the means ± standard deviations are also provided. Caloric demand (kcal/day), Calories consumed (kcal/day), and Calories digested (kcal/day) along with means are standard deviations for each are also included.

Constituent	Digestibility (%)
Protein	92±3
Lipid	51±7
Soluble Carbohydrate	80±3
Neutral Detergent Fiber	52±3
Acid Detergent Fiber	43±4
Total Organic Matter	50±2

Table 1.2: Mean (±standard deviation) digestibility (%) of protein, lipid, soluble carbohydrates, neutral detergent fiber, acid detergent fiber, and total organic matter of a 90% seagrass, 10% squid diet by the bonnethead shark.

	Ala	Asp	Glu	Gly	Ile	Leu	Lys	Met	Phe	Pro	Thr	Tyr	Val
Seagrass1	42.42±0.15	6.77±0.12	21.56±0.42	55.59±1.19	11.46±0.47	21.08±0.42	31.72±0.64	23.47±0.44	48.43±0.26	15.14±0.71	38.52±0.11	35.66±0.25	14.54±0.09
Seagrass2	41.74±0.17	7.52±0.33	21.82±0.16	54.92±0.7	13.01±0.4	20.60±0.23	31.43±1.18	21.59±1	46.46±0.45	15.78±0.04	37.22±0.34	34.29±0.32	14.44±0.15
Seagrass3	44.38±0.11	8.14±0.4	21.79±0.84	57.68±0.42	11.89±0.32	21.40±0.09	36.14±0.08	23.40±0.43	48.95±0.33	16.20±0.05	36.81±1.75	34.92±0.3	16.45±0.16
Shark1	10.72±0.68	8.27±0.26	8.38±0.44	6.91±1.05	13.93±0.17	21.12±0.1	12.86±0.3	24.35±0.07	22.67±0.49	11.45±0.13	3.52±0.43	22.25±0.03	19.17±0.5
Shark2	11.23±0.28	6.59±0.67	9.74±0.04	7.43±1.9	13.05±0.44	20.94±0.54	13.85±0.02	24.96±0.38	22.50±0.05	9.58±0.35	2.77±0.31	23.05±1.02	20.04±0.14
Shark3	11.78±0.33	9.48±1.39	8.80±0.03	3.77±0.89	12.74±0.04	20.07±0.45	13.89±0.37	24.65±0.58	21.55±0.31	18.59±0.89	3.50±1.34	19.29±0.05	19.48±0.06
Shark4	5.90±0.42	2.88±0.42	5.43±0.63	12.17±2.07	10.75±0.52	16.64±0.51	10.56±0.3	19.98±0.15	18.66±0.08	5.97±0.63	1.05±0	18.67±0.27	14.03±0.21
Shark5	8.41±0.04	6.54±1.08	11.99±0.39	7.18±1.5	13.75±0.3	20.76±0.42	13.38±0.79	24.23±0.39	22.72±0.23	11.73±1.05	2.22±0.07	21.41±0.28	18.42±0.28

Table 1.3: The means and standard deviations for the ratio of the mean $\delta^{13}C$ essential amino acid signatures in the ^{13}C- labeled seagrass used in the feeding trial over the mean $\delta^{13}C$ essential amino acid signatures in the lab-fed bonnethead shark livers**.**

	Essential AA's						Non-essential AA's						
	Asp	**Ile**	**Leu**	**Met**	**Pro**	**Val**	**Ala**	**Glu**	**Gly**	**Lys**	**Phe**	**Thr**	**Tyr**
Mean	1.302	0.951	1.064	0.972	1.565	0.844	4.7617	2.620	8.58	2.59	2.230	17.5	1.680
Standard Deviation	0.738	0.103	0.112	0.095	0.661	0.133	1.508	0.827	3.78	0.31	0.195	10.5	0.154

Table 1.4: The means and standard deviations of the ratio of the seagrass amino 10 acid δ13C values as compared to the lab-fed shark δ13C for the essential and non-essential amino acids.

Chapter 2

Gut Microbial Diversity and Function Highlights Digestive Strategies of an Omnivorous Shark

Introduction:

Vertebrates host an assortment of gastrointestinal microbes that play crucial roles in their digestive physiology as well as in other aspects of their life history that contribute to their overall health (e.g., development, immune protection, behavior; Van Soest 1994; Stevens & Hume 1998; Ley et al. 2008; de Paula Silva et al. 2011; Nicholson et al. 2012; Clements et al. 2014; Egerton et al. 2018). This is true of organisms across trophic levels; however, organisms consuming plant material (herbivores and omnivores) have been shown to possess a greater abundance and diversity of microbes in their guts (Van Soest 1994 Bryant 1997; Mackie 1997; Ley 2008; Sullam et al. 2012; Clements et al. 2014). Many herbivores and omnivores rely on these microbes to assist with the digestion and assimilation of plant components (i.e., fiber, secondary metabolites), which cannot be processed endogenously (by the host). Plants are sheathed in fibrous cell walls, and as such, fermentative digestion by microbes is often critical to successful herbivory (e.g. Choat and Clements 1998; Karasov and Martinez del Rio 2007). Digestive strategies can be interpreted within the "Rate vs. Yield" theoretical framework (Sibly 1981; Clements & Raubenheimer 2006; German et al. 2015). One on end of the spectrum, "rate maximizers" tend to have high intake of low-quality food, rapid digesta transit rates, and little microbial fermentation occurring along the gut (Crossman et al. 2005; German, 2009; German and Bittong 2009; Clements et al. 2014; German et al. 2015; Clements et al. 2017). As such, rate-maximizers tend to assimilate easily digestible nutrients via

endogenous mechanisms and pass the rest as waste in their feces (Crossman et al. 2005; Clements and Raubenheimer 2006; German, 2009). Pandas are a prime examples of a rate maximizers that rely on high intake of bamboo, have rapid gut transit and low levels of microbial fermentation in their guts, and thus, overall low digestive efficiency (Dierenfield et al. 1982). Any shortcomings are made up by simply eating more (Stevens and Hume 1998). Grass carp (*Ctenopharyngodon idellus*) represent an aquatic example of a similar digestive strategy: they rely on high intake of low quality food rather than low intake of high quality food (Hao et al. 2016). At the opposite end of the spectrum, "yield maximizers" are usually represented by herbivores that tend to have measured intake, slower digesta transit rates, higher levels of microbial fermentation occurring in their guts, and higher overall digestibility (Hofmann 1989; Mountfort et al. 2002; Crossman et al. 2005; German et al. 2015). This allows organisms to access nutrients (i.e., fiber; Itoi et al. 2006; Sugita & Ito 2006) that might otherwise be indigestible via endogenous mechanisms. Carnivores tend to also fit within a yield-maximizing strategy since they also have relatively low rates of digesta transit due to their overall low intake of food (but they consume high-quality food). However, carnivores are generally thought to be less reliant on microbial fermentation to meet their energetic demands since protein can be endogenously digested (Stevens & Hume 1998; German 2009a; Karasov and Douglas 2013).

Recent studies of nutritional physiology have implemented chemical reactor theory (CRT), a concept historically used in chemical engineering, to study animal digestion. CRT involves the interaction of chemical reactants (in this case, substrates and enzymes) to a system in which biochemical reactions can occur (digestive tract) to

generate products (e.g., glucose, amino acids, fatty acids) that can be absorbed and used by the animal for growth (Penry and Jumars 1987; Horn and Messer 1992; Wolesensky and Logan 2006; Leigh et al. 2017). The various regions of the gut (e.g., stomach, proximal intestine, middle intestine, distal intestine) may each function differently in terms of CRT, which will affect transit time of material through the system (German 2011). In the context of the "Rate vs. Yield" framework, herbivorous "yield maximizers," who are more reliant on microbial fermentation, would benefit from increasing their digesta transit rate, increasing their gut size (both length and absorptive surface area), and increasing their production of enzymes that are specific to breaking down components of plant matter in order to optimize their digestive efficiency according to CRT. However, few studies have investigated the functional roles of the microbiome in the context of CRT, particularly for aquatic organisms.

There are a rapidly growing number of studies addressing the roles of the microbiota in terrestrial vertebrates (e.g. Ley 2008; Russel et al. 2009; Kohl et al. 2011; Zhao et al. 2013; Kohl and Dearing 2014); however, there are far fewer studies investigating this topic in fishes (e.g. Rimmer & Wiebe 1987; Nayak 2010; Givens et al. 2015; Egerton et al. 2018; Earley et al. 2018). Fishes represent the largest taxonomic group of vertebrates on the planet and thus, their impact on ecosystem functions around the globe are vast (e.g. Choat and Clements 1998; Karasov & Martinez del Rio 2007). In the limited studies focusing on the role of microbial symbionts in the guts of fishes, the majority of them target either species that are highly relevant to aquaculture (Clements and Choat 1995; Ringø et al. 1995; Harpaz & Uni 1999; Hovda et al. 2007; Zhou et al. 2009; Ringø et al. 2006; Nayak 2010; Estruch et al. 2015; Ringø et al. 2016) or

representative species from lower trophic levels (Rimmer et al. 1987; Mountfort et al. 2002; Moran et al. 2004; Fidopiastis et al. 2005; Clements et al. 2007; Wu et al. 2012; Hao et al. 2016) since such animals may be the most likely to be more reliant on microbial digestion to make a living. There are few studies focusing on the function of the gut microbiome in predatory fishes, such as sharks (Sullam et al. 2012; Givens et al. 2015; Freund 2019). Sharks play an important role in energy fluxes in marine environments (Buddington 1997; Bucking 2016; Leigh et al. 2017). Although carnivores are generally thought to rely less on their microbiota for digestive purposes, there is one species of shark that has been shown to potentially function as an omnivore. The bonnethead shark (*Sphyrna tiburo*; Fig. 1), has been previously shown to consume a diet consisting of up to 62% seagrass (*Thalassia testudinum*) by gut content mass (Bethea et al. 2007; Bethea et al. 2011). Furthermore, they have been shown to digest approximately 50% of the total organic matter in seagrass, as well as assimilate components of seagrass into their blood (Leigh et al. 2018b). Additionally, digestive enzymes that are used in the degradation of components of cellulose (i.e. cellobiose) have been found in bonnethead shark hindguts, indicating likely involvement from enteric microbes (Jhaveri et al. 2015; Leigh et al. 2018b). Bonnethead sharks have been previously identified to fit a yield-maximization strategy (Jhaveri et al. 2015), but there is limited information regarding the potential functional role that their gut microbiome plays in this digestive strategy.

In this study, we further explore the digestive mechanisms in the bonnethead shark. To do this, we took a multi-faceted approach. First, we measured the activity levels of β-glucosidase (BG) and N-acetyl-β-D-glucosaminidase (NAG) activities, which are indicative of the digestion of fibrous materials found in their diet (cellobiose from plant

material, and chitobiose from crustacean exoskeletons, respectively). Given the high volume of seagrass in their diet, it would be expected that the bonnethead shark would possess relatively high activity levels of enzymes specific to degrading components of cellulose. Second, we used histological imaging to investigate the absorptive surface area of the epithelial lining of their digestive tract. Bonnethead sharks possess a scroll intestine in the mid-region of their digestive tract that is thought to increase their absorptive surface area (Leigh et al. 2018a), but to date, this has not been quantified. Third, using gas chromatography, we measured the levels of short-chain fatty acids (SCFAs) in their spiral and distal intestine regions to confirm whether there were active fermentations occurring; a clear sign of microbial activity since SCFAs are the end products of microbial fermentation. Generally, omnivores and herbivores are known to have diverse and abundant microbial communities in their hindguts, and therefore, the bonnethead shark should have fermentation levels comparable to other plant-consuming organisms. Finally, we used 16S rDNA sequencing of their gut bacterial communities to identify possible OTUs that may be assisting the bonnethead shark with the digestion of seagrass material. Ultimately, this study represents one of the most rigorous investigations of a marine predator gut microbiome and its potential function to date and shows how important it is to combine research of microbial community composition with physiological and morphological data in order to better understand how microbes contribute to the physiological functions of the host.

Materials and Methods

Shark Collection and Tissue Preparation

Bonnethead sharks were caught off the coast of Layton, FL, on Long Key (24º50'2.6" N 80º48'32.2" W) and off the southwestern coast of Key Biscayne (25º41'05.9" N 80º10'41.0" 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 Florida International University (FIU) to undergo feeding trials (henceforth the 'laboratory-fed' sharks). 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 hrs. Each shark was fed a 90% seagrass, 10% squid (*Doryteuthis opalescens*) diet equaling 5% of their initial body weight daily for three weeks. Feedings were divided into three feeding events per day. Sharks were moved into nearby individual 946 L circular, closed-system, tanks during the day for feedings in order to ensure that all sharks received the appropriate amount of food. Since the smaller 946 L tanks were closed systems, the sharks were moved back into the larger (40,337 L) tank in the evening and overnight so that they could be exposed to fresh, flowing seawater and oxygen. The smaller 946 L tanks were drained and cleaned at the conclusion of each day and filled with fresh seawater the following morning to repeat the feeding process. 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 esophagus 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 distal intestine (DI; German 2009a; Leigh et al. 2018a). The length and mass of each individual gut region was measured. The gut contents were removed from each section by pushing along the intestine with the edge of a glass microscope slide, placed into a 1.5 mL microcentrifuge tube, and frozen on dry ice before storage at -80°C. The remaining tissue from each gut region was weighed and 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. The mucosal layer was scraped from the internal tissue of each intestine region using the edge of a glass microscope slide, placed into a 1.5 mL microcentrifuge tube, and frozen on dry ice before storage at -80°C. Further details about shark collection, husbandry, and tissue preparation can be found in Leigh et al. (2018b).

Digestive Enzyme Assays

Intestinal homogenates were produced as described by Leigh et al. (2018a; 2018b). In order to determine the activity of enzymes that digest chitin, we assayed N-acetyl-β-D-glucosaminidase (NAG) activity for all intestinal regions. All enzyme assays were carried out at 22ºC in duplicate or triplicate using a BioTek Synergy H1 Hybrid fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). 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 NAG with gut tissues from bonnethead sharks. Enzyme activity was measured in each subdivision of 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. NAG activities were measured following German et al. 2011 using 200 μM solutions of the substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, 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. Methods and results for additional enzymes are reported in Leigh et al. 2018b.

Histology

Upon removal from the body, the digestive tracts of each individual shark (both laboratory-fed and wild-caught) were gently removed and three 1-cm sections were excised from each of the proximal, spiral, and distal intestine and placed in their own individual vials containing fresh Trump's fixative, pH 7.5 (4% formaldehyde, 1% glutaraldehyde, in 10 mM sodium phosphate [monobasic] and 6.75 mM sodium hydroxide; McDowell and Trump 1976). These tissues were then allowed to fix for at least one week at 4ºC. Following fixation, the tissues were removed from the fixative and rinsed in 0.1 M phosphate buffered saline (PBS), pH 7.5, for 3 x 20 min, and a final rinse overnight at 4ºC. Following rinsing in PBS, the tissues were rinsed for 40 min in running DI water, and prepared following German (2009). Intestinal tissues were serially sectioned at 7 μm, stained in hematoxylin and eosin (Presnell and Schreibman 1997), and

photographed at 40X, 60X, and 120X with a Cannon EOS Rebel T6i digital camera attached to a Zeiss Axioskop2 plus light microscope. Image J analytical software (Abramoff et al. 2004) was used to measure the mucosal surface area of each gut region for both the laboratory-fed and wild-caught sharks (n=2 per intestinal region, per individual shark; 6 images per shark).

Microbial Fermentation

Measurements of symbiotic fermentation activity were based on the methods of Pryor and Bjorndal (2005), as described in German and Bittong (2009). Fermentation activity was indicated by relative concentrations of short-chain fatty acids (SCFA) in the fluid contents of the spiral and distal intestines of the sharks. As described above, spiral and distal intestine contents were frozen in sterile centrifuge vials. Gut content samples were weighed, thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4 ◦C) at 16,000 × g for 10 min. The supernatant was then pipetted into a sterile centrifuge vial equipped with a 0.22 μm cellulose acetate filter (Costar Spin-X gamma sterilized centrifuge tube filters; Coming, NY, USA) and centrifuged under refrigeration at 13,000 × g for 5 min to remove particles from the fluid (including bacterial cells). The filtrates were collected and frozen until they were analyzed for SCFA and nutrient concentrations.

Concentrations of SCFA in the gut fluid samples from SI and DI gut regions were measured using gas chromatography. Samples were hand-injected into an Agilent Technologies 7890A gas chromatograph system equipped with a flame ionization detector. Two microliters of each sample were injected onto a 2 m-long stainless steel column (3.2 mm ID) packed with 10% SP-1000 and 1% H3PO4 on 100/120 Chromosorb

W AW (Supelco, Inc., Bellefonte, PA, USA). An external standard containing 100 mg l:1 each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate was used for calibration. A 20% phosphoric acid solution was used to clear the column between samples, followed by rinses with nanopure water. The SCFA concentrations are expressed as mM of gut fluid.

Gut Microbiome Sample Processing

The sample DNA was isolated from the gut contents and musosal scrapings for all gut regions (PI, SI, and DI) for both the laboratory-fed and wild-caught sharks using the Zymobiomics DNA mini kit from Zymo Research. 16S rRNA amplicon PCR was performed targeting the V4 - V5 region using the EMP primers (515F [barcoded] and 926R; Caporaso 2012; Walters 2016). The library was sequenced at the UC Irvine Genomics High Throughput Facility using a miseq v3 chemistry with a PE300 sequencing length. Sequencing resulted in 24,085,008 reads passing filter. The raw sequences were imported into qiime2 (qiime2.org). After initial sample quality check and trimming (DADA2 in qiime2) there were 3,003,501 paired-end merged reads. From the sequences the first 5 bp were trimmed and the forward reads were truncated at 299 bp and the reverse reads were truncated at 242 bp. Both single-end and paired-end reads were evaluated, but only single-end read results are reported. The sequences were assigned a taxonomic classification using the September 2016 Ribosomal Database Project (RDP; rdp.cme.msu.edu), trained with the primer pairs that were used to amplify the 16S region. Sequences were confirmed using the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi). Analyses of the sequences were completed using qiime2 software and Rstudio (v.1.0.136).

Statistical Analysis

Comparisons of enzymatic activities were made among gut regions with analysis of variance (ANOVA) followed by a Tukey's honest significant difference with a family error rate of $p < 0.05$. Comparisons of enzymatic activities and SCFA concentrations 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 of SCFA concentrations between spiral and distal intestine regions were made in the same manner. Comparisons of intestinal epithelial surface area were completed using an ANCOVA (with body mass as a covariate as done by German et al. 2014 and Leigh et al. 2018a) followed by a Tukey's honest significance difference with a family error rate of $p < 0.05$ to compare among gut regions and an unpaired t-test with a Bonferroni-corrected error rate of $p < 0.006$ was used to compare laboratory-fed sharks to wild-caught sharks. All statistical tests described above were performed in R studio (v. 1.0.136). Alpha diversity (faith's phylogenetic diversity) significance was determined using a Kruskal-Wallis pairwise test ($p < 0.05$). Beta diversity (Bray-Curtis dissimilarity) significance was determined using a PERMANOVA ($p < 0.05$) with 999 permutations. All statistical tests used to analyze 16S rDNA sequencing results were run in qiime2.

Results

No NAG or β-glucosidase (BG) activity was detected in the PI for either shark group. Both BG and NAG activity levels were significantly higher in the distal intestine compared to other gut regions (PI and SI) for both laboratory-fed and wild-caught sharks ($p=0.014$; Fig. 1). Full results for BG are reported in Leigh et al. (2018b). There are no significant differences between laboratory-fed or wild-caught sharks in terms of their

mucosal epithelial surface area ($p > 0.006$; Fig. 2; Supplementary Fig. S1). Surface area in the SI (3,057cm^2 for lab-fed; 2,904cm^2 for wild-caught) was significantly higher than either the PI (1,402cm^2 for lab-fed; 1,009cm^2 for wild-caught) and DI (1,646cm^2; 1,416cm^2 for wild-caught) regions ($p=0.023$ and $p=0.031$ respectively; Supplementary Fig. S1). Total short-chain-fatty-acid measurements were 18mM/L (laboratory-fed SI), 10.8mM/L (laboratory-fed DI), 8.5mm/L (wild-caught SI), and 8.1mM/L (wild-caught DI; Fig. 3). For the SI and DI for both the laboratory-fed and wild-caught sharks, acetate was the most abundant SCFA (31.7% of total SCFA concentration for lab-fed SI, 28.9% for lab-fed DI, 35.9% for wild-caught SI, and 36.7% for wild-caught DI), followed by propionate (17.5% of total SCFA concentration for lab-fed SI, 16.9% for lab-fed DI, 21.8% for wild-caught SI, and 21.7% for lab-fed DI) and butyrate (14.5% of total SCFA concentration for lab-fed SI, 14.7% for lab-fed DI, 12.8% for wild-caught SI, and 12.2% for wild-caught DI; Supplementary Table S1). There were no significant differences between the laboratory-fed and wild-caught sharks in terms of their alpha (faith's phylogenetic diversity; $p=0.8$) and beta (Bray-Curtis dissimilarity; $p=0.6$) microbial diversity. The PI showed significantly lower microbial abundance when compared to both the SI and DI (permanova: $p=0.003$; Fig. 4). SI and DI showed no significant differences when compared to each other ($p=0.8$). The top ten most abundant OTUs present in the samples were *Photobacterium damselae*, *Closdridiaceae sp*., *Peptostreptococcaceae sp*., *Pseudomonas veronii*, *Photobacterium sp*., *Vibrio sp*., *Mycoplasma sp*., *Candidatus Heptoplama sp*., *Clostridium perfingens*, and *Phyllobacterium sp*. (Fig. 5; Table 1). The top five orders were Vibrionales, Clostridiales, Rhizobiales, Pseudomondales, and Mycoplasmatales (Table 1). A full list of the OTUs

identified and their occurrence in each gut region for each shark can be found in Supplemental Table S2.

Discussion

The results of this study show that the gut microbiome of the bonnethead shark is likely contributing to the digestion and assimilation of seagrass and chitinous material. The presence of BG and NAG in the distal intestine suggests that components of cellulose and chitin breakdown products (i.e., cellobiose and chitobiose, respectively) can be digested (Jhaveri et al. 2015; Leigh et al. 2018b), and these enzyme activities coincide with the microbial communities of the hindgut. The activity levels of BG in the bonnethead SI and DI are discussed in detail in Leigh et al. (2018b). Elevated levels of NAG in the distal intestine are consistent with previous studies on wild bonnethead sharks (Jhaveri et al. 2015) and suggest that an active microbial population in their hindguts may be aiding with chitin digestion as well, indicating some capacity to breakdown the chitinous exoskeletons of crustaceans. Although bonnetheads eat large amounts of seagrass (Bethea et al. 2007), most of their diet is still composed of crustaceans (crab, shrimp; Cortés et al. 1996), which have chitinous exoskeletons. Interestingly, with billions of metric tons produced annually, chitin is the most common biopolymer in the ocean (Souza et al. 2011), so observations that marine organisms can digest chitin (Alliot 1967; Danulat and Kausch 1984; Fange et al. 1979; Gutowska et al. 2004), even with the aid of microbial symbionts, isn't surprising.

High levels of SCFAs confirm the presence of anaerobic microbes, since SCFAs are the end products of microbial fermentation. In the laboratory-fed sharks, we found a total of 18mM of SCFAs per L of gut content fluid in the SI and 10.8 in the DI. This is

comparable to omnivorous, and in some cases even herbivorous, fish species. For example, German et al. (2015) found total SCFA levels of 11.68mM/L in a benthic, relatively sluggish, herbivorous species of prickleback fish (*Cebidichthys violaceus*). The same study by German et al. (2015) looked at a carnivorous prickleback species as well and found SCFA levels around 2.7mM/L. Clements and Choat (1995) revealed much higher concentrations (>40 mM) in the guts of herbivorous fishes from tropical environments, but still found relatively high concentrations in planktivorous species as well (>15mM in nasid surgeonfish). Additionally, German (2009b) and German et al. (2010) found that carnivorous species of minnows had SCFA concentrations of 16mM and 14mM respectively. In the wild-caught sharks, whose diets presumably consisted of less seagrass than our lab-fed ones, SCFA levels were 8-8.5mM/L, which is what you might expect in a carnivore, indicating that the higher levels of fermentation in the laboratory-fed individuals can likely be attributed to the larger concentrations of seagrass in their diet. Acetate, propionate, and butyrate, all of which are end products of carbohydrate catabolism via microbes, when combined, make up over half of the total fermentation product produced by microbes in the shark's intestine (Fig. 3). This is clear evidence of microbial fermentation occurring in order to digest various carbohydrate forms. However, the total SCFA levels of the laboratory-fed sharks in particular do not align with species of bony fishes that are known carbohydrate fermenters nor known protein-fermenters (Fig. 3). Instead, the sharks appear to have high levels of both carbohydrate and protein fermentation occurring, indicating their capacity to efficiently digest an omnivorous diet consisting of proteins and both soluble and non-soluble carbohydrates (Fig. 3).

The results of the 16S rDNA sequencing of the gut microbiome further confirm this claim. There were significant differences when the PI diversity and abundance was compared to the SI and DI (Fig. 4), indicating, as predicted by Chemical Reactor Theory, that the majority of microbial activity occurs in the hindgut. Vibrionales was the most common order present (specifically *Photobacterium damselae*, another *Photobacterium sp*., and a *Vibrio sp.*). Vibrionales in general are common in both fresh and salt water and several are pathogenic (Clements et al. 2014). Various Vibrionales sp. have been found throughout the gut of a carnivorous fish (cod; Egerton et al. 2018). Additionally, Vibrionales (specifically *Vibrio* and *Photobacterium*) accounted for 70% of sequence reads according to a meta-analysis of the gut communities of marine fish (Sullam et al. 2012). Strains of *Vibrio* specifically have been found to produce hydrolytic enzymes (amylase, lipase, cellulose, chitinase, and others) responsible for the breakdown of various dietary components (Hamid et al. 1979; Gatesoupe et al. 1997; Henderson and Millar 1998; Itoi et al. 2006; MacDonald et al. 1986; Ray et al. 2012). Overall, *Vibrio* and *Photobacterium* are commonly found in carnivores, while Closdridiales, the second most abundant order present in the guts of the bonnethead sharks, is linked to an herbivorous diet (Sullam et al. 2012). Closdridiales are another common member of the gut microbiome across species. They have been shown to provide numerous specific and essential functions related to gut maintenance (Clements et al. 2007; Sullam et al. 2015). *Clostridiaceae sp*., *Peptostreptococcaceae sp*., and *Clostridium perfringens* were among the top ten most common OTUs present in the bonnethead shark gut. *Clostridium perfringens* is a common resident of the animal digestive system. For instance, it has been shown to make up over 55% of the sequence reads for the clownfish (*Premnas*

biaculeatus; Parris et al. 2019). It is particularly associated with marine herbivorous fish species (Clements et al. 2007; Kim et al. 2007; Givens et al. 2015), but its exact function is unknown. Pseudomondales, the third most abundant order in the bonnethead shark gut, has been shown to increase in the guts of rainbow trout (*Oncorhynchus mykiss*) when levels of plant material are increased in the diet, although their exact role digestion is unknown (Michl et al. 2017). In the bonnethead sharks, the most abundant Pseudomondales OTU was *Pseudomonas veronii,* which has been associated with the degradation of numerous organic materials (Michl et al. 2017). Rhizobiales has been shown to be present in the guts of herbivorous ant species, while absent in carnivorous ant species (Stoll et al. 2007; Russell et al. 2009). Rhizobiales has also been found in zebrafish (*Danio rerio*; Earley et al. 2018) and have been associated with nitrogen fixation (Stoll et al. 2007; Russell et al. 2009). Finally, Mycoplasmatales have been found to make up a large proportion of the gut microbiota in numerous organisms, but their function has been explored mostly in mice models and has been associated with aiding in immune responses (Zhao et al. 2013). The fact that there were no significant differences between the laboratory-fed and wild-caught sharks shows that future studies can bring sharks into the lab, at least for short periods of time, without the risk of altering the microbiome significantly. We acknowledge that within each order we have discussed, there are far more specific OTUs that have various functions depending on their environment and that exact function can not be known with 16S rDNA sequencing alone. Using programs like PICRUSt, while beneficial in analyzing the functional role of microbes in human studies, are risky in unknown environments (Langille et al. 2013), like the guts of sharks. Future studies should incorporate metagenomics in order to

further understand microbial functions within the guts of the bonnethead shark specifically. However, the results presented here are a critical first step to beginning to classify and understand the gut microbiome in this unique shark species.

Overall, these results show that the gut microbiome of the bonnethead shark is likely involved in their ability to digest seagrass material. They possess the enzymes necessary to breakdown components of cellulose, they have increased surface area for maximum absorption in their spiral intestine (although this is likely true of all sharks), and they have microbial fermentation occurring in their spiral and distal intestines which coincides with some of the possible functions of the orders of taxa present in these gut regions. These results also further support that the bonnethead shark is taking a yield maximization strategy to digestion as an omnivore. Indeed, the sharks can digest ~52% of the neutral detergent fiber in seagrass (Leigh et al. 2018a), likely with assistance from their active gut microbiome. Furthermore, with the presence of their spiral intestine (which has a scroll shape; Leigh et al. 2017, 2018a), they have increased absorptive surface area and likely slow the rate of digesta transit in this gut region (Supplementary Table S3; Leigh et al. unpublished data). Overall, in the context of Chemical Reactor Theory, the bonnethead shark does have a digestive tract that is functionally well suited to efficiently possess large quantities of seagrass. In addition to being one of the most informative investigations of a shark gut microbiome to date, our results highlight the importance of combining studies of microbial community composition with an informed context of host ecology and physiology. This opens the door to investigating these topics in other fish species and other vertebrates in general so that we can better understand the complex relationship between microbe and host.

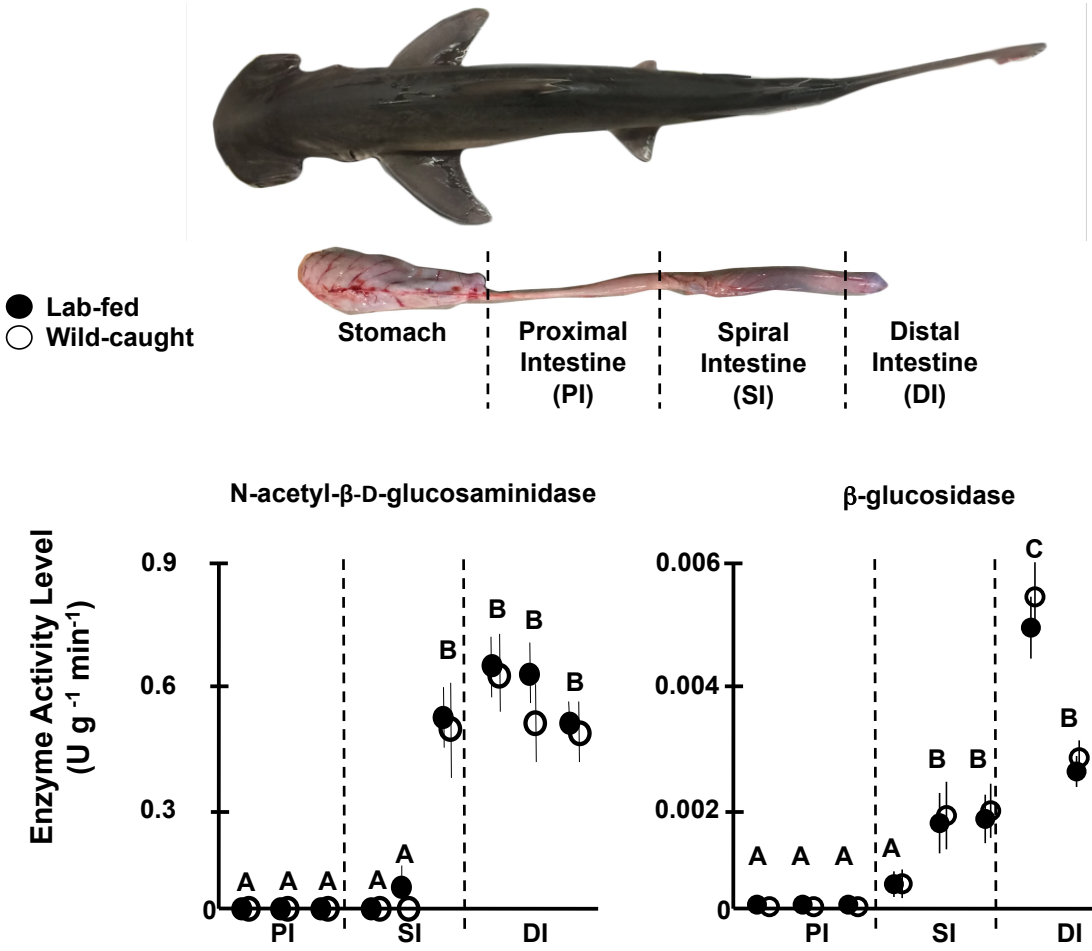

Figure 2.1: N-acetyl-β-D-glucosaminidase and β-glucosidase activities in the digestive tracts of bonnethead sharks. Open circles represent mean ± standard deviation values for wild-caught sharks, while filled circles represent laboratory-fed sharks. No significant differences were found between laboratory-fed and wild-caught sharks for any of the enzymes assayed ($p < 0.05$). Differing letters above data points indicate significant

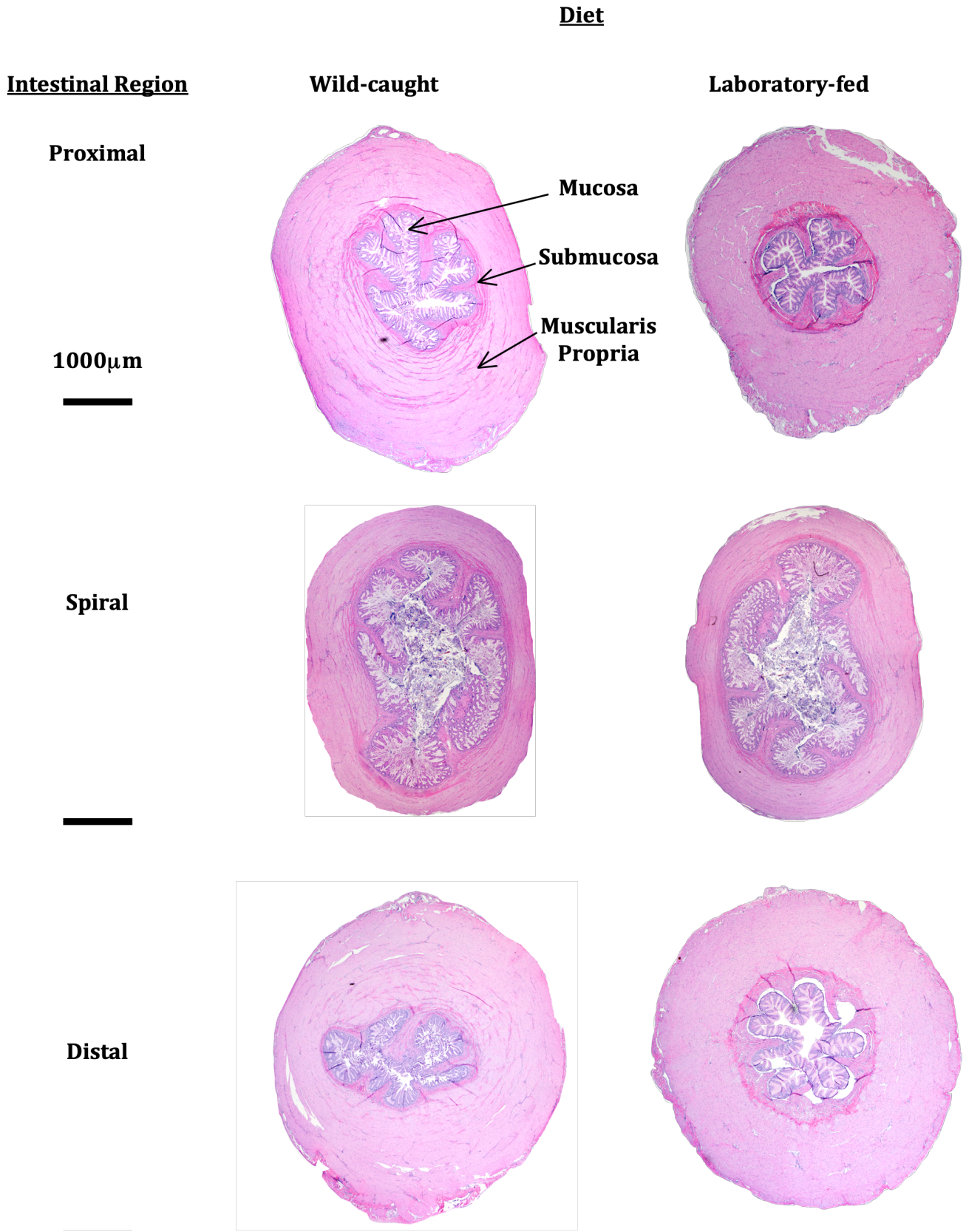

Figure 2.2: Histological cross-sections of proximal, spiral, and distal intestinal tissue of wild-caught and laboratory-fed sharks. Tissues were stained with hematoxylin and eosin. Scale bars are 1000 µm for each row of images. No significant differences between lab-fed and wild-caught for any gut region ($p>0.05$). SI has significantly larger intestinal surface area than PI and DI for both groups ($p<0.05$).

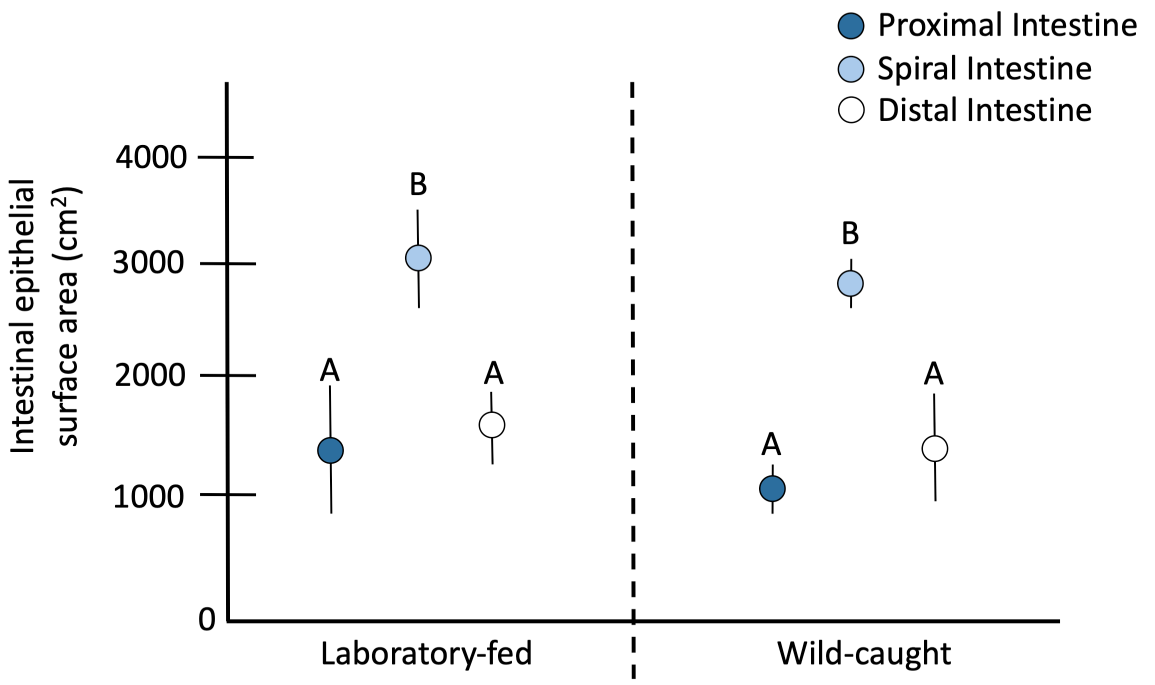

Figure 2.3: Mean ± standard deviation intestinal epithelial surface area for the PI, SI, and DI of laboratory-fed and wild-caught sharks. Letters above data points indicate differences among gut regions ($p < 0.05$).

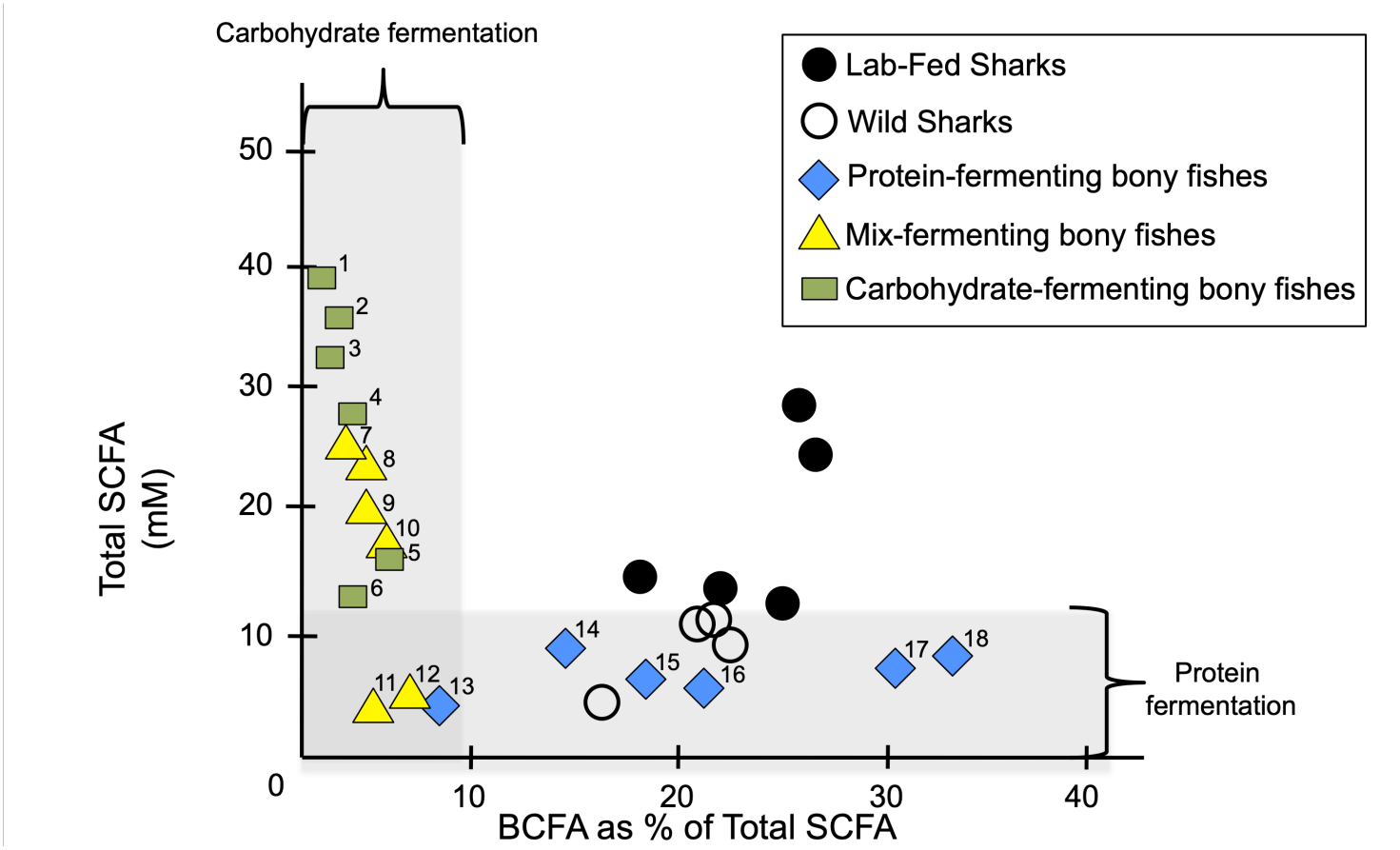

Figure 2.4: Total short-chain fatty acid (SCFA) vs. branched-chain fatty acids (isobutyrate and isovalerate summed) as a percentage of total SCFA. Black circles represent individual laboratory-fed sharks. Open circles represent individual wild-caught sharks. Diamonds, triangles, and rectangles represent data on protein-fermenting, mix-fermenting, and carbohydrate-fermenting bony fishes, respectively, from Clements et al. 2017 and Clements et al. 1995. 1) *Naso lituratus*, 2) *Naso unicornis*, 3) *Zebrasoma scopas*, 4) *Acanthurus nigricans*, 5) *Acanthurus nigrofuscus*, 6) *Acanthurus lineatus*, 7) *Naso vlamingii*, 8) *Naso hexacanthus*, 9) *Naso annulatus*, 10) *Naso brevirostris*, 11) *Abudefduf septemfasciatus*, 12) *Abudefduf sordidus*, 13) *Bolbometopon muricatum*, 14) *Scarus niger*, 15) *Chlorurus spilurus*, 16) *Scarus flavipectoralis*, 17) *Scarus schlegeli*, 18) *Scarus rivulatus*.

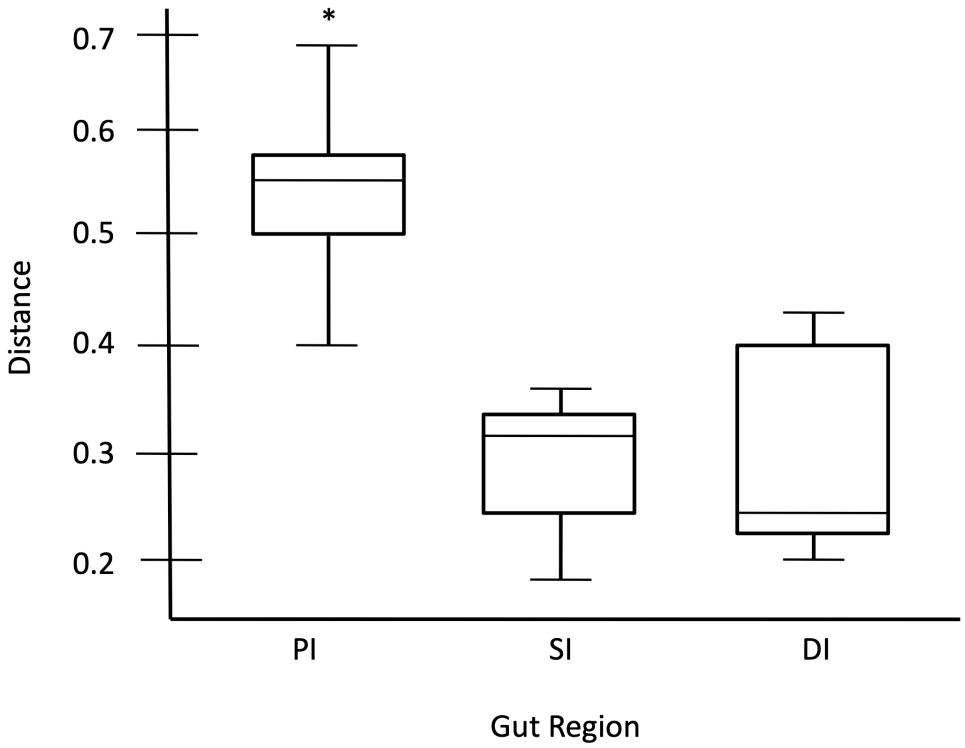

Figure 2.5: Bray-Curtis group significance plot depicting differences in microbial abundances between PI, SI, and DI gut regions. A distance of 0 indicates that all samples share the same species at the exact same abundances and a distance of 1 means that the samples have complete difference species abundances. *Indicates significance from a pairwise permanova ($p < 0.05$).

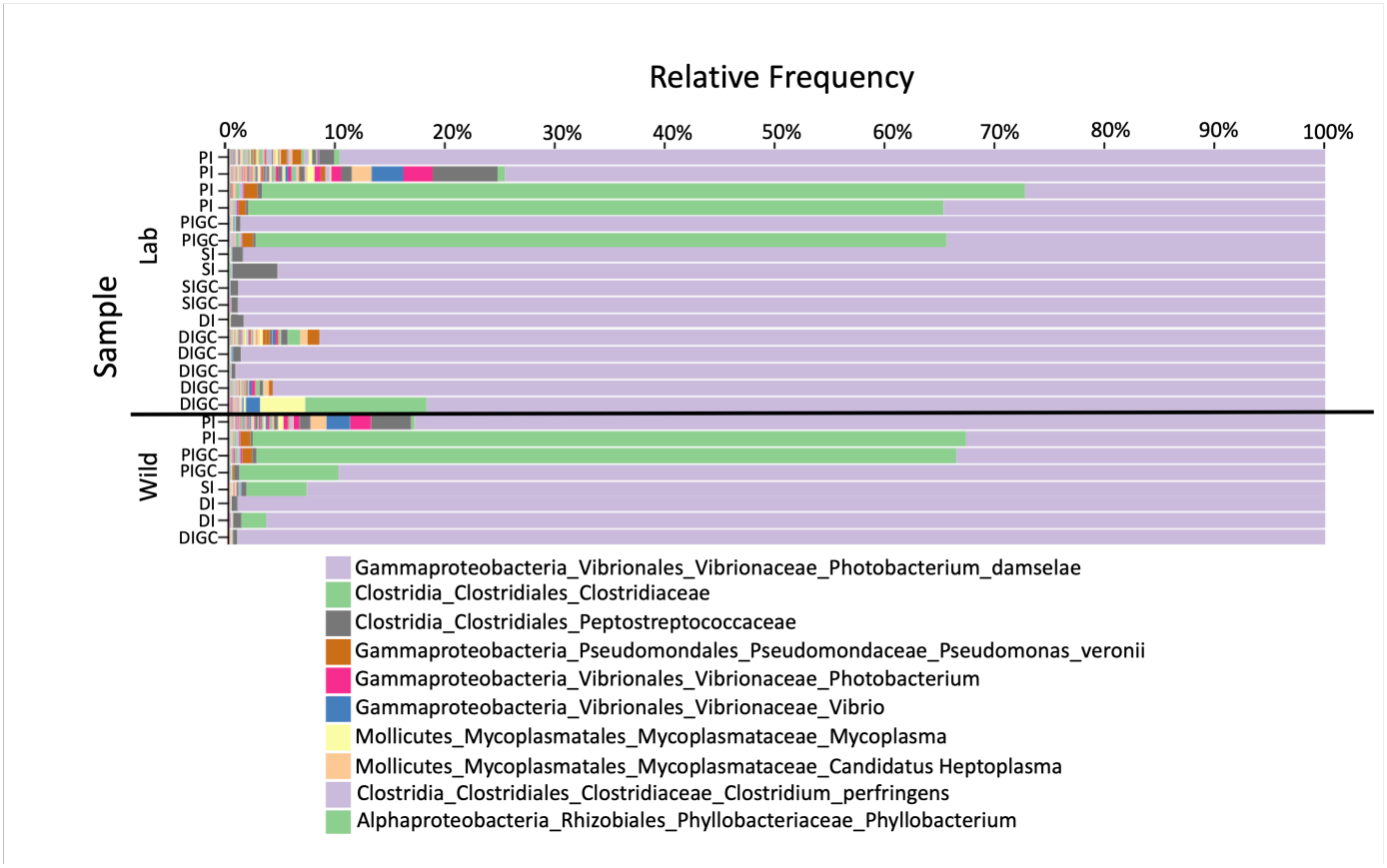

Figure 2.6: Taxonomy bar plot for PI, PIGC, SI, SIGC, DI, and DIGC gut regions and contents of both the laboratory-fed and wild-caught sharks depicting the relative frequency of each bacterial Operational Taxonomic Units (OTUs) detected from 16r DNA sequencing results. Only the top 10 OTUs are included in the legend.

	PI	PIGC	SI	SICG	DI	DIGC
Vibrionales	44.3	51.6	96.2	99.3	98.5	97.6
Clostridiales	54.5	47.4	3.62	0.687	1.40	1.42
Rhizobiales	0.222	0.220	0.113	0.015	0.014	0.318
Pseudomonadales	0.774	0.743	0.051	0.027	0.057	0.088
Mycoplasmatales	0.277	0.009	0.057	0.018	0.004	0.544

Table 2.1: The average percent abundance of the top five bacterial orders for each gut region and gut contents of each region. Since no significant differences were found between laboratory-fed and wild-caught sharks, the sequences were combined when determining average percent abundance.

	Total (mM/L)	% Acetate	% Propionate	% Butyrate
Lab-Fed SI	**18**	31.7	17.5	14.5
Lab-Fed DI	**10.8**	28.9	16.9	14.7
Wild SI	**8.5**	35.9	21.8	12.8
Wild DI	**8.1**	36.7	21.7	12.2

Table 2.2: Total short-chain fatty acid concentrations for the SI and DI of the laboratory-fed and wild-caught sharks. Percent concentrations included for acetate, propionate, and butyrate.

Operational Taxonomic Unit

k__Bacteria;p__;c__;o__;f__;g__;s__
k__Bacteria;p__Acidobacteria;c__AT-s2-57;o__;f__;g__;s__
k__Bacteria;p__Acidobacteria;c__Acidobacteria-6;o__CCU21;f__;g__;s__
k__Bacteria;p__Acidobacteria;c__Acidobacteriia;o__Acidobacteriales;f__Acidobacteriaceae;g__;s__
k__Bacteria;p__Acidobacteria;c__RB25;o__;f__;g__;s__
k__Bacteria;p__Acidobacteria;c__Sva0725;o__Sva0725;f__;g__;s__
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__AKIW874;g__;s__
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__C111;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae;g__Dietzia;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Frankiaceae;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Geodermatophilaceae;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Geodermatophilaceae;g__Blastococcus;s__aggregatus
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Intrasporangiaceae;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Kineosporiaceae;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Kineosporiaceae;g__Kineococcus;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Agrococcus;s__jenensis
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Curtobacterium;s__
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k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__;s__
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k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae;g__Actinomycetospora;s__

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Streptomycetaceae;g__Streptomyces;s__aculeolatus
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k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Gaiellales;f__;g__;s__
k__Bacteria;p__Actinobacteria;c__Thermoleophilia;o__Solirubrobacterales;f__;g__;s__
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k__Bacteria;p__Bacteroidetes;__;__;__;__;__
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k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Lutimonas;s__
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k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__;s__
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k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;__;__;__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__Crocosphaera;s__

k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__Cyanobacterium;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Xenococcaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Xenococcaceae;g__Xenococcus;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae;g__Geitlerinema;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;__;__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;__;__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;g__Synechococcus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;__;__;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Anoxybacillus;s__kestanbolensis
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__flexus
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__marisflavi
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Listeriaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Pasteuriaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Jeotgalicoccus;s__psychrophilus
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__[Exiguobacteraceae];g__Exiguobacterium;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Haloplasmatales;f__Haloplasmataceae;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Carnobacterium;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Trichococcus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Vagococcus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus;s__iners
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Lactococcus;s__

k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;s__infantis
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridiisalibacter;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__acetobutylicum
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__butyricum
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__neonatale
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__perfringens
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Natronincola_Anaerovirgula;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Oxobacter;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Garciella;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Gracilibacteraceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;s__producta
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Epulopiscium;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Tepidibacter;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus;s__bromii
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__SBYG_4172;g__;s__

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Acidaminobacteraceae];g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Acidaminobacteraceae];g__WH1-8;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__Dethiosulfatibacter;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Halanaerobiales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Halanaerobiales;f__Halobacteroidaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__OPB54;f__;g__;s__
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Propionigenium;s__
k__Bacteria;p__OP11;c__WCHB1-64;o__d153;f__;g__;s__
k__Bacteria;p__OP8;c__OP8_2;o__;f__;g__;s__
k__Bacteria;p__Planctomycetes;c__;o__;f__;g__;s__
k__Bacteria;p__Planctomycetes;c__Phycisphaerae;o__Phycisphaerales;f__;g__;s__
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__;s__
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Planctomycetales;f__Planctomycetaceae;g__Planctomyces;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Mycoplana;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Phenylobacterium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Kiloniellales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Aurantimonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Cohaesibacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Hyphomicrobium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__hispani
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__komaga

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Phyllobacterium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Shinella;s__granuli
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__Hirschia;s__baltica
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Nautella;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Paracoccus;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Paracoccus;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodovulum;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Roseovarius;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Azospirillum;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Inquilinus;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Anaplasmataceae;g__Neorickettsia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__Erythromicrobium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingobium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__az
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__ya
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Aquabacterium;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Pelomonas;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Rubrivivax;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Herbaspirillum;s__

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Janthinobacterium;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae;g__Hydrogenophilus;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfarculales;f__Desulfarculaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae;g__Desulfococcus;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobulbaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;f__Pelobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__NB1-j;__;__;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__NB1-j;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Spirobacillales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__[Chromatiaceae];g__Rheinheimera;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Chromatiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Ectothiorhodospiraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Citrobacter;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Salmonella;s__enteric
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__HOC36;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Endozoicimonaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter;s__lwoffii
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__alc
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__ver
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiohalorhabdales;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Piscirickettsiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Thiotrichaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Thiotrichales;f__Thiotrichaceae;g__B46;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium;s__damselae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium;s__rosenbergii
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Vibrio;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Lysobacter;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;s_
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__[Marinicellales];f__[Marinicellaceae];g__;s__
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__;s__
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__[Borreliales];f__[Borreliaceae];g__Spironema;s__
k__Bacteria;p__Tenericutes;c__;o__;f__;g__;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__;f__;g__;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae;g__;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae;g__Candidatus Hepatoplasma;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae;g__Mycoplasma;s__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RsaHF231;f__;g__;s__
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__Puniceicoccales;f__Puniceicoccaceae;g__Coraliomargarita;s__

Table 2.3: Complete list of all operational taxonomic units found within the digestive tract of the bonnethead shark.

Chapter 3

Spiraling Into Control:

The Function of the Spiral Intestine in Elasmobranchs

Introduction

Sharks have long been considered one of the most diverse groups of upper trophic level consumers in the ocean (Compagno 1984; Bucking 2016; Leigh et al. 2018). As such, they play a crucial role in the biodiversity of lower trophic levels that humans depend on for food and economic resources (Wetherbee et al. 1990). However, beyond prey capture methods and dietary analysis, the nutritional physiology of sharks is woefully understudied. Sharks consume a broad range of diet types (smaller sharks, marine mammals, teleosts, crustaceans, zooplankton, seagrass etc.; Leigh et al. 2018) and are also generally known for eating large meals on an infrequent basis, potentially going days, or even weeks, without a meal (Wetherbee et al. 1987; Cortes et al. 2008; Papastamatiou et al. 2015).

As most sharks are carnivorous with relatively low intake of food, they have relatively "short" guts that are equal to, or shorter than, their body lengths (Leigh et al. 2017). However, elasmobranchs (including sharks, skates, and rays) have spiral intestines (Fig. 3.1), which effectively expand the surface area and volume of the intestine over shorter lengths of the gut, allegedly increasing gut residence time and nutrient absorption, although there is little quantitative evidence of these claims (Holmgren and Nilsson 1999; Chatchavalvanich et al. 2006; Theodosiou et al. 2012; Jhaveri et al. 2015; Dezfuli et al. 2018; Leigh et al. 2019). The evolution of the spiral intestine itself is interesting when put in the context of the Adaptive Modulation Hypothesis (AMH; Karasov and Martínez del

Rio 2007). The AMH suggests that gut structure and function should largely match the dietary habits of an animal in order to optimize energy uptake. Based on economic principles, and the fact that the digestive tract is energetically expensive to maintain (Cant et al. 1996; Martine and Fuhrman 1995; Secor et al. 2012; Karasov and Douglas 2013), gut structure and function should line up with what is consumed in terms of quantity and biochemical composition. With the costs and limitations of maintaining the gut, the spiral intestine is a logical solution to thrive on infrequent meals observed in elasmobranchs, yet it is intriguing that this gut morphology is restricted to elasmobranchs and a few boney fishes amongst vertebrates (Leigh et al. 2017).

The spiral intestine is located posterior to the stomach and proximal intestine, and anterior to the rectum (Fig. 3.1). It consists of a varying number of intestinal tissue folds (2-50) and has been observed in four main morphological forms: columnar, scroll, funnels oriented posteriorly, and funnels oriented anteriorly (Fig. 3.2; Holmgren and Nilsson 1999; Wilson and Castro 2011). To date, these differing morphologies have been depicted in the literature using illustrations, photographs from dissections, or histological images; however, none of these methods provides an adequate means of analyzing the structures as they sit in the intestine, or the potential functionality of the different structures using 3-Dimensional models (Fig. 3.2; originally from Parker 1885, reproduced in Bertin 1958; Holmgren and Nilsson 1999; Wilson and Castro 2011).

Given that previously used techniques are damaging to the spiral structure, it is clear that a new method is needed in order to accurately depict and quantify the morphology of spiral intestines. Therefore, in this study, we investigated the morphology of the various spiral intestine shapes using a novel method: 3D reconstructions of

computerized tomography x-ray scan images (CT scans). We collected and scanned spiral intestines from at least one representative species for most families of known sharks (with a few exceptions; Table 3.1), generating functional images of these important structures. Based on the AMH, we would expect differing gut morphologies to coincide with dietary shifts. Hence, as a test of the AMH, we mapped the various spiral intestine structures onto a phylogenetic tree to gain insight into how the diverse spiral intestine structures may have evolved and whether the structures correlated with diet (Table 3.1, Fig. 3.3).

From a functional perspective, as we examined the scanned spiral intestines, it became apparent that the spiral intestine structures may passively (i.e., without any muscular contractions) affect digesta flow, favoring an anterior to posterior flow axis, and preventing back flow. That is, spiral intestines may act as natural Tesla valves, which prevent backflow using passive structures (Fig. 3.4.) To test this prediction, we measured flow rate through fixed spiral intestines of shark species that represent each of the four morphological forms (columnar, funnels pointed anteriorly, funnels pointed posteriorly, and scroll), showing that spiral intestines do in fact behave like Tesla valves. Finally, because spiral intestines actually function with muscular contractions, we quantified the contraction rate of the intestinal smooth muscle used to transport digesta through the columnar spiral intestine in *Squalus suckleyi* (Pacific Spiny Dogfish). Overall, this project seeks to understand the potential evolutionary trajectory of spiral intestine structures, and quantify the function of the spiral intestine by (1) comparing spiral intestine morphology across shark families and known diet types using 3D reconstructions of CT scans and (2) evaluating the movement of material through the

spiral intestines using quantifications of flow rate and intestinal muscle contraction rate. This study is the first to quantify spiral intestines in this manner and in this many taxa, thus providing insight on the evolution and function of this interesting gut structure.

Materials and Methods

Specimen Collections and CT Scanning

Spiral intestines were either dissected out from preserved shark specimens (in formalin) from the Natural History Museum of Los Angeles County (Table 3.2) or from previously frozen spiral intestines from donated shark specimens. To dissect out the spiral intestine, the ventral body cavity was opened using a razor blade (from anus to mouth) and cuts with dissecting scissors were made at the distal end of the proximal intestine and the anus. The spiral intestines from all specimens were flushed out with de-ionized water to remove any residual gut contents. They were then put through an ethanol series (30%, 50%, 70%). They stayed submerged in each ethanol concentration for a minimum of four hours and were stored in 70% ethanol. The intestines then underwent iodine staining for a minimum of four hours (using Lugol's Solution). After the stain, each intestine was tied off at one end with fishing line. They were then filled with 70% ethanol and tied off at the other end with fishing line. They were placed into individually labeled plastic 15mL or 50mL vials (depending on the size of the specimen) to be scanned.

CT scanning was done at two different facilities. One set was done at Friday Harbor Laboratories (University of Washington, Friday Harbor, WA, USA) to generate high-resolution images. These samples underwent lyophilization (Table 3.1). The lyophilizer (SP Scientific: VerTis, Warminster, PA) was set to -40°C for two hours prior

to use. The caps of the sample vials were loosened and then the vials were placed into the vacuum chamber of the lyophilizer. The vacuum pump was turned on and decreased the pressure in the chamber to 30 millitorr. The samples were left in the vacuum for a minimum of 12 hours to ensure complete freeze-drying. At the end of 12 hours, the samples were removed from the lyophilizer and kept dry in their individual vials until they could be prepped for the CT scanner. Each individual sample was removed from its vial and wrapped in dry cheesecloth. All of the individually cheesecloth-wrapped specimens were then wrapped together in more dry cheesecloth. This bundle was then placed into a plastic cylinder (size varied based on size of intestine sample) and packed so that no movement of the specimens could occur during the scan. The cylinder was wrapped with plastic wrap and then secured tightly inside the CT scanner (Bruker Skyscan 1173, Kontich, Belgium). The scanner had a 1mm filter type and x-ray detector resolution was 1120x1120 voxels (61.4μm pixels) and ran for 4 and a half hours. SkyScan1173 software was used to manage the scanning parameters. After the scanning was complete, the samples were removed and returned to their individual vials to be kept dry. The image files created by the scan were separated by species using Data Viewer and CT Vox. The files were converted in Dicom files and uploaded into Horos (version 1.1.7). Horos was used to create the 3D renderings of each of the spiral intestines. Each of the spiral intestine types (column, funnel (anterior), funnel (posterior) and scroll) were scanned using this lyophilization method. The remaining spiral intestines were immediately scanned once filled with 70% ethanol, which produced highly contrasted, useful images, at University of California (Irvine, CA, USA) using a Gamma Medica X-SPECT scanner (50kVp, 1000uA). The image files created by the scan were

reconstructed using exxim COBRA (2006 version). This latter set of scanned spiral intestines allowed us to identify the morphological type of each spiral intestine, but the images were not as high-quality as those produced at Friday Harbor Laboratories.

Flow Rate

A 50 L carboy was filled with 5L of de-ionized water. As a control, a clear plastic tube (15cm long, 0.75cm in diameter) was attached to the outflow valve of the carboy and a bucket was placed below the outflow. When the outflow valve was opened, time was recorded until 1L flowed completely through the clear plastic tubing. This was repeated five times. The plastic tubing was removed and replaced with the proximal and spiral intestines of *Squalus suckleyi*, *Centrophorus squamosus*, *Mustelus canis*, and *Sphyrna tiburo* (previously fixed overnight in 70% ethanol) were attached to the carboy individually. These species were chosen to represent each of the four spiral shapes (column, funnels pointed anteriorly, funnels pointed posteriorly, and scroll, respectively). They were attached (individually) onto the carboy outflow (so water would flow from the anterior end to the posterior end of each intestine), and the process was repeated five times for each intestinal section. Flow rate was initially recorded as liters per second and was converted in m^3s^{-1} in order to calculate resistance. Resistance was calculated as the change in pressure divided by flow rate ($R = \Delta P/Q$) (Mearin et al. 1990). P_1 was calculated as height of the water column (0.17m) multiplied by the density of the water (assumed to be $1000kg/m^3$) multiplied by the force of gravity ($9.8m/s^2$) ($P_1=h\rho g$). P_2 was determined to be zero since the height of the water column at P_2 was zero, and therefore, $\Delta P = 0.00166MPa$. Resistance ($MPa*s/m^3$) was calculated for the plastic control tube, as well as each proximal intestine and each spiral intestine. To examine whether flow was

impeded when moving from the posterior to the anterior end of the intestinal sections, the entire process was repeated with the posterior ends of both the proximal and spiral intestines attached to the carboy outflow so that the flow of water was moving from the posterior end to the anterior end of each intestinal section.

Intestinal Smooth Muscle Contractions

In order to examine the smooth muscle contractions of a spiral intestine, five *S. suckleyi* were collected alive from an otter trawl in Friday Harbor, WA. They were transported in live wells to Friday Harbor laboratories on San Juan Island where they were held in two large round tanks (one meter deep and two meters in diameter) with flow-through seawater systems (University of Washington IACUC #4239-03 to Adam Summers). Contraction experiments were performed only on the *S. suckleyi*. Each shark (n=5) was individually euthanized using 1% MS-222 in buffered (NaOH) seawater. The shark remained submerged in the MS-222 for 20 minutes to ensure death. Immediately after death, the ventral body cavity was opened and the proximal and spiral intestines were identified. Corn syrup with a known viscosity (20 poise) was mixed with green food coloring. A 16-gauge needle attached to a 3mL syringe was slowly filled to 1mL with the green corn syrup. The same step was repeated with corn syrup mixed with blue food coloring. The corn syrup with the green food coloring was injected into the lumen of the anterior proximal intestine. The corn syrup with blue food coloring was injected into the lumen of the anterior spiral intestine. 3mL of 1M acetylcholine in saline solution (containing 102.7mM NaCl, 1.61 mM KCl, 1.36mM $CaCl_2$, and 1.19mM $NaHCO_3$) was injected into the smooth muscle layer of both the anterior proximal and anterior spiral intestine using a 21-gauge needle (Jensen and Holmgren 1985; Kitazawa et al. 1990). A

timer was started upon the injection of the acetylcholine and a camera (Panasonic lumix DMC-FZ200) recorded the contractions of the intestines until the food coloring previously injected into the spiral intestine lumen began to emerge from the colon. At this time, the timer was stopped. Throughout this process, seawater was dripped onto the exterior of the intestines using a transfer pipette until the intestines ceased to contract. The video was used to calculate the average number of contractions that occurred per minute, the average length of time (s) that a single contraction took to occur, and to confirm the total time for the dye to pass through the entire length of the spiral intestine. This information was then used to determine the average number of contractions required to move the corn syrup from the anterior of the intestine to the colon. However, the proximal intestine did not contract in response to acetylcholine and therefore the material injected into the proximal intestine never moved through the intestine to the colon. As such, the average number of contractions required to move corn syrup through the intestine was calculated for the spiral intestine only.

Statistical Analyses

A phylogenetic generalized least squares (PGLS) test was performed to determine phylogenetic relationships of shark species with respect to their spiral intestine morphology. Correlations between diet and spiral intestine type were determined using a logistic regression test (diets were reduced to numerical categories, i.e. primarily bony fish diet = 1). Comparisons of flow rate were made between proximal and spiral intestines (anteriorly to posteriorly only) for each species using paired t-tests with a Bonferroni-corrected error rate of $p=0.004$. Comparisons of flow rate among the proximal intestine, the spiral intestine with flow anteriorly to posteriorly, and the spiral

intestine posteriorly to anteriorly were made using an ANOVA ($p<0.05$). All statistical analyses were run in R (1.1.383).

Results

A full list of the shark species analyzed, their spiral intestine morphology types, and their diets can be found in Table 3.1. There is not a significant correlation between diet type and spiral intestine morphology ($p < 0.05$). For families with multiple species included in the analyses, different species within a single family tended to have the same spiral intestine morphology; however, different families within a single order can have differing spiral intestine morphologies among the families (Fig. 3.3). Generally speaking, the columnar spiral intestine morphology seems to be the ancestral condition, but the evolution of the other morphologies doesn't appear to follow any specific sequence of one spiral morphology (e.g., column) preceding the other types (Fig. 3.3). Volumetric flow rate (m^3/s) was compared to resistance ($MPa*s/m^3$) for all four species. Higher resistance led to significantly slower volumetric flow rate (Fig. 3.5). The spiral intestines for all species exhibited a significantly higher resistance and slower volumetric flow rate than the proximal intestines or control tubing ($p < 0.01$). The control tubing flow rate and resistance was not significantly different from any of the proximal intestines ($p > 0.05$), showing that the proximal intestine functions as a bore tube. When the flow rate of the proximal intestine is compared to the spiral intestine as a ratio, it is found that the spiral intestine has a flow rate three and a half times slower than the proximal intestine. When flow rate is compared between the spiral intestines oriented anteriorly/posteriorly to spirals oriented posteriorly/anteriorly it was found that flow rate was slower and resistance was higher in the spirals oriented posteriorly/anteriorly (Fig. 3.5). In other

words, there was less resistance to flow in the anterior to posterior direction, showing that the structures of the spiral intestine passively engender anterior to posterior flow. The average number of contractions that occurred per minute for *S. suckleyi* was 0.74 (±0.33). The average amount of time necessary for the dyed corn syrup to move from the anterior end of the spiral intestine to the posterior end was 35.65 minutes (±13). This was used to calculate the average number of contractions necessary to transport the dyed corn syrup through the spiral intestine, which was found to be 48.17 contractions (±3.9). The proximal intestine never contracted upon injection of acetylcholine, and never moved the dyed corn syrup through to the spiral intestine. However, upon dissection after the experiments, green dye was found in the esophagus of *S. suckleyi*, indicating that the proximal intestine is subject to back-flow of digesta material. Previous histological data (Leigh, Chapter 2) of the proximal, spiral, and distal intestines reveals that the muscular layer of the spiral intestine is significantly thinner than either the proximal or distal intestines ($p<0.05$), perhaps contributing to this result.

Discussion

This investigation produced the first 3D images of spiral intestines, which is important because two-dimensional histological images and sketches are what we have had to work with for over 130 years (e.g., Parker 1885; Wilson and Castro 2011; Theodosiou et al. 2012; Dezfuli et al. 2018). We can now confirm the spiral intestine structures for species within the majority of the existing shark families. However, families within a single order do have differing spiral intestine structures. Also, there is no clear correlation between shark diet types and spiral intestine morphology. For example, *Sphyrna tiburo* has a scroll intestine and consumes a diet consisting of up to

62% (by gut content mass) of seagrass material, along with crustaceans, cephalopods, and small bony fishes (Cortés et al. 1996; Bethea et al. 2007). However, the closely related *Sphyrna zygaena* also has a scroll intestine, but consumes smaller elasmobranchs, a variety of bony fishes, and various invertebrates (e.g. Smale and Cliff 1998; Table 3.1). This same trend occurred throughout various families of sharks containing multiple species (Figure 3.3). While the most basal groups (those that arose prior to Selachimorpha) all possess the columnar spiral intestine shape, there does not appear to be any phylogenetic reason for why different families of sharks evolved different spiral intestine shapes according to the PGLS analysis (Figure 3.3). Thus, a potential functional reason for the differing spiral shapes remains a possibility. Nevertheless, by mapping the various spiral structures onto the phylogentic tree, assuming the tree is correct, we can qualitatively see the general order in which the different structures evolved, beginning with the columnar morphology. After the columnar shape, the scroll intestine becomes present within the family Hexanchidae. Next, we see the funnels pointed posteriorly arise in Etmopteridae, followed by the funnels pointed anteriorly arising in Somniosidae. However, we still see the columnar and scroll intestine morphologies in some of the most derived orders, such as the Carcharhiniformes, again indicating that structure may play an important functional role rather than following linear evolutionary changes. For instance, greater resistance, and thus, slower flow rates along the posterior to anterior axis, is more likely to occur for all spiral morphologies as digesta viscosity is increased. However, at lower viscosities, only the funnel morphologies showed significantly slower flow rates along the posterior to anterior axis in comparison to the anterior to posterior axis (Fig. 3.5), thus suggesting that families with these funnel morphologies may have lower

digesta viscosities, perhaps relating to water absorption in the spiral intestine (Theodosiou and Simeone 2012). There are also some non-elasmobranch fishes that possess a spiral intestine such as Acipenseridae (sturgeon; Buddington and Doroshov 1986), Dipnoi (lungfish; Argyriou et al. 2016), and Lepisosteidae (gar; Frías-Quintana et al. 2015) indicating that the spiral intestine is a characteristic that appeared early in the evolution of vertebrates, but also that it has independently evolved for various fish species (Kikugawa et al. 2004). The roles of these structures in the digestive process should be explored further.

Investigating the genes involved in spiral intestine development may also be crucial in understanding how the different morphologies evolved. For example, roles in gut patterning and subsequent intestinal epithelial and smooth muscle differentiation have been identified for *Hox* genes in *Danio rerio* (zebrafish; Jiang et al. 2015). Interestingly, genes *Hoxa13* and *Hoxd13* implicate posterior *Hox* gene function during development of the skate spiral intestine (Theodosiou and Simeone 2012; Theodosiou et al. 2007; Warot et al. 1997). Future investigations should focus on determining if mutations to these genes or shifts in their expression patterns during the developmental process can lead to changes in the morphological development of the spiral intestine in sharks. Perhaps simply changing the timing of expression of some genes leads to subtle changes that result in the different spiral intestine morphologies.

In addition to providing evolutionary insights, the 3D images we generated allow us to visualize the actual structure of the tissue folds in the spiral intestine and compare the morphology between species without needing to physically cut it open, which damages the structure. These images can also be used to quantify the number of intestinal

folds, the volume of the lumen, and the surface area of tissue provided by the spiral shape that may lead to increased levels of nutrient absorption. These 3D renderings also allow us to visualize how flow may occur through the spiral intestine For instance, the spiral intestine of *S. suckleyi* and others appears to have a central lumen (separate from the spirals), meaning that digesta could pass directly through and bypass the spirals, or travel through the spirals to allow more time for nutrient absorption. The topic of a central lumen in a spiral intestine has not been discussed in the literature, showing how these 3D renderings can provide insight to the morphology. It was these observations that prompted us to test whether the spiral intestines could function as natural Tesla valves (Cieri and Farmer 2016). A Tesla Valve allows fluid to move unidirectionally, without any moving parts (Nobakht et al. 2013). The general idea is that currents flow along different paths, in different directions, and that these differences have a disproportionate effect on the resistance of the tube (Figure 3.4). The spiral intestine may be working in a similar fashion, which would allow segmental contractions to better mix digesta in the SI without the risk of much backflow. Spiral intestines evolved approximately 450 million years ago (e.g. Williams 1972) (before the existence of insects, mammals, birds, etc.), suggesting that it is a successful structure in the digestive process. Hence, spiral intestine morphologies should be explored further as mechanisms to produce one-way flow without the use of mechanical parts or energy. While we cannot know for sure what spiral intestine morphology was like 450 million years ago, there is evidence of fossilized columnar shaped coprolites (e.g. Williams 1972), agreeing with our phylogenetic analysis that the columnar shape may be the ancestral phenotype (Fig. 3.3).

We also provide the first quantitative analysis of the functional flow rate and contraction rate of the spiral intestine (SI) in a shark. The flow rate results also provide support for the idea that the spiral intestine is acting similarly to a Tesla Valve, We have established, quantitatively, that flow rate is slowed in the spiral intestine due to the high resistance produced by the tissue folds. Additionally, the flow rate was significantly slowed further when the two funnel-shaped spiral intestines (anterior and posterior funnels) were subjected to flow in the posterior to anterior direction. This indicates that at least the two funnel shaped spiral intestines are capable of producing unidirectional flow, similarly to a Tesla Valve design. The proximal intestine (PI), which is a relatively straight lumen lacking in any additional internal tissue, provided very little resistance to flow. The PI also did not produce contractions when stimulated with acetylcholine while the SI produced an average of 48.17 contractions per minute. This indicates that digesta can flow freely through the PI and needs to be pushed through the SI via contractions, and the spiral morphology prevents back flow. This type of quantitative data could explain why digesta transit rates vary among species and among different SI structures (Aedo and Arancibia 2001; Bush and Holland 2002; Papastamatiou and Lowe 2004; Papastamatiou et al. 2007). It is time to start investigating these unique intestinal structures as a key component to the digestive success of sharks. Until now, very little was known about their functional morphology. The new techniques produced by this project lays the groundwork for future investigations involving the spiral intestine, and for understanding the functional role of the digestive tract in vertebrates in general.

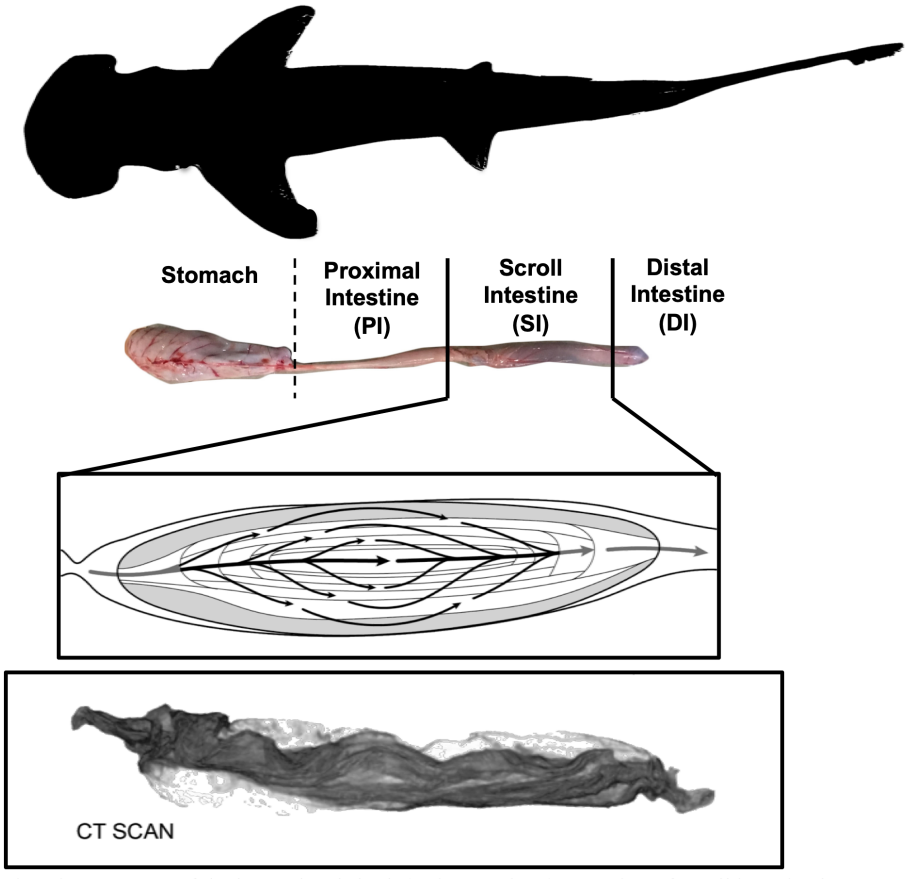

Figure 3.1: Digestive anatomy of the bonnethead shark (*Sphyrna tiburo*). Drawing of scroll intestine by A. Dingeldein. CT scan reconstruction of scroll intestine by SC Leigh.

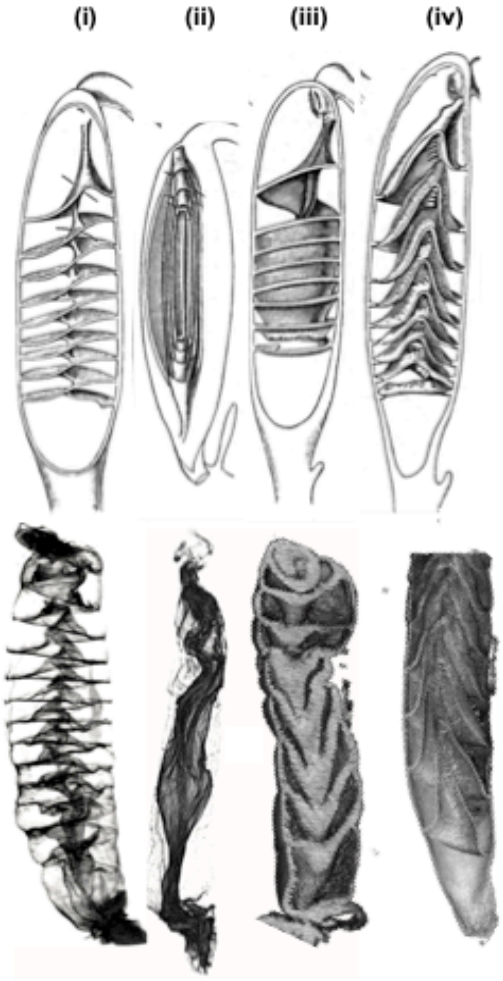

Figure 3.2: The four spiral intestine structures: (i) columnar, (ii) scroll, (iii) funnels pointed posteriorly, and (iv) funnels pointed anteriorly. Sketches adapted from Parker 1885. CT scans by SC Leigh.

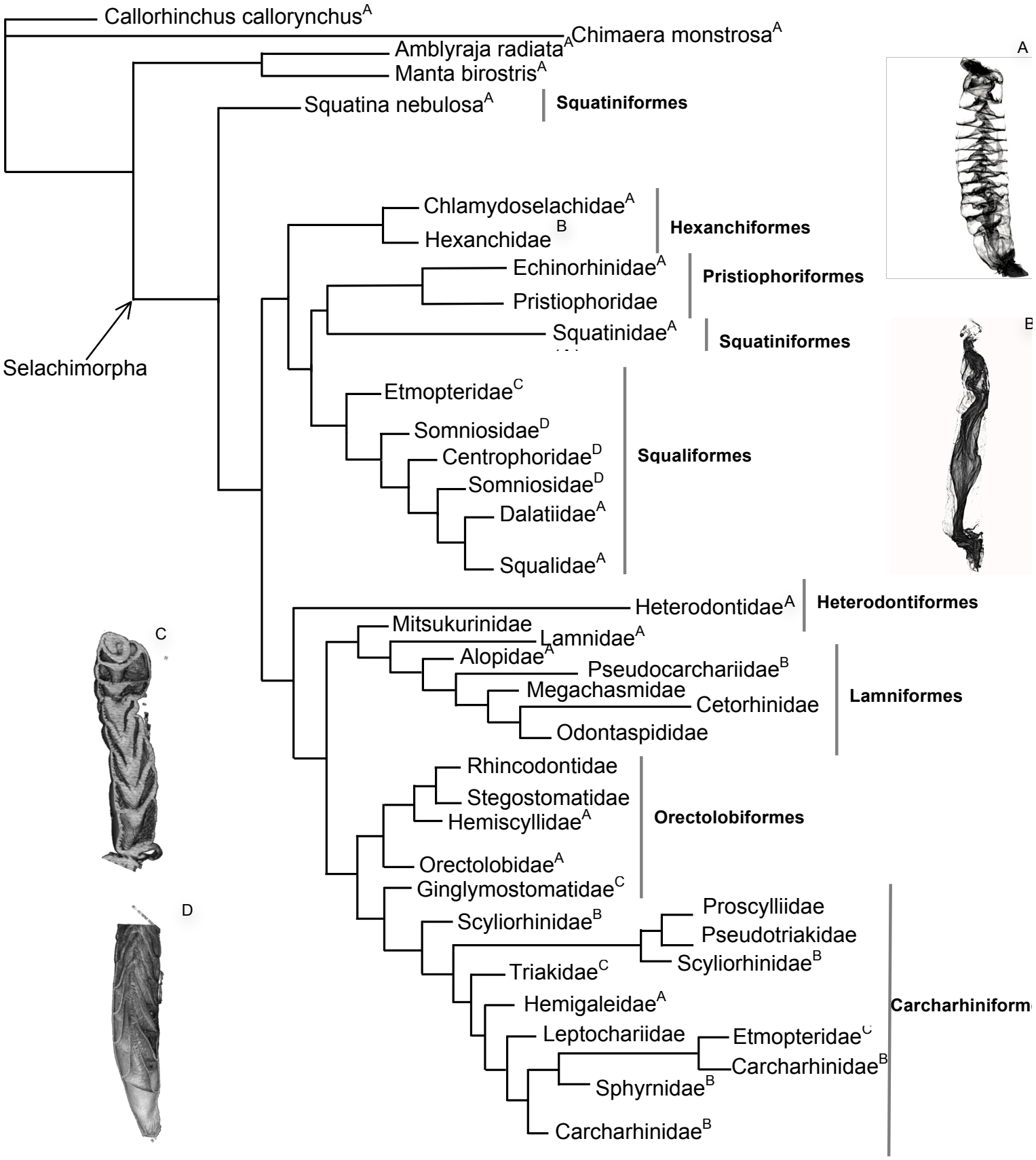

Figure 3.3: Phylogeny of sharks to the family level based on the tree from Vélez-Zuazo & Agnarsson (2011). Adapted from Leigh et al. (2018). Depicts which spiral intestine morphology corresponds to each family. Light gray lines show which families belong to certain orders. A) column, B) scroll, C) funnels pointed posteriorly, and D) funnels pointed anteriorly. Most basal categories (prior to Selachimorpha) do not have CT scans. Information about their spiral intestine structure came from previous literature.

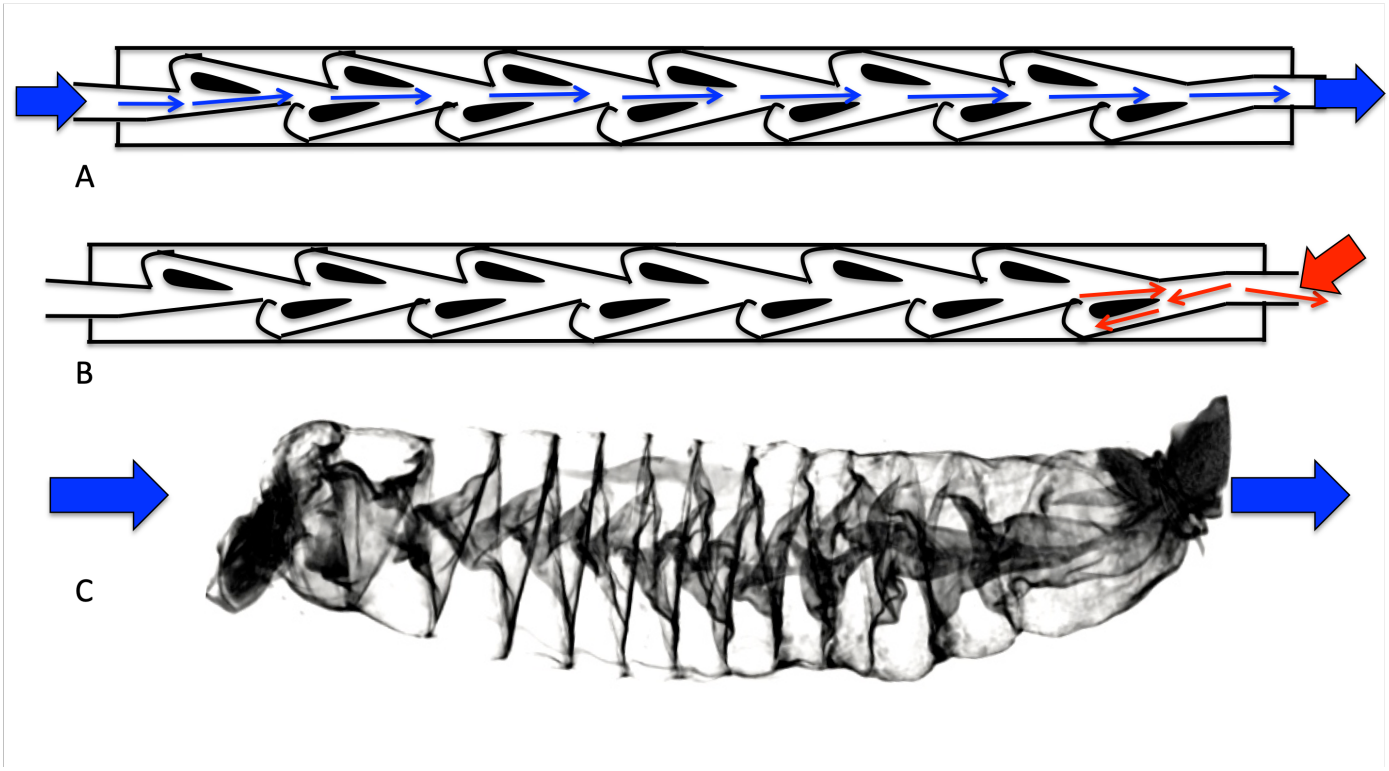

Figure 3.4: A Tesla valve (A and B) produces unidirectional flow without the use of mechanical parts. A spiral intestine (C) appears to have a similar structure.

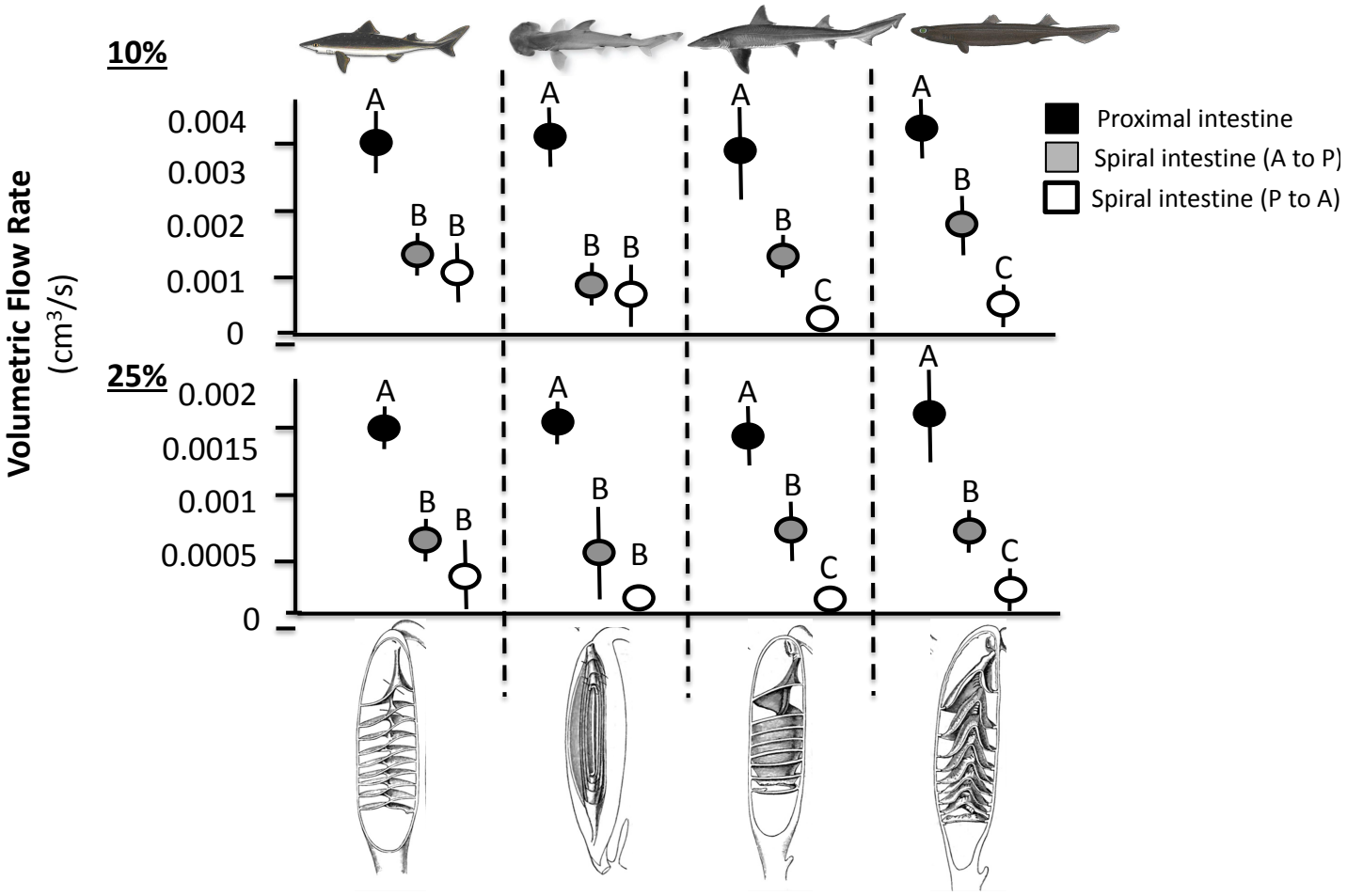

Figure 3.5: Average volumetric flow rate in the proximal and spiral intestine (both oriented anteriorly to posteriorly and posteriorly to anteriorly) for *Squalus suckleyi*, *Sphyrna tiburo*, *Mustelus canis*, and *Centrophorus squamosus*, and using a 10% glycerol solution and 25% glycerol solution. Letters above data points signify significance among intestine sample type for each species (ANOVA, $p<0.05$).

Table 3.1: Families, species, spiral intestine shape, and diet type for all samples CT scanned. *= Lyophilized prior to scanning.

Family	Species	Spiral Intestine Shape	Diet
Alopiidae	Alopias vulpinus	Column	Schooling bony fishes (i.e. menhaden, anchovies)
Carcharhinidae	Carcharhinus leucas	Scroll	Bony fishes (i.e. skipjack tuna), small elasmobranchs cephalopods, turtles
	Carcharhinus melanopterus	Scroll	Small bony fishes (i.e. mullet, groupers)
	Carcharhinus amblyrhynchos	Scroll	Small bony fishes, small elasmobranchs, cephalopods, crustaceans
	Carcharinus plumeus	Scroll	Small bony fishes, small elasmobranchs, cephalopods, crustaceans
	Carcharinus taurus	Scroll	Small bony fishes, small elasmobranchs, cephalopods, crustaceans
Centrophoridae	Centrophorus granulosus*	Funnels (anterior)	Bony fishes (i.e. mackerels), cephalopods
	Deania calcea	Funnels (anterior)	Bony fishes (i.e. lanternfishes), cephalopods
Chlamydoselachidae	Chlamydoselachus anguineus	Column	Small elasmobranchs, cephalopods, bony fishes
Dalatiidae	Squaliolus laticaudus	Column	Cephalopods, shrimp, bony fishes (i.e. lanternfishes)
Echinorhinidae	Echinorhinus cookei	Column	Small elasmobranchs, bony fishes, cephalopods
Etmopteridae	Centroscyllium nigrum	Funnels (posterior)	Bony fishes, variety of invertebrates
Ginglymostomatidae	Ginglymostoma cirratum	Funnels (posterior)	Mollusks, crustaceans, bony fishes
Hemigaleidae	Chaenogaleus macrostoma	Column	Small bony fishes, cephalopods, crustaceans
Hemiscyllidae	Hemiscyllium ocellatum	Column	Polychaete worms, crustaceans
Heterodontidae	Heterodontus francisci	Column	Benthic invertebrates, small bony fishes
Hexanchidae	Notorynchus cepedianus	Scroll	Elasmobranchs, marine mammals, bony fishes
Lamnidae	Isurus oxyrinchus	Column	Bony fishes (i.e. tuna), elasmobranchs, cephalopods
Orectolobidae	Orectolobus maculatus	Column	Crustaceans, cephalopods, bony fishes
Pseudocarchariidae	Pseudocarcharias kamoharai	Scroll	Cephalopods, crustaceans, small bony fishes
Scyliorhinidae	Apristurus brunneus	Scroll	Bony fishes (i.e. lanternfishes), crustaceans
	Cephaloscyllium ventrosum*	Scroll	Benthic mollucks, crustaceans, small bony fishes
Somniosidae	Somniosus pacificus	Funnels (anterior)	Bony fishes, cephalopods, crustaceans, carrion
Sphyrnidae	Sphyrna lewini*	Scroll	Bony fishes (i.e. sardines), variety of invertebrates
	Sphyrna tiburo*	Scroll	Cephalopods, crustaceans, seagrass
	Sphyrna zygaena	Scroll	Small elasmobranchs, bony fishes, invertebrates
Squalidae	Squalus acanthias	Column	Bony fishes (i.e. jack mackerel), cephalopods
	Squalus suckleyi*	Column	Bony fishes (i.e. herring), invertebrates
Squatinidae	Squatina dumeril	Column	Mollusks, crustaceans, fishes (i.e. flounders/stingrays)
Stegostomatidae	Stegostoma fasciatum	Column	Mollusks, small bony fishes
Triakidae	Mustelus canis*	Funnels (posterior)	Crustaceans, polychaetes, mollusks
	Triakis semifasciata	Funnels (posterior)	Variety of benthic invertebrates, small bony fishes

Table 3.2: Sample names and ID#s from the Natural History Museum of Los Angeles.

Sample Name	Sample ID#
Aliopias vulpinus	36227-1
Ginglymostoma cirratum	9045-6
Hemiscyllium ocellatum	39985-36
Apristurus brunneus	39985-36
Cephaloscyllium ventrosum	24508
Galeus area	42328-1
Carcharhinus nesiotes	1948
Carcharhinus melanopterus	54163-1
Sphyrna lewini	36277-5
Sphyrna zygaena	9500-1
Squalus acanthias	23100
Centroscyllium nigrum	11156-1
Notorynchus cepedianus	42298-1
Heterodontus francisci	45685-1
Chlamydoselachus anguineus	43793-1
Squaliolus laticaudus	36021-1
Squatina californica	57653-1
Echinorhinus cookei	33827-31
Deania rostrata	42154-1
Isurus oxychinus	30830-2
Somniosus pacificus	39568-1
Stegostoma fasciatum	38125-2
Chaenogaleus macrostoma	38145-2
Orectolobus maculatus	42624-16
Pseudocarcharias kamoharai	45857-1

DISSCUSSION

The chapters of this dissertation represent one of the most conclusive investigations into the nutritional physiology of sharks to date. I have provided 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. I have also provided one of the most informative investigations of a shark gut microbiome to date, and my results highlight the importance of combining studies of microbial community composition with an informed context of host ecology and physiology. This opens the door to investigating these topics in other fish species and other vertebrates in general so that we can better understand the complex relationship between microbe and host. Finally, I show how new methodology can be used to investigate the digestive morphology and function of sharks and fishes in general. I provide qualitative data using CT scan images to understand how the unique spiral structures evolved and how they correlate with diet type, as well as quantitative data with respect to the flow rate of digesta through the spiral intestine as well as information on the contraction rate of the smooth intestinal muscle.

Few studies have explored the role of digestive enzymes in the guts of sharks. There is much to be learned from identifying and classifying the enzymes in each region of the digestive tract, or what enzymes are even present in shark genomes (e.g., Castro et al. 2014; Venkatesh et al. 2014). Such information can be used to pinpoint exactly which nutrients are being used by sharks and where their breakdown is occurring within the gut. This may also provide insight about shark vitamin and mineral requirements. It has been assumed that their needs are similar to other vertebrates (iron, calcium, B vitamins, lipid

soluble vitamins, etc.) but this has yet to be explored in a shark species (Halver 2002; Teles 2012). Many of the techniques to measure enzymatic activity are already being used to explore the digestive physiology of other organisms, and therefore, the methodology could be readily applied to sharks as well (German 2011), especially using incidental mortalities from survey work (e.g., Bethea et al. 2007; Jhaveri et al. 2015; Newton et al. 2015). Understanding which enzymes are of microbial origin versus endogenously derived would also aid in developing the field of digestive physiology from a biochemical standpoint. Since microbiomes are prone to change based on the surrounding environment and individual physiology, it is likely that sharks, particularly migratory sharks, have access to variable sources of exogenous enzymatic activity and nutrient input at varying points throughout their lifetime. This could greatly impact their digestive success and food choices as they develop. As for endogenous enzyme production, exploration in gene expression (transcriptomics) would provide insight to which genes activate the secretion of different enzymes for different shark species. There are many studies of teleost genomics and transcriptomics (Whitehead et al. 2011; Qian et al. 2014; German et al. 2016; Calduch-Giner et al. 2016), but few in sharks (Dowd et al. 2008; Pinhal et al. 2012; Wyffels et al. 2014; Venkatesh et al. 2014; Mulley et al. 2014), and none on the gut in sharks. Understanding which genes are expressed in various shark species would reveal the molecular underpinnings leading to dietary specialization. Ontogenetic shifts in gene expression and enzyme activity have also not been explored (except in teleost fishes) and could be extremely informative given that many species have dramatic shifts in diet as they grow. More genomic studies of sharks should be a top priority given that there is currently very little genomic information available for

elasmobranchs (Leucoraja erinacea; Wyffels et al. 2014) and holocephalans (Callorhinchus milii; Venkatesh et al. 2014).

Despite years of scientists citing reviews that state that the spiral intestine is the most important organ in the nutrient absorption process and that it slows the rate of transit through the gut, there is little quantitative evidence to support these contentions (Wilson and Castro 2011; Jhaveri et al. 2015; Chatchavalvanich et al. 2006; Hart et al. 2016). This needs to be addressed if we are to further the field of shark digestive physiology. We need to move beyond dissection photographs and illustrations and use CT scan technology to create 3D renderings of the various spiral intestine structures. These renderings give us the ability to visualize flow, and make quantitative assessments of the intestinal volume and tissue surface area. This information, paired with quantification of the contractive capabilities, volumetric flow rate, and absorptive properties of the entire shark gut, and the spiral intestine in particular, would reveal how this unique gut type contributes to the economical design of the gastrointestinal tract as a whole. I propose that the ‘‘rate vs. yield’’ theoretical framework currently used to describe teleost digestive strategies should be applied to sharks as well (e.g., Jhaveri et al. 2015). Like teleosts, sharks consume a broad range of diet types, inhabit a broad range of habitats, and feed with different frequencies. As such, they likely encompass a broad range of digestive strategies and efficiencies that could be described using this framework. Dietary specialization within this framework could be coupled to stable isotope analysis (e.g., Lujan et al. 2011) and fatty acid profiling (e.g., Clements et al. 2017) to further our understanding of shark dietary diversity (Bucking 2016).

Additionally, more studies of isotopic turnover rates and tissue-diet discrimination in sharks are necessary in order to make sense of field isotopic data.

Sharks are undeniably still a mystery in many respects, especially with regards to their nutritional physiology. We know that they share some similarities with other, well-studied, carnivorous vertebrates, such as teleosts. However, there are still knowledge gaps in the topics of feeding mechanics, functional morphology of the digestive tract (the spiral intestine in particular), digestive biochemistry, and gastrointestinal/microbiota relationships. As important as sharks are presumed to be ecologically, there is a definite need for future research to investigate their nutritional physiology.

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