UNIVERSITY OF CALIFORNIA, IRVINE

From whole animal physiology to gene expression and the microbiome: how do fishes specialize to thrive on different diets

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Michelle Joni Herrera

Dissertation Committee:
Professor Donovan P. German, Chair
Professor Nancy Aguilar-Roca
Professor J.J. Emerson

2023

DEDICATION

To my mother,
Grace Herrera,
for supporting my dreams, believing in me, and always
encouraging me. Thank you for the many sacrifices you have
made so I could focus on studying and achieving my goals. You
always ignite the ambition and passion in everything I set my mind to.

To my sister,
Elizabeth Herrera,
for always being there for me and encouraging me to do
anything I want to do, and constantly celebrating accomplishments
and being there for me through disappointments. You are my best friend.

To my grandparents,
Grandpa Rolly and Grandma Remy,
for making the greatest sacrifice of leaving your home country
for the unknown to give your family greater opportunities, and
for watching over me every day. I hope I have made you proud.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my dissertation advisor, Dr. Donovan German for his guidance over the past eight years as a graduate and undergraduate researcher. Donovan is a phenomenal advisor and mentor and has been instrumental in helping me grow and flourish into the researcher I am today. Through his mentorship as an undergraduate researcher, particularly at the Friday Harbor Labs REU program, he ignited my passion for fishes, physiology, and intertidal field work. Through his mentorship, he encouraged me to pursue research and a PhD program. He never doubted that I could handle myself in treacherous field work environments or multiple intense lab work days, and is always there to help me while giving me space to grow as an independent researcher. He has believed in my abilities since the beginning, and he constantly gives me the confidence to pursue integrative beasts of research projects. He encourages me to flourish in the passions and career endeavors that I have. Donovan has served as a role model for the values I would like to mirror as a scientist, advisor, mentor, and teacher. He constantly works to change the traditionally toxic environment of academia, and has never hesitated to be there to support me through it all. I am extremely grateful he has gone out of his way to create a safe and comfortable environment for me to study and pursue my research. He has been a trustworthy, kind, patient, and understanding mentor who has helped me reach my full potential, while also understanding the obligations I had in my personal life and always being graciously patient. I would not be where I am without his unwavering support as a mentor. My gratitude goes beyond words can describe. Thank you for your support.

I also especially thank my bioinformatics mentor, Dr. Joseph Heras. Joe has been there to support me since I first joined the German lab, and has been instrumental in conducting my dissertation research. I am grateful for his time and effort teaching me molecular techniques and bioinformatics. He took me, a person with no previous background in bioinformatics, genomics, or transcriptomics, and helped me grow into an evolutionary physiologist. He has constantly believed in my ability to learn and grow as a scientist, and I am indebted to him. Thank you for your unwavering friendship, support, and mentorship.

I sincerely thank my family, my mother Grace Herrera, my sister Elizabeth Herrera, and my beloved Tommy, Silverstream, Chinchi and Chance for their constant support throughout my life and for always being there to support me and my dreams. Thank you for being there through all of the good times and tough times and being a constant source of happiness and love.

My lab mates have been my second family and has been a constant source of support and happiness. I am especially thankful to Dr. Beck Wehrle and Dr. Alyssa Frederick, who have both served as role models for the scientist I have strived to be. They got me through tough times at the start of graduate school, and have always been there for me to provide support, especially in the form of cute animal bonding time. Karina Brocco French - Reeses Pieces - has been an incredible friend and neighbor, and has always been there when I needed someone to cry on the couch with. She is always there to remind me that the goal is not to just survive but also to flourish. Karina has supported me through so much, and I thank sincerely thank her for the confidence she has instilled in me. I sincerely thank my friend and labmate Nefertiti Smith for all of the pool and snack days to get my mind off the work, and for being there when I needed someone to chat with about anything. Thank you to Newton Hood for his friendship and helping me with field work along the coast of California, and enduring early mornings and difficult terrain to collect the fish. Thank you to Matea Djokic for her support. Thank you to Daniel Rankins, who kindly made delicious cakes and food to share, and for his constant support and

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Many thanks to my committee members, Dr. Nancy Aguilar-Roca and Dr. JJ Emerson, for their feedback and support in my dissertation research. Nancy has been instrumental in my journey into physiological research, and in facilitating my internship with the San Diego Zoo Wildlife Alliance. She has been an incredible role model and I strive to mirror her values that she encapsulates in her teaching. JJ has been incredibly helpful in expanding my knowledge of evolution and genomics. He has constantly supported me throughout my graduate career, and provided valuable feedback on my resarch. I also thank my advancement committee, with the additions of Dr. Kwasi Connor and Dr. Katrine Whiteson, for providing their inputs on transcriptomics and microbiome into my dissertation research.

A special thanks to all the members of the UCI Comparative Physiology Group, who has provided feedback on my research and professional development in a healthy and constructive manner. SACNAS at UCI was a strong source of support in my graduate career and I especially thank all of the founding members for their support. Thank you for UCI Microbiome Initiative, including Dr. Jennifer Martiny, Dr. Katrine Whiteson, and Claudia Weihe, for supporting my microbiome research and helping me learn microbiome techniques and analyses. Thank you to Ridge2Reef, particularly Dr. Steve Allison, Courtney Hunt, and Bri McWhorter, for helping me expand my science communication skills and providing academic and financial support. I also thank Friday Harbor Labs, and the mentors and fellow trainees of the 2016 REU program and Fish Functional Morphology Class, for their support in my research and aid in fish collection, especially to Dr. Adam Summers and Dr. Lisa Crummett for sharing lab space and resources at FHL, and Dr. Vikram Iyengar for support and guidance.

I acknowledge the traditional owners of the land on which we live and work, who have inhabited the region before the founding of the state and who continue to live on these lands. The work presented here was conducted primarily on the occupied and unceded land of the Acjachemen and Tongva Nations, where University of University of California, Irvine is located. Fishes for this research were collected from the ancestral lands and waters of the Salinan, Coastal Miwok, Amah Mutsun, and Coast Salish Nations. I recognize that I benefit from the continued denial of these nations' rights to their land and resources.

Chapter 2 of this dissertation is a reprint of the material as it appears in Herrera et al. 2022, used with permission from Journal of Comparative Physiology B (Herrera et al. 2022 J Comp Physiol B (2022). 192: 275-295 https://doi.org/10.1007/s00360-021-01426-1). The co-authors listed in this publication are Michelle J. Herrera, Joseph Heras, PhD, and Donovan P. German, PhD.

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1852096), Society for Integrative and Comparative Biology Grant-in-Aid of Research, UC Natural Reserve System Mildred E. Mathias Graduate Student Research Grant, American Society of Ichthyologists and Herpetologists Raney Fund Award, Sigma Xi Grant-in-Aid of Research and undergraduate research grants awarded to M.J.H. This work was conducted under University of California, Irvine, Institutional Animal Care and Use Committee Protocol 2011-2989, and University of Washington IACUC Protocol 4238-03.

VITA

Michelle Joni Herrera

EDUCATION

Sept. 2023	PhD Biological Sciences, Department of Ecology and Evolutionary Biology, School of Biological Sciences, University of California, Irvine, CA Advisor: Dr. Donovan P. German Research: Molecular, Biochemical, and Physiological Responses to Dietary Shifts in prickleback fishes (family Stichaeidae); cumulative GPA: 4.0
2020	M.S. Biological Sciences University of California, Irvine, CA Advisor: Dr. Donovan P. German
2017	B.S. Biological Sciences, University of California, Irvine, CA Advisor: Dr. Donovan P. German Research topics: The molecular underpinnings of digestive specialization in prickleback fishes and potential for plasticity; Phylogenetics of prickleback fishes in the family Stichaeidae.

RESEARCH INTERESTS

- Using evolutionary physiology as a tool for animal conservation
- Understanding how factors such as diet, environment, and genetics influence overall physiology, the gut microbiome, and patterns of gene expression
- I aspire to become a professor so that I may continue to dismantle barriers that impede underrepresented students in STEM education and develop my own physiological ecology research program with a focus on conservation and animal contributions to ecosystem functions.

PUBLICATIONS & MANUSCRIPTS IN ADVANCED PREP

- Herrera, M.J., Heras, J. & German, D.P. Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes. J Comp Physiol B (2022). 192: 275-295 https://doi.org/10.1007/s00360-021-01426-1
- Rankins, D.R., M.J. Herrera, M.P. Christensen, A. Chen, N.Z. Hood, J. Heras, D.P. German. When digestive physiology doesn't match "diet": *Lumpenus sagitta* (Stichaeidae) is an "omnivore" with a carnivorous gut. *Comparative Biochemistry and Physiology, Part A.* A 285:111508
- Le, N., J. Heras, M.J. Herrera, D.P. German, L.T. Crummett. The genome of Anoplarchus purpurescens (Stichaeidae) reflects its carnivorous diet. Journal of Molecular Genetics and Genomics. (In press)
- Herrera, M.J., J. Heras, C. Catabay, J.D. Bastian-Salgado, M. Turken, K.M. Connor, D.P. German. Dietary-induced shifts in the hindgut microbiome of a marine herbivorous fish leads to subtle changes in gut and liver function. (In prep) to be submitted to *Molecular Ecology* in October 2023.

- Herrera, M.J., J. Heras, N.N. Smith Christman, N.Z. Hood, D.P. German. Digestive physiology and individual variation impact the hindgut microbiome of prickleback fishes (Stichaeidae) with different diets. (In prep) to be submitted in October 2023.
- Herrera, M.J., J. Buckner, N.N. Smith Christman, M. Hilleman, J. Heras, D.P. German. Gastric enzyme activities and stomach transcriptomics correlate with diet in prickleback fishes (Stichaeidae). In prep
- Lee, A.M., M.J. Herrera, D.P. German, A.R. Frederick. Digestive biochemistry from opposite ends of the Pacific: red abalone and pāua show differences in digestive enzyme activities, microbial diversity, and gastrointestinal fermentation. In Prep- to be submitted to *Marine Biology*
- Herrera, M.J. and German, D.P., 2016. Digestive specialization in prickleback fishes (Family Stichaeidae). Friday Harbor Laboratories, University of Washington.
- Herrera, M.J. and German, D.P., 2017. Digestive specialization in prickleback fishes (Family Stichaeidae): molecular underpinnings and potential for plasticity. UCI Journal of Undergraduate Research in Biological Sciences.

INVITED SEMINARS

- How does diet and environment influence an animal? University of California, San Diego Microbiome Consortium Invited Seminar (Dorrestein/Raffatellu labs)
- Are you what you eat? Herbivorous fish can digest more than plants. Scripps Institution of Oceanography Marine Biology Department Invited Seminar. April 2023
- What does it mean to be specialized for a certain diet? CSU San Bernardino Biology Department Invited Seminar. April 2023
- How diet influences animal physiology and health. CSU San Bernardino Ichthyology Class Invited Seminar. March 2023
- Unraveling the relationship between genetics, diet, environment and animal health: My journey through science. CSU Dominguez Hills Invited Seminar. September 2022.

PUBLIC COMMUNICATIONS PUBLICATIONS

- Herrera, M. (2019) "Extending night hours at zoos: How are animals affected?" *Society for Integrative and Comparative Biology Student Journalism*. (link to story)
- Cat, L.A., A. Frederick, M. Herrera, E. Valdez-Ward (2018) "Diverse ecosystems require diverse ecologists" Rapid Ecology. (link to story)

SELECTED RESEARCH GRANTS (Total Awarded: $7,650)

2021	Society for Integrative and Comparative Biology (SICB) Grant-in-Aid of Research: $1000
2020	UC Natural Reserve System Mildred E. Mathias Graduate Student Research Grant: $1,000
2020	American Society of Ichthyologists and Herpetologists Raney Fund Award: $1,000
2017	UCI Microbiome Initiative Pilot Project Award
2017	Sigma Xi Grant-in-Aid of Research: $1,000

2016-17	Undergraduate Research Opportunities Program (UROP) Grant: $350
2015-16	Undergraduate Research Opportunities Program (UROP) Grant: $300

SELECTED SCHOLARSHIPS, AWARDS, & HONORS (total scholarship awarded: $215,659)

2023	American Physiological Society Comparative & Evolutionary Physiology Section Steven M. Horvath Professional Opportunity Award: $500
2023	American Physiological Society Martin Frank Diversity Travel Award: $1,500
2022	UC Irvine Rose Hills Foundation Science and Engineering Fellowship: $10,000
2022-23	UC Irvine President's Dissertation Year Fellowship: $46,000
2021	Summer Inclusive Excellence Award
2020, 2021	American Physiological Society (APS) Porter Physiology Development Fellowship: $46,000
2020	UC Irvine Inclusive Excellence Ambassador Fellowship Award
2020	Grover C. Stephens Memorial Fellowship Award
2019	Society for the Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) Best Graduate Oral Presentation Award
2019	UCI Winter Ecology and Evolutionary Biology Graduate Student Symposium Best Oral Presentation Award
2019	UCI Latino Excellence and Achievement Dinner (LEAD) School of Biological Sciences Excellence in Research Award
2019	Ford Foundation Predoctoral Fellowship Alternate/Honorable Mention
2017&19	National Science Foundation: Graduate Research Fellowship Program Honorable Mention
2019	National Science Foundation: Ridge to Reef Fellowship: $52,000
2019	UCI School of Biological Sciences Graduate Fellowship Award
2019	Society for Integrative and Comparative Biology (SICB) Student Journalism Internship
2018,19,22	Diverse Educational Community and Doctoral Experience (DECADE) Student Travel Award: $1000
2018-22	Ridge 2 Reef Training Program (UCI)
2017&18	SICB Broadening Participation Travel Award: $500
2017&18	SICB Charlotte Magnum Student Support Program: $109
2017	Voth Family Trust Fellow
2017	Associated Graduate Student Travel Grant: $400
2017	ASIH Graduate Student Travel Grant: $400
2017	NSF Bio REU Travel Grant: $1,000
2017	Competitive Edge Summer Research Fellowship Award: $5,000
2017	Diversity Recruitment Fellowship Award: $5,000
2017	UC Irvine Excellence in Research in Biological Sciences
2016	Friday Harbor Laboratories NSF REU BEACON Summer Fellowship
2015&17	Deans Honor List
2015	ABRCMS Best Poster Presentation Award: $250
2014-16	Minority Biomedical Research Support (MSBRS-IMSD) funded by the National Institutes of Health (NIH)

SELECTED PRESENTATIONS & PUBLISHED ABSTRACTS (12 out of 32 total talks)

M. Herrera, J. Heras, C. Catabay, J.D. Bastian-Salgado, M. Turken, K.M. Connor, D.P. German (2023) Dietary-induced shifts in the hindgut microbiome and metabolism. *Invited Student Speaker for The Company of Biologists Workshop: Inside Out: New Frontiers in the Comparative Physiology of the Vertebrate Gut.* Oral Presentation.

M. Herrera, J. Heras, C. Catabay, J.D. Bastian-Salgado, M. Turken, K.M. Connor, D.P. German (2023) How does the gut respond to a changing world? Dietary-induced changes in the hindgut microbiome and metabolism of a marine herbivorous fish. *American Physiological Society Summit.* Poster Presentation.

M. Herrera, J. Heras, C. Catabay, J.D. Bastian-Salgado, M. Turken, K.M. Connor, D.P. German (2022) How does the gut respond to a changing world? Dietary-induced changes in the hindgut microbiome of a marine herbivorous fish. *Southwest Regional Meeting of Organismal Biologists.* Poster Presentation.

M. Herrera, J. Heras, C. Catabay, J.D. Bastian-Salgado, M. Turken, K.M. Connor, D.P. German (2022) How does the gut respond to a changing world? Dietary-induced changes in the hindgut microbiome of a marine herbivorous fish. *Orange County Coastal Marine Research Symposium*. Poster Presentation.

M. Herrera, J.M. Gauglitz, K. Kerr, K.L. Marshall, K. Weldon, P.C. Dorrestein, C. Singleton, and C.L. Williams (2022) Examining the potential of fecal microbiota transplants to treat gastrointestinal imbalance in greater one-horned rhinoceros. *International Symposium on Microbial Ecology*. Lausanne, Switzerland. Poster Presentation.

M. Herrera and D.P. German (2022) Unraveling the relationship between genetics, diet, environment, and animal health. *UCI Physiology Group Seminar.* Oral Presentation.

M. Herrera, J.M. Gauglitz, K. Kerr, K.L. Marshall, K. Weldon, P.C. Dorrestein, C. Singleton, and C.L. Williams (2022) Gut Feelings: How can fecal microbiota transplant impact overall health? *Microbial Ecology and Conservation Symposium*. Oral presentation.

M. Herrera, J.M. Gauglitz, K. Kerr, K.L. Marshall, K. Weldon, P.C. Dorrestein, C. Singleton, and C.L. Williams (2021) Fecal microbiota transplants and their potential to treat chronic gastrointestinal disease in greater one-horned rhinoceros. *World Microbe Forum*. Oral/Poster Presentation.

M. Herrera and D.P. German (2020) What Chooses our gut friends? Host identity and proximate diet influence enteric microbiome community and function in prickleback fishes. *Comparative Nutrition Society*. Oral/Poster Presentation.

M. Herrera and D.P. German (2019) Gut Microbes and Diet: Testing Phylosymbiosis in Closely-Related Prickleback Fishes with Different Diets. *Society for the Advancement of Chicanos/Hispanics and Native Americans in Science*. Oral Presentation.
Awarded Best Graduate Student Oral Presentation

M. Herrera (2019) From oceans to guts. *SACNAS at UCI Outreach Event: Lightning Talk to Godinez High School*. Oral Presentation.

M. Herrera and D.P. German (2019) Changes in the microbiome in response to dietary shifts in prickleback fishes. *Winter Ecology and Evolutionary Biology Graduate Student Symposium*. Oral Presentation.
Awarded Best Graduate Student Oral Presentation

SELECTED OUTREACH AND LEADERSHIP ACTIVITIES

2015-present	Targeted Instruction Generating Excitement about Research and Science
April 2021-April 2022	Stanford University: Diversity in Environmental Careers Panelist and Mentor
June 2020-June 2021	UCI Inclusive Excellence Ambassador
July 2018-July 2019	UCI Associated Graduate Students President
2019	Inclusive Excellence Forum Co-facilitator
2019	NSF Research Traineeship Meeting Participant
2019	Ridge to Reef Summer Institute
2019	"What can I do with my PhD" Symposium (UCI Ecology and Evolutionary Biology)
June 2018-September 2018	Competitive Edge Summer Research Program Peer Mentor
June 2018-September 2018	Mentoring Excellence Program and Certificate (UCI)
2018-2020	"Turn of the Tide" Podcast, Co-host (2018-present)
2018-2019	UCI Public Policy Prep (P3) Program
Feb 2018-July 2018	UCI Associated Graduate Students Council Member
2018	Irvine Unified School District Science Fair Santa Ana Unified School District MacArthur Science Fair
2017-2022	Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) – UC Irvine Chapter
2017	How to Find a STEM related Summer Research Opportunity: NSF REU Workshop Panelist
2017-2022	Diverse Educational Community and Doctoral Experience (DECADE at UCI)

TEACHING EXPERIENCE

Guest Lecturer, UCI Ecology and Evolutionary Biology Department

- Human Nutrition, Topic: Lipid Synthesis and Metabolism (Winter 2019)
- Human Nutrition, Topic: Vitamins: Their roles in metabolism (Winter 2023)

Transcriptomics/Bioinformatics Workshop, UCI Ecology and Evolutionary Biology Department Physiology Group (2023)

- I designed and led weekly workshops on transcriptome concepts and analysis
- I individually helped participants outside of workshop hours, with the goal of understanding steps in transcriptome analysis and conducting analysis.

Microbiome Workshop, UCI Ecology and Evolutionary Biology Department Physiology Group (2021)

- I designed and led weekly workshops on microbiome concepts and analysis
- I individually helped participants outside of workshop hours, with the goal of understanding steps in microbiome analysis and conducting analysis.

Graduate Teaching Assistant, UCI Ecology and Evolutionary Biology Department

- Biology: From Organisms to Ecosystems (Winter 2018)
  Responsible for designing and leading weekly discussion sections to review concepts from lecture. I incorporated creative presentation into discussion activities.
- Human Physiology Laboratory (Spring 2018, Fall 2018, Spring 2019)

Responsible for leading laboratory lecture, functions, quizzes, lab reports, and grades

- Human Nutrition (Summer 2018, Winter 2019, Summer 2023)
  Responsible for functions, exams, and grades (evaluated performance and provided feedback for up to 30 students per quarter)

ADDITIONAL RESEARCH EXPERIENCE

San Diego Wildlife Alliance Research Fellowship, Dr. Pieter Dorrestein, Dr. Chris Tubbs and Dr. Candace Williams
Title: Visiting Scholar/Research Fellow (March 2020-present)
Projects:

1) Examine how fecal microbiota transplants can help ameliorate gastrointestinal states in greater one-horned rhinocerous
2) Examine the effects of captivity over time on the gut microbiome of rhinocerous

University of California, Irvine: Dr. Luis Mota-Bravo Laboratory
Title: Undergraduate Intern (March 2014-April 2015)
Projects:

2) Assessed the presence and range of beta-lactam antibiotic resistance in *Aeromonas* species from aquatic environments
1) Characterized the occurrence of extended-spectrum beta-lactamases (ESBLs) in *Aeromonas* species.

ABSTRACT OF THE DISSERTATION

From whole animal physiology to gene expression and the microbiome:

how do fishes specialize to thrive on different diets

By

Michelle Joni Herrera

Doctor of Philosophy in Biological Sciences

University of California, Irvine 2023

Professor Donovan P. German, Chair

What an animal eats and how it digests its food determines that animal's contribution to its ecosystem. However, the nutritional physiology (including digestion) of animals is commonly overlooked, even though such data are needed for conservation of many ecosystems. This is particularly true for wild fishes in comparison to terrestrial vertebrates or even fishes farmed for aquaculture. The goal of my dissertation is to understand how prickleback fishes specialize to use specific resources, and how their nutritional physiology impacts their roles in their environment. I aimed to address the research gap in our understanding of dietary specialization and fish nutritional physiology, which are topics that I briefly review in the first chapter of my dissertation.

In the second chapter, I integrated nutritional physiology and transcriptomics to further our understanding of digestive system plasticity in response to dietary perturbations. This study showed that prickleback fishes with different diets can respond to dietary perturbations in different ways. Our dataset elegantly shows how gut length of fishes change with dietary perturbations, even within a single species over a four-week feeding experiment. Although there were hundreds of genes differentially expressed among the three diet groups in the intestine

(pyloric ceca and mid-intestine), three of the four species didn't appreciably alter gene expression in the liver in response to different diets in the laboratory. Thus, we find a species-specific pattern in liver gene expression and metabolic pathways within prickleback fishes.

In the third chapter of my dissertation, I aimed to advance our understanding of the factors that can influence gut physiology and function to provide insight into ecological adaptations and potential impacts on ecosystem dynamics. I investigated the effects of different diets that vary in protein content, on the physiology, host gene expression, and gut microbiome of *Cebidichthys violaceus*, a marine herbivorous fish. This integrative study highlighted the complex interactions between diet, gut physiology, gene expression and the hindgut microbiome in a marine herbivorous fish.

Interactions between gut microbes and animal hosts impact the physiology and health of animals, yet we lack a thorough understanding of the determinants of gut microbial community structure. In chapter four of my dissertation, I examined the factors that shape the gut microbial communities of prickleback fishes within the context of their environment, diet, and genetics. We discovered that changes in the gut microbiome were linked more to individual differences and species identity within closely-related fishes living in the same area, rather than being primarily influenced by diet or location.

In summary, dietary perturbations can alter the gut physiology, transcriptome, and microbiome of prickleback fishes. My dissertation work advances our understanding of dietary specialization in vertebrates and fish nutritional physiology.

Chapter 1: Introduction

What does it mean to be specialized for a certain diet? Examining dietary specialization and nutritional physiology of prickleback fishes

Vertebrate animals eat a dizzying array of food items, and their digestive tracts reflect a complexity influenced by diet and genetics (Karasov and Douglas 2013; Karasov and Martínez del Rio 2007). Because different vertebrate taxa consume different diets, there tends to be variation in the morphology, size, pH, and enzyme biochemistry of their digestive systems (German 2011; Karasov and Martínez del Rio 2007; Starck 2005; Stevens and Hume 1995). As the supply organ of nutrients to an animal, the digestive system can also be plastic in its responses to dietary perturbations, ranging from changes in gene expression (De Santis et al. 2015a; De Santis et al. 2015b; Gawlicka and Horn 2006; He et al. 2013; Kim et al. 2014; Król et al. 2016; Le et al. 2019; Parris et al. 2019; Wang et al. 2015), to changes in the gut microbiome (Kohl et al. 2018b; Ley et al. 2008a; Muegge et al. 2011), digestive tract size (Fuentes and Cancino 1990; German and Horn 2006; He et al. 2013; Leigh et al. 2018a), digestive enzyme activities (German et al. 2004; German et al. 2010; Harpaz and Uni 1999; He et al. 2013), and nutrient transporter activity (Buddington et al. 1987; Day et al. 2014; Verri et al. 2017).

Although plasticity of digestive tract function is well investigated on many levels in model terrestrial systems (Karasov and Douglas 2013; Karasov and Martínez del Rio 2007), and in a handful of (mostly carnivorous) aquaculture species (reviewed in (Grossel et al. 2011)), plasticity of fish digestive systems remains poorly investigated, particularly in an evolutionary

context relating to dietary specialization (German and Horn 2006; German et al. 2004; German et al. 2010). Fishes compose the largest vertebrate group, and yet, it isn't clear what dietary specialization means on the gut level for various taxa (German 2011; German et al. 2016), beyond what isn't tolerated in aquaculture feed formulation (e.g., (Król et al. 2016)). The plasticity displayed in some fish guts (e.g., (German et al. 2010; Harpaz and Uni 1999; Leigh et al. 2018a; Wang et al. 2015)) suggests that the guts of some fish species may be equally generalized and able to respond to dietary shifts, regardless of natural diet (but there are exceptions; (German and Horn 2006; German et al. 2004)). Thus, we do not fully understand the general principles of fish nutritional physiology and what constitutes dietary specialization for them.

Prickleback fishes (Family Stichaeidae) provide an excellent system in which to investigate fish nutritional physiology. With dietary variation, ontogenetic dietary shifts, convergent evolution of herbivory, and sister taxa with different diets, the Stichaeidae offers multiple opportunities to understand how fishes thrive on their specific diets and the mechanisms underlying potential digestive specialization (German et al. 2015; Kim et al. 2014). Moreover, there is a rich literature developing on the digestive physiology (German et al. 2014; German and Horn 2006; German et al. 2004; German et al. 2015; Kim et al. 2014) and genomics (German et al. 2016; Heras et al. 2020) of these species, providing ample opportunity to test for dietary specialization. There are five sympatric stichaeid species with different diets: *Xiphister mucosus* (herbivore), *Cebidichthys violaceus* (herbivore), *X. atropurpureus* (omnivore), *Phytichthys chirus* (omnivore), and *Anoplarchus purpurescens* (carnivore). The first three all belong to the Xiphisterinae clade, which is one of the clades that evolved algal consumption (the other being the one including *Cebidichthys violaceus*). *Anoplarchus purpurescens* is part of the Alectriini

clade, which is wholly carnivorous, like much of the family (German et al. 2015; Kim et al. 2014). These species are sympatric, meaning they experience similar environmental conditions in their intertidal habitat, with diet being one of the only differences among them in the wild (German and Horn 2006; German et al. 2004; German et al. 2015).

Based on previous investigations of prickleback digestive physiology (German et al. 2014; German and Horn 2006; German et al. 2004; German et al. 2015), genetics of amylase genes (German et al. 2016; Kim et al. 2014), and a genome of the herbivorous *C. violaceus* (Heras et al. 2020), we expected to find signatures of dietary specialization in the different prickleback fishes. Beyond the intestinal tissues, in which we predicted to observe differences in gene expression and evidence of selection on digestive enzyme genes, the liver may provide insight into metabolic pathways favored by the different species with different diets, and whether those can shift when the animals are consuming different nutrient loads in the laboratory (Yang et al. 2017; Merkin et al. 2012). Thus, for my dissertation, I investigated how specialized these animals are for their respective diets and provide insight into the underpinnings of their abilities (or lack thereof) to use a broader base of resources than they would naturally.

For my dissertation research, I investigated dietary specialization in prickleback fishes using integrative studies to determine factors that influence fish physiology, the gut transcriptome, and the gut microbiome. In the second chapter of my dissertation "Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes", I pursued the following questions: Does the expression of genes involved in digestion and metabolism vary with dietary perturbations? Are there growth and fitness consequences as a result of differing digestibility of diets that vary in biochemical composition? Thus I had two aims for this chapter: first, to compare the morphology, gene expression patterns (transcriptomics), and genes under

selection from the digestive system and liver of the different fish species with different diets captured from the wild, and second, examine these same parameters in the face of dietary shifts in the laboratory. The most fascinating result was that the liver exhibits a more tissue-specific response when comparing different diets and tissues, and the most responsive pathway is lipid metabolism.

In the third chapter of my dissertation "Diet shifts affect gut and liver function and the distal intestine microbiome of an herbivorous fish", I aimed to answer the following question: How do the diet and microbiome interact to influence host gut and liver gene expression and overall physiology, particularly for herbivorous fishes? I integrated multiple parameters, including gut physiology, digestive enzyme activity, short-chain fatty acid concentrations, gut microbiome, and hindgut and liver transcriptomics to examine the response of *Cebidichthys violaceus*, a marine herbivorous fish, to dietary shifts. Fish successfully assimilated the laboratory diets, and there were diet-dependent shifts in SCFA levels and digestive enzyme activity levels. The host hindgut gene expression patterns closely resembled those of the gut microbial diversity patterns. Additionally, the hindgut and the liver displayed different responses to dietary shifts in terms of the transcriptome. This chapter's findings provided insight into how fish acclimate to shifting resources, which helps inform efforts in marine resource management and the potential development of new aquaculture species.

In the fourth chapter of my dissertation "Digestive physiology and individual variation impact the hindgut microbiome of prickleback fishes (Stichaeidae) with different diets", I aim to answer the following questions: How does geography or species identity influence the gut microbiome in prickleback fishes with different diets? How do dietary changes influence the fish gut microbiome? In addition to our limited understanding of dietary specialization and nutritional

physiology among fishes, the factors that shape the vertebrate gut microbiome are unclear. Thus, I aimed to unravel the relationship between the host genetics, diet, environment, and the gut microbiome by sequencing their gut microbiome, measuring aspects of community function, including fermentative and enzyme activity, and conducting laboratory-controlled feeding experiments. Our results highlighted that individual variation and species identity play a crucial role in molding the gut microbiome, affirming the existence of distinct microbial community patterns for each species.

Beyond the basic research on the evolution of dietary specialization, this dissertation provides data on how these fish contribute to ecosystem fluxes of energy and nitrogen, which is useful to marine protected area (MPA) managers; pricklebacks are common denizens of MPAs across California. Additionally, my research informs biomedical science by advancing our understanding of factors that influence the gut function and how microbiomes change under different circumstances.

Chapter 2

Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes

ABSTRACT

Beyond a few obvious examples (e.g., gut length, amylase activity), digestive and metabolic specializations towards diet remain elusive in fishes. Thus, we compared gut length, $\delta^{13}C$ and $\delta^{15}N$ signatures of the liver, and expressed genes in the intestine and liver of wild-caught individuals of four closely-related, sympatric prickleback species (family Stichaeidae) with different diets: *Xiphister mucosus* (herbivore), its sister taxon *X. atropurpureus* (omnivore), *Phytichthys chirus* (omnivore) and the carnivorous *Anoplarchus purpurescens*. We also measured the same parameters after feeding them carnivore or omnivore diets in the laboratory for four weeks. Growth and isotopic signatures showed assimilation of the laboratory diets, and gut length was significantly longer in *X. mucosus* in comparison to the other fishes, whether in the wild, or in the lab consuming the different diets. Dozens of genes relating to digestion and metabolism were observed to be under selection in the various species, but *P. chirus* stood out with some genes in the liver showing strong positive selection, and these genes correlating with differing isotopic incorporation of the laboratory carnivore diet in this species. Although the intestine showed variation in the expression of hundreds of genes in response to the laboratory diets, the liver exhibited species-specific gene expression patterns that changed very little (generally <40 genes changing expression, with *P. chirus* providing an exception). Overall, our

results suggest that the intestine is plastic in function, but the liver may be where specialization manifests since this tissue shows species-specific gene expression patterns that match with natural diet.

INTRODUCTION

Vertebrates consume a large array of food items, and their digestive tracts reflect a complexity influenced by diet and genetics (Karasov and Douglas 2013; Karasov and Martínez del Rio 2007). Because different vertebrate taxa consume different diets, there tends to be variation in the morphology, size, pH, and enzyme biochemistry of their digestive systems (German 2011; Karasov and Martínez del Rio 2007; Starck 2005; Stevens and Hume 1995). As the supply organ of nutrients to an animal, the digestive system can also be plastic in its responses to dietary perturbations, ranging from changes in gene expression (De Santis et al. 2015a; De Santis et al. 2015b; Gawlicka and Horn 2006; He et al. 2013; Kim et al. 2014; Król et al. 2016; Le et al. 2019; Parris et al. 2019; Wang et al. 2015), to changes in digestive tract size (Fuentes and Cancino 1990; German and Horn 2006; He et al. 2013; Leigh et al. 2018a), digestive enzyme activities (German et al. 2004; German et al. 2010; Harpaz and Uni 1999; He et al. 2013), and nutrient transporter activity (Buddington et al. 1987; Day et al. 2014; Verri et al. 2017).

Although plasticity of digestive tract function is well investigated on many levels in model terrestrial systems (Karasov and Douglas 2013; Karasov and Martínez del Rio 2007), and in a handful of (mostly carnivorous) aquaculture species (Grossel et al. 2011), plasticity of fish digestive systems remains poorly investigated, particularly in an evolutionary context of dietary specialization (German and Horn 2006; German et al. 2004; German et al. 2010). Fishes compose the largest vertebrate group, and yet, it is not clear what dietary specialization means on

the gut level for various taxa (German 2011; German et al. 2016), beyond what is not tolerated in aquaculture feed formulation (Król et al. 2016). For instance, in terms of ecomorphology, the oral jaws of cichlid fishes show incredible diversity leading to resource specialization in various species, yet the pharyngeal jaws of these same species show marked generality and plasticity, suggesting that the true masticatory apparatus of the oral cavity (i.e., the pharyngeal jaws) maintains the ability to process a wide-array of ingested foods (Gunter et al. 2013; Liem 1973; Meyer 2015; Stiassny and Jensen 1987; Burress et al. 2020). The plasticity displayed in some fish digestive systems suggests that the guts of some fish species may be equally as generalized and able to respond to dietary shifts (German et al. 2010; Harpaz and Uni 1999; Leigh et al. 2018a; Wang et al. 2015) but there are exceptions (German and Horn 2006; German et al. 2004). Thus, we do not fully understand the general principles of fish nutritional physiology and what constitutes dietary specialization for them. To address this research gap, we took a systems approach by integrating nutritional physiology and transcriptomics to better understand digestive system plasticity in response to dietary perturbations. In addition to changes in gene expression, fishes can certainly have mutational or gene copy number differences that can help explain physiological and biochemical variation among them, thus highlighting the importance of a modern molecular approach, like transcriptomics (German et al. 2016; Heras et al. 2020; Betancor et al. 2018). RNA-seq using the Illumina high-throughput sequencing platform can provide whole *de novo* transcriptome information, gene functional information, and the molecular mechanisms of biological processes, including those related to digestion and metabolism, without requiring a reference genome (Martin et al. 2016; Martin and Król 2017; Qi et al. 2011).

For this study, we used prickleback fishes (Family Stichaeidae) since they provide an excellent system in which to investigate fish nutritional physiology. With dietary variation, ontogenetic dietary shifts, convergent evolution of herbivory, and sister taxa with different diets, the Stichaeidae offers multiple opportunities to understand how fishes thrive on their specific diets and the mechanisms underlying digestive specialization (German et al. 2015; Kim et al. 2014). Moreover, there is a rich literature developing on the digestive physiology (German et al. 2014; German and Horn 2006; German et al. 2004; German et al. 2015; Kim et al. 2014) and genomics (German et al. 2016; Heras et al. 2020) of these species, providing ample opportunity to test for dietary specialization. We studied four closely-related, intertidal stichaeid species with different diets: *Xiphister mucosus* (herbivore), *X. atropurpureus* (omnivore), *Phytichthys chirus* (omnivore), and *Anoplarchus purpurescens* (carnivore). Thorough gut content analyses of all target species dating back approximately four decades confirm that *X. mucosus* has greater than 98% algal material composing their diets, and the omnivores have at least 50% algae composing theirs (Horn et al. 1982; Horn et al. 1986; Setran and Behrens 1993; Chan et al. 2004; German and Horn 2006; German et al. 2014; German et al. 2015). The herbivorous and omnivorous species clearly have greater carbohydrate digestive capacity and positive allometry of gut length in comparison to the carnivores (German et al. 2004; German et al. 2014; German et al. 2015; German et al. 2016). All of these species are sympatric, meaning they experience similar environmental conditions in their intertidal habitat, with diet being one of the only differences among them in the wild (German and Horn 2006; German et al. 2004; German et al. 2015).

This study had two main objectives: (1) an evaluation of differences in diet, gut length, and genes under selection from the digestive system and liver among wild-caught fishes with different diets (Table 2.1); and (2) an evaluation of gut length, gene expression patterns of the

digestive system and liver, growth rates, and metabolic rates of the same species experiencing dietary shifts in the laboratory (Table 2.2). Objective one allows us to examine baseline differences among species with different diets in the wild, whereas objective two allows us to see how flexible these parameters are in the face of laboratory dietary perturbations. We focused on gut length (i.e., the length of the entire digestive system; German and Horn 2006), which can show plasticity, and tends to be longer in fishes consuming lower quality foods, such as algae (Farrell A.P. 2011; German and Horn 2006; Davis et al. 2013). Although detailed gut content analyses have been performed on pricklebacks in previous investigations (German and Horn 2006; German et al. 2004; German et al. 2015), there are limited studies using stable isotopic analyses to examine trophic relationships in these species (Saba 2004), and thus, we measured the $\delta^{13}C$ and $\delta^{15}N$ signatures of the fishes' livers from the wild to discern dietary differences among wild-caught fishes (Guelinckx et al. 2007). We also used liver stable isotopic signatures to confirm that the laboratory-reared fishes were assimilating the assigned diets. In the laboratory, as measures of performance on the different diets, we measured growth rate across a four-week feeding trial, and the routine metabolic rates of the fish to observe whether different diets altered their metabolic rates (Reardon and Chapman 2010).

Based on previous investigations of prickleback digestive physiology, genetics of amylase genes, and a genome of the herbivorous *Cebidichthys violaceus*, we expected to find signatures of dietary specialization in the different fish tissues (German et al. 2014; German and Horn 2006; German et al. 2004; German et al. 2015) . Beyond the intestinal tissues, in which we predicted to observe evidence of selection on digestive enzyme genes (Table 2.1), and differences in gene expression in response to laboratory diet shifts (Table 2.2), the liver may provide insight into metabolic pathways favored by the different species with different diets, and

whether those can shift when the animals are consuming different nutrient loads in the laboratory (Yang et al. 2017; Merkin et al. 2012). Thus, we investigated how specialized these animals are for their respective diets and provide insight into the underpinnings of their abilities (or lack thereof) to use a broader base of resources than they would naturally.

MATERIALS AND METHODS

Fish capture and tissue preparation of wild individuals

Juveniles of *X. mucosus*^{H}, *X. atropurpureus*^{O}, *P. chirus*^{O}, and *A. purpurescens*^{C} (112 individuals total) were collected by hand and dipnet in June 2016 at low tide from rocky intertidal habitats on San Juan Island (Dead Man Bay 48.51° N, 123.14° W and Cattlepoint; 48.45° N, 122.96° W). Superscript letters denote their natural diets: H=Herbivore, O=Omnivore, and C=Carnivore. Fifteen juveniles of each species were transported live in seawater to Friday Harbor Laboratories (Friday Harbor, WA) where they were placed in wet table aquaria with flow through seawater (held at approximately 13° C) to be used in a feeding experiment. The remaining individuals of each species (at least 11 of each species), abbreviated as WF (wild-caught fish), were euthanized with an overdose of tricaine methanesulfonate (MS-222 in 1 g L^{-1} seawater), measured [standard length (mm)], weighed (g), and dissected on a cutting board kept on ice (4° C) within 4 hours of collection. The digestive system of each fish was removed by cutting at the esophagus and at the anus. The gut was removed, uncoiled, and the total gut length (mm) measured as the distance from the pyloric sphincter to the distal-most end of the intestine. The measured digestive systems were used to calculate relative gut length, which is the ratio of gut length/standard length (German and Horn 2006). The liver, stomach, and pyloric ceca were excised. The intestine was divided into three sections of equal length and the sections were designated as the proximal, mid, or distal intestine. The contents of the stomach and intestine

were emptied into their own vials. Approximately 100 mg of each of the tissues were immediately placed in 0.5-mL centrifuge vials containing RNAlater, and stored overnight at 4° C, and subsequently transferred to a -80° C freezer for storage until further processing (less than one week). The remaining portions of the tissues, stomach, and intestinal contents were frozen on dry ice and transferred to -80° C freezer for storage for stable isotopic analysis, digestive enzyme activity assays, and other uses.

Food preparation and feeding experiment

The remaining 15 individuals of *X. mucosus*^{H}, *X. atropurpureus*^{O}, *P. chirus*^{O}, and *A. purpurescens*^{C} were individually placed in cubicles (approximately 1.5-L in volume) within wet table flow-through aquaria and used for a feeding experiment. Each individual fish was anesthetized (0.1 g L^{-1} MS-222), measured and weighed, and assigned to a carnivore, abbreviated as LC (Lab Carnivore), or omnivore diet, abbreviated as LO (Lab Omnivore), at the start of the experiment. All individuals of *X. atropurpureus*^{O} and *P. chirus*^{O} were fed the LC diet, as none would consume the LO diet in the laboratory. The fishes were acclimated to laboratory conditions and the formulated diet for two weeks. Fresh thalli of the algal species *Ulva lobata* (Chlorophyta), *Mazzaella splendens* (Rhodophyta), and *Porphyra* sp. (Rhodophyta), all of which are common in the diets of *X. mucosus*^{H}, *X. atropurpureus*^{O}, *P. chirus*^{O} (Horn et al. 1986; German et al. 2004; German et al. 2014; German et al. 2015), were collected from the intertidal zone from which the fish were collected, and initially dried in the sun. Sundried algae were transferred into a 60° C drying oven and dried overnight. Flatfish (several species) were collected by seining and otter trawl around San Juan Island, WA, and were mortalities from fish surveys. Dead flatfish were decapitated and skinned to produce fillets, which were dried to a constant weight at 60° C. Vitamin and mineral premixes were obtained from Zeigler Bros.

Aquafeed, whereas other ingredients (Fish oil, casein, soybean meal, methyl cellulose) were purchased from various vendors. Dried algae and flatfish were ground to pass through a 1-mm screen with a food processor followed by mortar and pestle. The omnivore and carnivore diets created in the laboratory were composed of varying concentrations of carbohydrates (dried algae) and protein (fish) and constant concentrations of lipids, vitamins and minerals (Table 2.3). Once combined, ingredients were wetted with deionized water and mixed by hand with a whisk, spread onto a cafeteria tray, and dried to a constant weight at 60° C. The food was then crumbled and offered to the fish, which they readily consumed. Fish were fed their respective diets two-three times daily to satiation for four weeks. Feces were collected just before each feeding and the debris in each tank was siphoned out after each feeding. Proximate analyses of the diets were performed following methods of the Association of Official Analytical Chemists (AOAC International, 2006). The total fat, organic matter, carbohydrate, total protein, and energetic content were quantified for the omnivore and carnivore diets (German et al. 2010).

At the conclusion of the feeding trials (four weeks on the prescribed diets), the routine metabolic rates of each fish were measured in a respirometer and taken over a short period of time. Negative control runs (i.e., without a fish in the system) validated that there was little oxygen consumption in the system itself across the time frames of measurement (~15 min intervals). The fish were fed their normal morning feeding because we wanted to examine any instantaneous effects of the different diets on their metabolic rates. The closed chamber respirometer resembled that described by Reardon and Chapman (Reardon and Chapman 2010), featuring a 400 mL chamber that housed the fish, and the system contained a total of 1.9L with a flow rate of 5L per minute set with a pump and flow meter (Supplemental Figure S1). Prickleback fishes are benthic (e.g., Ralston and Horn 1986), and the chosen flow rate did not

force them to swim within the chamber. The fishes sat on the bottom of the chamber, unencumbered, for the measurements. Decreases in oxygen concentration (% O_2 saturation) were used to estimate the rate of $\dot{V}O_2$ (volume of oxygen consumed per unit time) of the fish. Oxygen and temperature data were recorded every 30 s during the trial with Ocean Optics FOXY probes and thermistors, respectively. The temperature was maintained at 14ºC (±0.2ºC) by submerging the chamber in flow-through seawater pumped directly from Friday Harbor (Supplemental Figure S1). The fish were allowed to acclimate to the chamber for at least 30 minutes before starting measurements. Once the O_2 concentrations dipped below 90% saturation (approximately 15 min in the closed system), valves were manually opened, flushing the system with ambient seawater for five minutes, then manually closed again for the next measurement period. Each fish was measured three times (see Supplemental Figure S1 panel B for a representative trace). The nature of the setup did not allow us to keep the fish in the system for extended periods of time without causing significant further stress on the fish. Thus, we did not determine basal metabolic rate or specific dynamic action.

At least one full day following the metabolic rate measurements, including being fed, the fish were euthanized, measured, weighed, and dissected as described above under "*Fish capture and tissue preparation of wild individuals*". Tissues were subsampled for transcriptomic and stable isotopic analyses (see below under "*RNA Isolation and Library Preparation*"), with the remainder used for a separate study of digestive enzyme activity levels, gut ultrastructure, and gut microbiome. Growth of the individual fish was assessed as weight gained between the beginning and end of the experiment. There were no mortalities throughout the feeding experiment.

Stable Isotopic Analyses

To assess carbon and nitrogen assimilation from the diets, we measured $\delta^{13}C$ and $\delta^{15}N$ signatures of liver tissue from wild-caught (four individuals for each species), LO-fed fishes (three individuals for each species) and LC-fed fishes (3 individuals for each species) and of the omnivore and carnivore diets made in the laboratory. Liver tissue and diets were dried overnight at 60°C, and ground into powder. Approximately 0.7 mg of individual liver or diet samples were then transferred into individual 5 mm x 9 mm tin capsules (Costech Analytical Technologies). Samples were run through a Fissions NA 1500NC elemental analyzer interfaced to a ThermoFinnigan-DeltaPlus CF (Bremen, Germany) isotope ratio mass spectrometer in the Center for Isotope Tracers in Earth Science facility at UC Irvine. Stable isotope abundances are expressed in delta (δ), defined as parts per thousand (‰) relative to the standard as follows:

$$\delta = [(R_{sample}/R_{standard}) - 1] (1000) \quad (1)$$

where R_{sample} and $R_{standard}$ are the corresponding ratios of heavy to light isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample and standard, respectively. $R_{standard}$ for ^{13}C was Vienna Pee Dee Belemnite (VPDB) limestone formation international standard. $R_{standard}$ for ^{15}N was atmospheric N_2. Analyses were performed following German and Miles (2010).

RNA isolation and library preparation

Total RNA from the tissue samples (20-50mg) of the pyloric ceca, mid-intestine, and liver from two individual fish of each of the four species were isolated using TRIzol reagent (Thermo Fisher Scientific) following the manufacture's protocol. We chose to evaluate more tissue types (three) as opposed to more replicates (two) of the same tissue type to get more coverage of expressed genes. We used Principal Component Analysis (PCA) to examine the appropriateness of our replicates, which appear sufficient, as each tissue is similar to itself as opposed to being more similar to other tissues (Supplemental Figures S2-S11). All samples were

extracted and prepared within days of each other. Samples were quantified (ng/µl) using an RNA Nanodrop and RNA quality was determined by Bionalyzer (RNA Integrity > 7) at the UC Irvine Genomics High Throughput Facility. Samples were prepped for Illumina Sequencing using a TruSeq RNA sample prep kit (Illumina, San Diego, CA) to prepare individual cDNA libraries. Agencourt AMPure XP magnetic beads were used to re-purify the samples (Beckman Coulter Genomics, Danvers, MA). The Bioanalyzer again was used to conduct a quality control check of the cDNA. The cDNA pools were normalized to 10 nM and samples were scattered, as to not have a lane or batch effect, with the pyloric ceca and mid-intestine samples run across four lanes and two runs, and a separate run containing the liver samples to constitute three paired-end 100 bp runs on a HiSeq 2500 (Illumina, San Diego, CA) by the UCI Genomics High-Throughput Facility. All data generated were deposited into NIH Archive with accession number PRJNA738880.

Assembly of Sequence Reads and Gene Annotation

Raw data files were filtered and trimmed with Trimmomatic v0.32 (Bolger et al. 2014) implemented in UCI's High Performance Cluster (HPC), in order to make certain that trailing bases have a phred score of a minimum of 30. Reads were then normalized to low systematic coverage to remove errors and reduce data set size using the Trinity v r2015-2.1.1 normalize_by_kmer_coverage.pl script (Haas et al. 2013). Such normalization reduces among-sample bias (Abrams et al. 2019). A *de-novo* assembly using Trinity v r2015-2.1.1 was conducted, where one "wild" individual as selected as the reference assembly and used the RNA-seq by Expectation Maximization (RSEM) package v1.2.31 to align RNA-Seq reads back to the Trinity transcripts (Grabherr et al. 2011; Li and Dewey 2011; Mandelboum et al. 2019). Annotation was conducted with Trinotate v3.0.0 annotation suite for genes under differential

expression, the full transcripts of the wild individuals, and ortholog pairs and clusters. Trinotate uses TransDecoder v2.0.1 (Haas et al. 2013) to identify open reading frames (ORF), then translated and untranslated ORFs are blasted (BLASTX) against the swiss-prot database, where the best hit and gene ontologies (GO) are used for annotation. Afterwards, HMMER v3.1 tool hmmscan (Finn et al. 2011) and the Pfam-A database (Punta et al. 2012) are used to annotate protein domains for the predicted protein sequences.

Quality Check Samples and Biological Replicates

We sequenced the transcriptomes of the liver, pyloric ceca, and mid intestine from two individuals from each diet group in each species, and we conducted a quality check to ensure our biological replicates are well correlated using the Trinity program "PtR" (Haas et al. 2013). Samples within a diet group were well correlated within their respective tissue when comparing across the liver, pyloric ceca, and mid-intestine samples. Principal Component Analysis (PCA) plots for each species and diet group comparing across the three different tissues we sequenced are displayed in Supplementary Figures S2-S11, showing that the tissue replicates are more similar to each other than any are to other sequenced tissues. We also conducted BatchQC analysis to check for any batch effects, and found that there were no strong correlations with batch as displayed in Supplementary Figure S12. Therefore, we are confident that our low sample sizes for transcriptomics are suitable for the level of analysis conducted here.

Ortholog identification and estimation of positive selection in wild-caught fishes

Assembled sequences were masked for repetitive elements with Repeatmasker v4.0.5 (Smit 2004) with teleost fish as the query species. Using the standalone Orfpredictor v3.0 (Min et al. 2005), the open reading frame was identified and sequences with a minimum length of 60 nucleotides were used to identify orthologous pairs through Inparanoid v.4.0 (O'Brien et al.

2005) with all pairwise comparisons of the four target species (6 possible pairwise comparisons). The ortholog pairs were used to identify ortholog clusters in all four species using Quickparanoid (http://pl.postech.ac.kr/QuickParanoid/). Then, perl scripts were used to obtain ortholog clusters comparing only one sequence per four target species, with a gene seed ortholog and confidence score of 1, with no tree conflict. Orthologs clusters with one orthologous gene from each species per cluster were used for the estimation of positive selection. Protein and nucleotide sequences of the orthologs were aligned using Muscle v3.7 (Edgar 2004) and pal2nal 12.2 (Suyama et al. 2006) based on translated coding sequences. *X. mucosus*^{H} was used as a reference dataset to represent the ortholog clusters identified from all four species and, therefore, was used to annotate orthologous clusters through the Trinotate annotation suite (see "Annotation of Genes"). A perl script was used to process multiple aligned ortholog clusters into CODEML as part of the PAML v4.8a package (Yang 1997) in order to estimate positive selection. To identify genes under positive selection from all four species of wild-caught fishes, we used the following site models: M0 (one omega), M7 (beta distributed variable selective pressure), and M8 (beta distributed with positive selection) in PAML v4.8a. Models M7 (neutral) and M8 (positive selection) were compared, in which the likelihood values were used to detect positive selection using Likelihood Ratio Test (LRT). Pchisq in R v3.4.4 was used to compare LRT values of M7 and M8 with a $\chi 2$ distribution with an α level of significance at 0.05. We used Benjamini-Hochberg corrected p-values that was calculated from the $\chi 2$ distribution values and an α level of significance at 0.05. We viewed only the first represented Gene Ontology (GO) for biological processes by using REViGO (http://revigo.irb.hr/) and their corresponding omega values from the M0 PAML results.

To identify genes under positive selection, we used the omega values from the M0 PAML results. Branch selection was examined using adaptive branch-site random effects likelihood (aBSREL) test for episodic diversification (Datamonkey v 2.0 web application), and curated manually using the PAML results (Weaver et al. 2018; Smith et al. 2015; Kosakovsky Pond et al. 2011).

Differential Expression Level Analysis

Relative expression levels of all genes expressed in tissue types of interest were standardized to constitutively expressed Ribosomal Protein L8 using FPKM ratios calculated with eXpress (Roberts and Pachter 2013). Then, relative gene expression levels were estimated using RSEM v1.2.31 (Li and Dewey 2011), which allows for the identification of gene and isoform abundance. Therefore, the calculated gene expression can be directly used for comparing differences among individuals of the same species experiencing different diet challenges. Then, we calculated differences in the abundance of expression of each gene within individuals of the same species across the diet groups and generated heatmaps using EdgeR (Bioconductor v3.2) with an FDR <0.001 and a dispersion value of 0.4. This was carried out for each tissue type and each species separately.

For clarity purposes, heatmaps were broken into clusters based on expression profile, which are described in Table 2.5 and Supplemental Materials and Methods (online).

Statistical analyses

For study objective one, interspecific comparisons of relative gut lengths were made among the species of wild-caught fishes with Analysis of Covariance (ANCOVA), using body mass as a covariate. A Tukey's Honest Significant Difference (HSD, with an α=0.05) was used to evaluate what species had longer guts than the others. $\delta^{13}C$ and $\delta^{15}N$ values were compared

(separately) among the wild-caught fishes using ANOVA followed by a Tukey's HSD. Ortholog comparisons and genes under positive selection are described above (see "Ortholog identification and estimation of positive selection in wild-caught fishes"). For study objective two, intraspecific comparisons of relative gut length were made among individuals fed the various diets in the laboratory and the wild-caught fish consuming their natural diets, using ANCOVA with body mass as a covariate. Outliers that were more than twice the 1.5 interquartile range were removed from statistical analyses. For *X. mucosus*^{H} and *A. purpurescens*^{C}, intraspecific comparisons of growth rates and metabolic rates among individuals fed the LO and LC diets were performed with a *t*-test. Intraspecific comparisons $\delta^{13}C$ and $\delta^{15}N$ values amongst wild-caught and lab-fed fishes were made with ANOVA followed by Tukey's HSD. In addition to intra-specific comparisons of differentially expressed genes among fishes fed the different diets in the laboratory and wild-caught fishes, we also examined the similarity of expressed genes in different tissues of all of the fishes using PCA, which allowed us to qualitatively state which tissues showed the most plasticity in gene expression among the species and diet treatments. We also used the PCA vectors to estimate what made the species different from one another in terms of expressed genes. All statistics were run in R (version 3.6.0).

RESULTS

Objective 1: Comparisons of Wild-Caught Fishes

Relative Gut Length

Significant differences in relative gut length were detected among wild fishes (WF) of the four species (ANCOVA species: $F_{3,44}$=8.98, P<0.001; body mass: $F_{1,44}$=11.80, P<0.01, Species x Body Mass interaction: $F_{3,44}$=0.736, P=0.537; Fig. 2.2), with *X. mucosus*^{H} possessing the longest guts, and no significant differences detected amongst the other species.

Stable Isotopic Analyses

When comparing wild-caught individuals of the different species to each other, WF *X. atropurpureus*^{O} showed enriched $\delta^{13}C$ and $\delta^{15}N$ signatures in comparison to WF *X. mucosus*^{H} (ANOVA: Carbon $F_{3,12}$=4.428, p=0.0258; Nitrogen $F_{3,12}$=3.963, p=0.0355, Supplemental Table S1; Figure S13), isotopically confirming that these sister taxa have different diets. Wild *P. chirus*^{O} also showed a significantly enriched $\delta^{13}C$ signature compared to WF *X. mucosus*^{H} (ANOVA: Carbon $F_{3,12}$=4.428, p=0.0258, Fig. 2.3; Supplemental Table S1; Figure S13). Wild *A. purpurescens*^{C} had $\delta^{13}C$ and $\delta^{15}N$ signatures intermediate to the other species.

Orthologous Genes and Ortholog Clusters

There are not as many shared orthologs among all four wild species in the liver (870 orthologs) compared to the pyloric ceca (3,787 orthologs) and the mid-intestine (3,267 orthologs, Table 2.4). The closer phylogenetically related species, such as the *X. mucosus*^{H} and *X. atropurpureus*^{O}, share more orthologs with each other (e.g., pyloric ceca: 17,111 ortholog pairs) compared to a more distantly related species, such as *X. mucosus*^{H} and *A. purpurescens*^{C} (e.g., pyloric ceca: 13,079 ortholog pairs; Table 2.4).

Positively Selected Genes

Liver: We found fatty acid-binding protein, mitochondrial import inner membrane translocase subunit (TIM21), and endothelial lipase under positive selection in the liver and the transcripts contained high (100%) to medium coverage (~50%) of the full gene from the swissprot database (Supplemental Table S2, Fig. 2.4). Glucose-6-phosphate 1-dehydrogenase (G6PD), which is part of the pentose phosphate pathway, is under positive selection in *P. chirus*^{O}. There is branch selection in G6PD for *A. purpurescens*^{C} and *P. chirus*^{O} (Fig. 2.4). Looking at the sites under selection, we found that many sites in the transcriptome of *P. chirus*^{O} are significantly different

from the other three species. For fatty acid-binding protein, TIM21, and lipase, we find branch selection in *P. chirus*^{O} and significant selection at multiple sites in the transcriptome of *P. chirus*^{O}*,* making this species stand out from the other three species (Fig. 2.4).

Pyloric ceca: The pyloric ceca featured genes involved in protein and fatty acid metabolism under positive selection, including aminopeptidase, phospholipase, elastase, and tubulin alpha chain (Supplemental Table S3, Supplemental Figure S14). The transcripts contained high to medium coverage. Serine protease 27 is under positive selection in *P. chirus*^{O} and *A. purpurescens*^{C}, and there is branch selection in *A. purpurescens*^{C} as well (Supplemental Figure S14). For tubulin alpha chain, there is branch selection for *X. mucosus*^{H} (Supplemental Figure S14). There is also branch selection in *X. mucosus*^{H} and *A. purpurescens*^{C} for the protease elastase.

Mid-intestine: The mid-intestine featured genes involved in carbohydrate digestion and metabolism, including alpha-mannosidase, succinate dehydrogenase, and NADH dehydrogenase (Supplemental Table S4).

Objective 2: Dietary Flexibility in the Laboratory

Relative Gut Length

Wild *X. mucosus*^{H} had significantly longer guts than individuals of this species fed a carnivore diet in the laboratory (LC), yet the gut lengths of the fish fed an omnivore diet in the laboratory (LO) were not statistically different from WF fish or LC fish of this species (ANCOVA diet: $F_{2,20}$=2.32, P=0.124; body mass: $F_{1,20}$=1.53, P=0.231). There were no significant differences among the relative gut lengths of WF *X. atropurpureus*^{O} (ANCOVA diet: $F_{1,21}$=0.013, P=0.912; body mass $F_{1,18}$=3.682, P=0.071) and WF *P. chirus*^{O} fish (ANCOVA diet: $F_{1,24}$=0.623, P=0.439; body mass $F_{1,21}$=3.322, P=0.08) and LC fish within each respective

species. LO *A. purpurescens*^{C} had a significantly longer gut length than WF fish and LC *A. purpurescens*^{C}, with the two latter groups not being significantly different in this species (ANCOVA diet: $F_{2,22}$=3.67, P<0.05; body mass: $F_{1,22}$=5.03, P<0.05, Diet x Body Mass interaction: $F_{2,22}$=0.148, P=0.863; Fig. 2.2).

Interspecific comparisons across species within a diet group showed that LO *X. mucosus*^{H} had significantly longer guts than LO *A. purpurescens*^{C} *(*ANCOVA species: $F_{1,11}$=12.89, P<0.01; body mass: $F_{1,11}$=0.72, P=0.41. Additionally, LC *X. mucosus*^{H} possessed the longest guts compared to the other three species (ANCOVA species: $F_{3,29}$=5.81, P<0.001; body mass: $F_{1,29}$=2.56, P=0.12; Fig. 2.2).

Growth Rate and Metabolic Rate

After four weeks of the feeding trial, LC *X. mucosus*^{H} (16.8 ± 2.6%) exhibited a significantly higher growth rate than LO fish (5.4 ± 5.2 %; t=4.552, df=11, p=0.001; Supplemental Tables S5 and S6). There was no significant difference in growth rate between LC (22.7 ± 12.0 %) and LO (20.5 ± 12.9 %) *A. purpurescens*^{C} (t=0.309, df=11, p=0.763). The growth rate of LC *P. chirus*^{O} individuals was 21.68 ± 8.37 % and for LC *X. atropurpureus*^{O} individuals it was 11.45 ± 5.8 % (Supplemental Table S6). The sizes of the fishes used in this study are in Supplemental Table S5, and it is worth noting that the individuals of *X. mucosus*^{H} were approximately double the masses of the other species.

The routine metabolic rate of LC and LO fishes of *X. mucosus*^{H} (t=0.741, df=9, p=0.478) and *A. purpurescens*^{C} (t=0.936, df=9, p=0.373, Supplemental Table S6) did not differ significantly within each species. There was no statistical difference among the metabolic rates of all species fed diets in the laboratory.

Stable Isotopic Analyses

The LC and LO fishes clearly assimilated the laboratory diets and are different from WF fish within the same species (Fig. 2.3). From the $\delta^{15}N$ perspective, WF *X. mucosus*^{H} (ANOVA: $F_{2,7}$=12.21, $p < 0.05$) and WF *A. purpurescens*^{C} (ANOVA: $p < 0.05$, $F_{2,7}$=18.73) differed significantly from LC and LO fish within the respective species, with LC and LO groups not being statistically different (Fig. 2.3, and see Supplemental Table S1 for more statistical detail). LC *P. chirus*^{O} showed a statistically significant enrichment in $\delta^{13}C$ (ANOVA: $F_{1,5}$=144.2, $p<0.001$) and $\delta^{15}N$ (ANOVA: $F_{1,5}$=29.58, $p<0.05$) signatures of their livers relative to WF *P. chirus*^{O} fish. LC *X. atropurpureus*^{O} showed a slight enrichment in $\delta^{15}N$ signatures (ANOVA: $F_{1,5}$=3.054, p=0.141) compared to the WF *X. atropurpureus*^{O} fish, but not significantly so (Fig. 2.3, Supplemental Table S1). When consuming the same diet in the laboratory, LO *X. mucosus*^{H} and LO *A. purpurescens*^{C} differed significantly for $\delta^{15}N$ (ANOVA: $F_{1,4}$=10.25, p=0.0328, Supplemental Table S1), but not for $\delta^{13}C$ (ANOVA: $F_{1,4}$=0.216, p=0.666, Supplemental Table S1). When consuming the carnivore diet in the laboratory, LC *P. chirus*^{O} stood out from the rest of the species and had statistically significant enriched $\delta^{13}C$ signatures (ANOVA: $F_{3,8}$=11.10, $p<0.05$, Supplemental Table S1). In terms of $\delta^{15}N$, LC *P. chirus*^{O} were enriched in comparison to LC *X. mucosus*^{H} (ANOVA: $F_{3,8}$=3.976, p=0.0526, Supplemental Table S1), but no other differences were detected.

Relative Gene Expression

We used RNA-seq data of the liver, pyloric ceca and mid-intestine for each of the four species to observe the suites of genes that changed with different diets and how species respond to dietary variation. Relative expression levels of all genes expressed in tissue types of interest, which included genes involved in digestion (e.g., digestive enzymes, nutrient transporters, metabolic pathways), were analyzed. Note that we are only reporting on pathways relevant to

digestion and metabolism of specific nutrient classes (Fig. 2.5, Table 2.5). If a cluster is not mentioned, yet depicted in the heatmap, then the genes within that cluster were not directly relevant to digestion and nutrient metabolism. For simplicity and space, we share heatmaps for *X. mucosus*^{H} only, and all other heatmaps, as well as details of these results, are available in the supplemental materials (Supplemental Tables S7-S9, Supplemental Figures S14-S22).

Liver: Overall, the most interesting finding for the liver with regards to transcriptomic analyses was how few genes showed changes in expression in the fishes fed the different diets. While 37 genes were differentially expressed between WF, LO and LC *X. mucosus*^{H}, 30% of genes were annotated (Fig. 2.5). Cluster 1 (elevated in wild fish) consisted of genes for lipid metabolism (Table 2.6, Supplementary Table S7). Cluster 2 (wild-omnivore genes) consisted of genes for gluconeogenesis, while Cluster 5 (carnivore genes) contained genes responsible for the cellular processes associated with protein digestion and metabolism.

Pyloric ceca: For most species, there were more differentially expressed genes (DEGs) in the pyloric ceca than the liver. For instance, there were 183 DEGs when comparing WF, LO and LC *X. mucous*^{H}, out of which 49.2% of genes were annotated (Fig. 2.5). Cluster 1 (elevated in wild fish) consisted of various genes involved in fatty acid and cholesterol biosynthesis, as well as in lipid, collagen, and protein metabolism (Table 2.6). Cluster 2 (wild-omnivore genes) consisted of various genes involved in fatty acid and cholesterol biosynthesis as well as collagen and bile acid metabolism.

Mid-intestine: There were consistently several hundred DEGs amongst the lab and wild-caught fishes when examining the mid intestine. There were 336 DEGs when comparing WF, LC, and LO *X. mucosus*^{H}, out of which 37.8 % were annotated (Fig. 2.5). Cluster 1 (elevated in wild fish) contained genes for collagen catabolism, chitin metabolism, and fatty acid biosynthesis (Table

2.6). Cluster 2 (wild-omnivore genes) contained genes for cholesterol biosynthesis. Cluster 3 (elevated in the lab genes) contained genes were involved in protein metabolism, glycolysis, and feeding behavior.

Comparisons among all tissues: We generated Principal Components Analysis (PCA) plots for each tissue based on the first two PCs, which explain most of the variation (Figure 6). In the liver, pyloric ceca, and mid-intestine, individuals group by species and within each species, by diet group. In the liver, there is less variation than the digestive tissues as shown by the relatively more constrained axes, and 3-hydroxyxyanthranilate 3,4-dioxygenase explains some of the variation in *X. mucosus*^{H}. In the pyloric ceca, the gene for plectin explains some of what sets *X. mucosus*^{H} apart from the other species, while ATP-citrate synthase explains some of the variation in *A. purpurescens*^{C} (Figure 6B, Supplementary Table S10). When all tissues are combined, we find grouping by tissue, with the liver being the least plastic and the mid-intestine exhibiting the most variation and plasticity followed by the pyloric ceca.

In addition, we generated a correlation matrix of the gene expression patterns of all the tissues we measured. The samples tended to cluster by tissue, species, and diet (WF, LO, LC), with the exception of liver, which did cluster by tissue and species, but not by diet, reflecting the lack of expression changes seen in the lab fishes *vs* the wild fishes (Supplemental Fig. S24). *P. chirus*^{O} showed the most divergent expression patterns, particularly in their livers (distinct cluster 1), where they did not group with any other species (Supplemental Fig. S24). Interestingly, *A. purpurescens*^{C} and *X. atropurpureus*^{O} group adjacent to each other for pyloric ceca, and liver, but *X. atropurpureus*^{O} grouped more with *P. chirus*^{O} for mid intestine gene expression patterns (Supplemental Fig. S24). *X. mucosus*^{H} tended to cluster more with itself,

representing its unique status as the herbivorous fish in this study. These data support what is shown in the PCA plots (Fig. 2.6).

DISCUSSION

This study showed that prickleback fishes have variable responses to dietary perturbations, thus indicating that natural diet and species identity affect digestion and metabolism in some predictable (Tables 2.1 and 2.2) and unpredictable ways. Gut length largely varied with diet quality (Fig. 2.2), agreeing with our expectations that herbivorous fishes generally have longer guts than carnivorous fishes. Moreover, although we did see differences in expressed genes in response to dietary shifts, the liver appears to be less flexible than other tissues, hinting that liver metabolism may be where dietary specialization manifests the most in prickleback fishes, and in a species-specific manner. We will address study objectives one and two in order.

Objective 1: Comparisons of Wild-Caught Fishes

One of the biggest determinants of gut length is intake: animals eating lower-quality foods (higher fiber, less soluble nutrients) have higher intake, and hence, more rapid transit of digesta through their guts (German 2011). This rapid transit of digesta requires a longer gut to allow for adequate nutrient absorption in terms of time and surface area (Al-Hussaini 1947; German and Horn 2006; Kapoor 1975; Horn 1989; Kramer 1995a; Leigh et al. 2018a; Davis et al. 2013). Indeed, wild herbivorous *X. mucosus*^{H} have longer guts than the other prickleback species (German and Horn 2006; German et al. 2014), and a longer gut often equates to greater absorptive surface area achieved through increased intestinal folding and more microvilli (Karasov and Hume 1997; Starck 2005; (Secor 2008) German et al. 2010; Leigh et al. 2018). Coinciding with these longer guts, tubulin alpha chain, which is a constituent of microtubules

that are critical in microvilli structure in the gut (Paradela et al. 2005), is under positive selection in the pyloric ceca of *X. mucosus*^{H} (Supplemental Table S4), and this species shows more positive gut length allometry as they grow than the other prickleback species we studied (German et al. 2014). The PCA vectors also show that plectin genes make *X. mucosus*^{H} stand apart from the other taxa in terms of genes expressed in the pyloric ceca (Fig. 2.6, Supplementary Table S10). Plectins are also involved with microvilli lengthening and structure (Wiche 1998; Weisz and Rodriguez-Boulan 2009). Hence, there is clear evidence that the herbivorous *X. mucosus*^{H} not only displays a longer gut, but has genes under selection that contribute to greater epithelial surface area, all of which allow them to thrive on an algal diet. The pyloric ceca and mid-intestine are similar in function, as both are highly absorptive (Buddington and Diamond 1987; Heras et al. 2020), and therefore, finding genes that express proteins involved with increasing surface area in the pyloric ceca agrees with the function of that tissue.

To our knowledge, only one other study (Saba 2004) examined how diet affects the $\delta^{13}C$ and $\delta^{15}N$ signatures of prickleback fishes. Examining the isotopic signature of tissues with a high protein turnover rate and that are metabolically active, such as the liver, allows us to track the isotopic composition of diet closely (Karasov and Martínez del Rio 2007). In wild fishes (Supplemental Figure S13), we isotopically confirmed that the sister taxa *X. mucosus*^{H} and *X. atropurpureus*^{O} consume different diets, with *X. atropurpureus*^{O} being enriched from the carbon and nitrogen perspective. As noted previously, WF *P. chirus*^{O} are more enriched from the carbon perspective compared to WF *X. mucosus*^{H} and *P. chirus*^{O} consumes the most crustaceans among the four studied species (German et al. 2015). Contrary to expectations (Barton 1982; German et al. 2004; German and Horn 2006; German et al. 2014), the stable isotopic signature of *A.*

purpurescens^{C} suggests a mixed diet that should be examined in more detail, especially since they do not appear to assimilate algal protein in the lab (Fig. 2.3). *Anoplarchus purpurescens*^{C} consume more worms in nature than the other pricklebacks, which may skew their $\delta^{15}N$ signatures to be lower than the other fishes consuming more crustaceans (German and Horn 2006), although the elevated expression of α-mannosidase in the mid-intestine of *A. purpurescens*^{C} (Supplemental Table S9) may be involved with digestion of glycoproteins in crustaceans (Kuballa and Elizur 2008).

The genes under positive selection among the prickleback species are mostly relating to lipid metabolism in the liver (Table 2.4; Fig. 2.4), and mostly in *P. chirus*^{O}, although niacin metabolism is also identified as an important gene in *X. mucosus*^{H} using the PCA vectors (Fig. 2.6). Fishes consuming higher carbohydrate diets seem to require more niacin, and herbivorous diets are lower in tryptophan (a niacin precursor) and niacin itself than carnivorous diets (Shiau and Suen 1992; Hansen et al. 2015). Because they naturally have a higher-carbohydrate diet in comparison to the other studied fishes, *X. mucosus*^{H} may have ramped up niacin synthesis pathways (specifically 3-hydroxyanthranilate 3,4-dioxygenase) to ensure they have enough NAD and NADPH for metabolism like the Citric Acid Cycle and lipid synthesis. With the strong interest in moving towards more sustainable, plant-based aquaculture feeds, in addition to examining how herbivorous fishes digest algal diets (e.g., Heras et al. 2020), understanding what metabolic pathways are ramped up in herbivores is also important, and these findings on niacin metabolism in *X. mucosus*^{H} may provide a new avenue to explore in herbivorous fish aquaculture (e.g., Hansen et al. 2015). Several proteolytic genes were under positive selection in the pyloric ceca and mid intestine of the fishes (Supplemental Tables S3 and S4; Supplemental Figure S14), but interestingly, they were not all in the carnivores, as *X. mucosus*^{H} showed selection on elastase

and trypsin. Although it is common to see elevated carbohydrase gene expression and enzyme activities in the guts of fishes eating more plant material (e.g., German et al. 2015; German et al. 2016; Heras et al. 2020), all fishes need protein, so it is not surprising to see selection on proteases in fishes consuming lower-protein diets, like *X. mucosus*^{H} (Heras et al. 2020).

Objective 2: Dietary Flexibility in the Laboratory

The flexibility of the prickleback gut length in response to the laboratory diets changed in accordance with intake, with LC fishes having shorter guts than LO fishes (Table 2.2, Fig. 2.2). Interestingly, changes in gut length are reflected on the molecular level, with the upregulation of genes involved in the generation of microtubules and muscle fiber in wild and LO fishes in comparison to LC fishes, particularly in *X. mucosus*^{H} (Table 2.6). This indicates changes on the molecular level to achieve a larger gut (Castoe et al. 2011). The changes in gut size and concomitant changes in gene expression support models of cellular hypertrophy (Starck 2005; Leigh et al. 2018a) or cellular proliferation (Riddle et al. 2020) in fishes, that can affect gut surface area (Leigh et al. 2018a).

The mid-intestine is the primary site of digestion of carbohydrates, fats, and proteins and it is a highly absorptive region of end products, vitamins, and minerals (Stevens and Hume 1995; Heras et al. 2020). As expected, we do see diet-dependent changes in gene expression profiles of the mid-intestine. When examining the genes showing differential expression, it is clear that *X. mucosus*^{H} (and to a lesser extent, *A. purpurescens*^{C}) increased expression of proteolytic enzymes in response to the high-protein laboratory diets (Table 2.6). Serine proteases (including trypsin and chymotrypsin) were increased in expression on the LO and LC diets, concurring with increases in tryptic activity in these same species raised on high-protein diets (German et al. 2004), and showing the flexibility of the gut in response to the laboratory diets. Why the two

natural omnivores, *X. atropurpureus*^{O} and *P. chirus*^{O}, did not show many increases in gene expression for proteolytic enzymes or amino acid transporters in response to the LC diet is unclear, but the mid intestines of these taxa were more similar to each other than any of the other species (Fig. 2.6; Supplemental Fig. S24), suggesting some shared function among them. Finally, trypsin, carboxypeptidase, chitinase, and lipase expression in the mid intestine confirms the broader distribution of pancreatic cells along the stichaeid intestine (Heras et al. 2020), showing it is not confined to the pyloric cecal region, as proposed previously (Gawlicka and Horn 2006).

The pyloric ceca of fish plays a key role in digestion and absorption, functioning in enzymatic digestion and nutrient absorption, including lipid digestion (Williams 2019; Buddington and Diamond 1987; Stevens and Hume 1995). Similar to our results, the pyloric ceca transcriptome of salmon fingerlings varying oil (Jin et al. 2018) or carbohydrate (Betancor et al. 2018) sources upregulated genes involved in lipid metabolism. In prickleback fishes, we found that wild fishes upregulate genes involved in protein, carbohydrate, and lipid metabolism and converting nutrients to energy storage in the pyloric ceca, yet we do not see this pattern in fishes fed the LO and LC diets. Although we changed the carbohydrate content in both lab-formulated diets (Table 2.3), we do not see fishes fed laboratory-formulated diets respond to the differences in carbohydrate content in the pyloric ceca (German et al. 2004; Kim et al. 2014). Instead, genes involved in key carbohydrate metabolism pathways, such as glycolysis, gluconeogenesis, and pentose phosphate pathway, which is a major pathway for glucose breakdown in fish, were upregulated in the pyloric ceca of wild *X. atropurpureus*^{O}, and this taxon has moderate chitin digestive capability (German et al. 2015). Elevated ability to digest chitin and protein may be why, for the

pyloric ceca, *X. atropurpureus*^{O} showed more similarity with *A. purpurescens*^{C} than other species when comparing the transcriptomics of all tissues (Fig. 2.6; Supplemental Fig. S24).

The pyloric ceca of *P. chirus*^{O} showed relatively few DEGs, and only wild individuals of this species upregulate genes to break down protein, even though we see many changes in the liver transcriptome and liver stable isotope signatures in *P. chirus*^{O}. Genes for protein and fatty acid metabolism are under positive selection in wild fishes as well. For instance, consistent with their high protein natural diets, serine protease 27 is upregulated and under positive selection in *P. chirus*^{O} and *A. purpurescens*^{C} (Supplemental Table S8, Supplemental Fig. S14).

Although metabolic rate didn't vary among the prickleback species fed different diets (see Supplemental Discussion for more information on metabolic rate), *X. mucosus*^{H} and *A. purpurescens*^{C} consuming the high-protein LC diet grew fastest in the laboratory, as was expected (Fris and Horn 1993; Horn et al. 1995; Leigh et al. 2018a). Thus, each of the prickleback species tolerated the carnivorous diet well.

The liver stable isotopic data (Fig. 2.3) showed that fishes assimilated and metabolized the formulated diets, agreeing with our expectations that fishes fed either an omnivore or carnivore diet in the lab would have stable isotopic signatures reflecting the laboratory diets. Given that fish liver tissue can isotopically turn over within a 28 day time frame (Guelinckx et al. 2007a; German and Miles 2010; Matley et al. 2016), the measured isotopic signatures likely reflect an equilibrium value for tissue-diet discrimination (German and Miles 2010). The fishes largely showed typical tissue-diet discrimination for $\delta^{13}C$ and $\delta^{15}N$. If the species were all the same in how they digested and metabolized the diets, they would completely overlap in the laboratory in terms of their $\delta^{13}C$ and $\delta^{15}N$ signatures (Saba 2004), but this was not the case. For one, *A. purpurescens*^{C} did not appear to assimilate much of the algal nitrogen in the omnivorous

diet, as their signatures were nearly identical on the two laboratory diets, suggesting this species was primarily digesting the fish protein within the LC and LO diets. Although not statistically significant, *X. mucosus*^{H} did show some variation in $\delta^{15}N$ on the two diets, with fish fed the LC diet trending upwards, suggesting that this species was indeed assimilating at least some algal protein on the LO diet, as would be expected for herbivorous pricklebacks (Horn et al. 1986; Fris and Horn 1993). The $\delta^{15}N$ of LO *X. mucosus*^{H} was also significantly lower than in LO *A. purpurescens*^{C}, suggesting these two species differed in how they digested and metabolized the LO diet. *P. chirus*^{O} stood out both in how much their isotopic signature and liver transcriptome changed when being fed the carnivore diet in the laboratory. These two factors may be related and are discussed below.

Liver exhibits species-specific responses

Although there were hundreds of genes differentially expressed among the three diet groups in the intestine (pyloric ceca and mid-intestine), three of the four species did not appreciably alter gene expression in the liver in response to different diets in the laboratory, and the liver showed fewer shared orthologs among wild-caught fishes than the other tissues. Consistent with previous studies that compared the liver with the intestine, there were few changes in the gene expression of the liver of prickleback fishes fed different diets, and the most responsive pathway is lipid metabolism in the laboratory-fed fishes (De Santis et al. 2015a). In our PCA plots (Fig. 2.6) and correlation matrix (Supplemental Fig. S24), it is clear that the liver shows the most species-specific expression patterns, and changed the least in response to the LO and LC diets. In the PCA plots, the liver axes were the smallest (i.e., covers the least amount of variable space), and the liver was in a tight space within the plot including all tissues and treatments (Fig. 2.6D). These liver expression patterns agree with previous investigations

showing more species and population-level liver gene expression patterns (Bernal et al. 2019; Merkin et al. 2012; Betancor et al. 2018).

Wild fishes of *X. mucosus*^{H}, *X. atropurpureus*^{O} and *A. purpurescens*^{C} showed elevated expression of genes for lipid metabolism and glucose metabolism in their livers, yet these same pathways were downregulated on the laboratory diets, similar to Atlantic Salmon fed plant-based diets in the laboratory (Król et al. 2016; De Santis et al. 2015a). The relatively few changes in liver gene expression in fishes fed formulated diets in the laboratory suggests that gene expression of the liver is not readily altered in the face of dietary perturbations, and instead, is more specialized by species and likely reflects natural diet. Merkin et al. (2012) also showed the liver is specialized based on species identity, when comparing liver gene expression profiles with other tissues in vertebrate animals. Similarly, Atlantic salmon fed high or low starch diets revealed population-level, not dietary, effects on liver metabolic pathway regulation (Betancor et al. 2018), whereas this same species showed few liver DEGs in response to dietary variation, unlike their pyloric ceca, stomach, or distal intestine, which showed increased expression of genes involved in lipid metabolism (Jin et al. 2018). Overall, only a handful of studies examine changes in liver gene expression in response to dietary differences, and several of them find that the liver exhibits a more tissue-specific response when comparing different diets and tissues, and we find that the prickleback livers' response is also species-specific and more tuned to natural diet. This is a truly novel result in this comparison of closely related species and argues that the liver may be an important aspect of dietary specialization.

While there are not many metabolic genes under positive selection in the liver, glucose-6-phosphate 1-dehydrogenase (G6PD), the enzyme involved in the first step of the pentose phosphate pathway, and endothelial lipase, an enzyme that breaks down plasma lipids for entry

into cells, are under positive selection in *P. chirus*^{O} (Fig. 2.4). Finding G6PD under positive selection in the liver agrees with previous studies that have found lipid metabolism pathways in the liver to be responsive to nutritional stress (De Santis et al. 2015a). Interestingly and contrary to what is found in the other three species, *P. chirus*^{O} shows large changes in gene expression in the liver when comparing wild fishes to fishes fed a carnivore diet in laboratory (Supplemental Figure S16). *P. chirus*^{O} on the LC diet upregulated genes involved in lipid metabolism, fatty acid synthesis, and bile acid biosynthesis, indicating this species ability to metabolize the LC diet. Further, the stable isotope signature of LC *P. chirus*^{O} fish livers were significantly more enriched from the carbon perspective compared to the other species (Fig. 2.3). While a typical tissue-diet discrimination factor for ^{13}C ($\Delta^{13}C$) in the liver ranges ±1.5‰ and most of the species studied here follow this expectation (Karasov and Martínez del Rio 2007; Caut et al. 2009), LC *P. chirus*^{O} fish livers show a $\Delta^{13}C$ of +4‰ . Additionally, when comparing only wild fishes, wild *P. chirus*^{O} only significantly differ in ^{13}C signature from wild *X. mucosus*^{H} fish, showing that they are not dramatically different from the other species in the wild (Supplemental Figure S13). It is worth noting that we did not extract lipids from the liver before stable isotope analysis. Lipids are typically depleted in $\delta^{13}C$ relative to their dietary source (Post et al. 2007), and in this study we found enrichment in δ13C in *P. chirus*^{O}, making the enrichment unlikely to do with liver lipid. We did try adjusting our data for lipid content with the equations in Post et al. (2007), which did not appreciably change anything. Instead, there may be a metabolic explanation for the large $\Delta^{13}C$ in *P. chirus*^{O}. Rito et al. (2019) found that an excess of ^{13}C in seabass could be explained by variability in pentose phosphate pathway activity (Jin 2014; Rito et al. 2019). It is possible that variability in the pentose phosphate pathway could explain the high $\Delta^{13}C$ in *P. chirus*^{O}, especially because G6PD, the enzyme involved in the first step of the pentose phosphate

pathway, is highly expressed in LC *P. chirus*^{O} (Supplemental Table S7, Supplemental Figure S16) and is under positive selection in wild *P. chirus* (Fig. 2.4). G6PD has previously been identified as influenced by diet and population in a feeding experiment on Atlantic Salmon (Betancor et al. 2018). Alternatively, a different aspect of their metabolism in the liver can selectively be routing in more ^{13}C to the liver, leading to an enriched signal in relation to the other fishes (Karasov and Martínez del Rio 2007), but still unique to this species.

Conclusions

In this study, we sought to understand dietary specialization in closely related, sympatric, prickleback fishes with different diets. We confirmed that gut length varies with diet quality, even intra-specifically, but there are limits to this since the herbivorous *X. mucosus*^{H} always had a longer gut than the other species, even when they consumed the same diet in the laboratory. Thus, gut length definitely has a genetic underpinning and is not just plastic (German and Horn 2006; Riddle et al. 2020). We observed positive selection on, and increased expression of, genes that would contribute to increased epithelial surface area in *X. mucosus*^{H}, but not the other taxa. Our transcriptomics data confirm the plasticity of the mid intestine in pricklebacks, affirming plasticity of digestive enzyme activities observed previously (German et al. 2004; Gawlicka and Horn 2005, Gawlicka and Horn 2006; Kim et al. 2014). Therefore, the gut itself can display enough plasticity to allow even an herbivore to digest a carnivorous diet, but the opposite is not true, as carnivorous fishes do not tolerate herbivorous diets (Król et al. 2016), and *A. purpurescens*^{C} did not appear to assimilate algal protein in this study. However, the real novel finding is how inflexible the liver gene expression patterns are. In fishes, true specializations may manifest with food acquisition (Burress et al. 2020) and then on the nutrient processing side of things once nutrients are absorbed into the blood stream (Wilmott et al. 2005; DeSantis et al.

2015a; Betancor et al. 2018). If the liver is inflexible, this means that although the gut itself can respond to shifts in nutrient concentrations entering the gut, the liver may not be equipped to process excesses of a nutrient class (e.g., glucose in carnivores, or amino acids in herbivores; Shiau and Suen 1992; Ferrais and Diamond 1997). Indeed, this may be related to the variance in signals received in the different tissues: the gut deals with large swings in nutrient concentrations, whereas the liver encounters much smaller changes in concentrations in the blood stream (Ferrais and Diamond 1997). Moreover, given the differences in niacin synthesis pathways we observed among the fishes, vitamin requirements may also be inflexible, and certainly vary among species based on natural diets (Shiau and Suen 1992; Hansen et al. 2015). Therefore, by taking more of a systems approach, we identify areas on which to focus in the quest to understand dietary specializations in fishes in ecological, evolutionary, and aquaculture contexts. Much work is still necessary to elucidate the mechanisms underlying liver specialization, which results in the liver being less plastic than digestive tissues in response to different diets. For instance, it is the loss of the uricase gene in primates that sets their liver function, particularly in response to fructose metabolism, apart from other mammals (Kratzer et al. 2014). Similar patterns, with different genes (e.g., G6PD), may emerge in fishes, and this study should help generate hypotheses for new directions in dietary specialization research. We do recognize that our low sample sizes for the transcriptomics analysis (n=2) may limit what we observed in this study, both, in terms of genes under selection (objective one), and for differentially expressed genes in response to a dietary perturbation (objective two), and thus, we need to sample more individuals in future studies. Nevertheless, the dataset presented here provides new directions in the field of fish nutritional physiology.

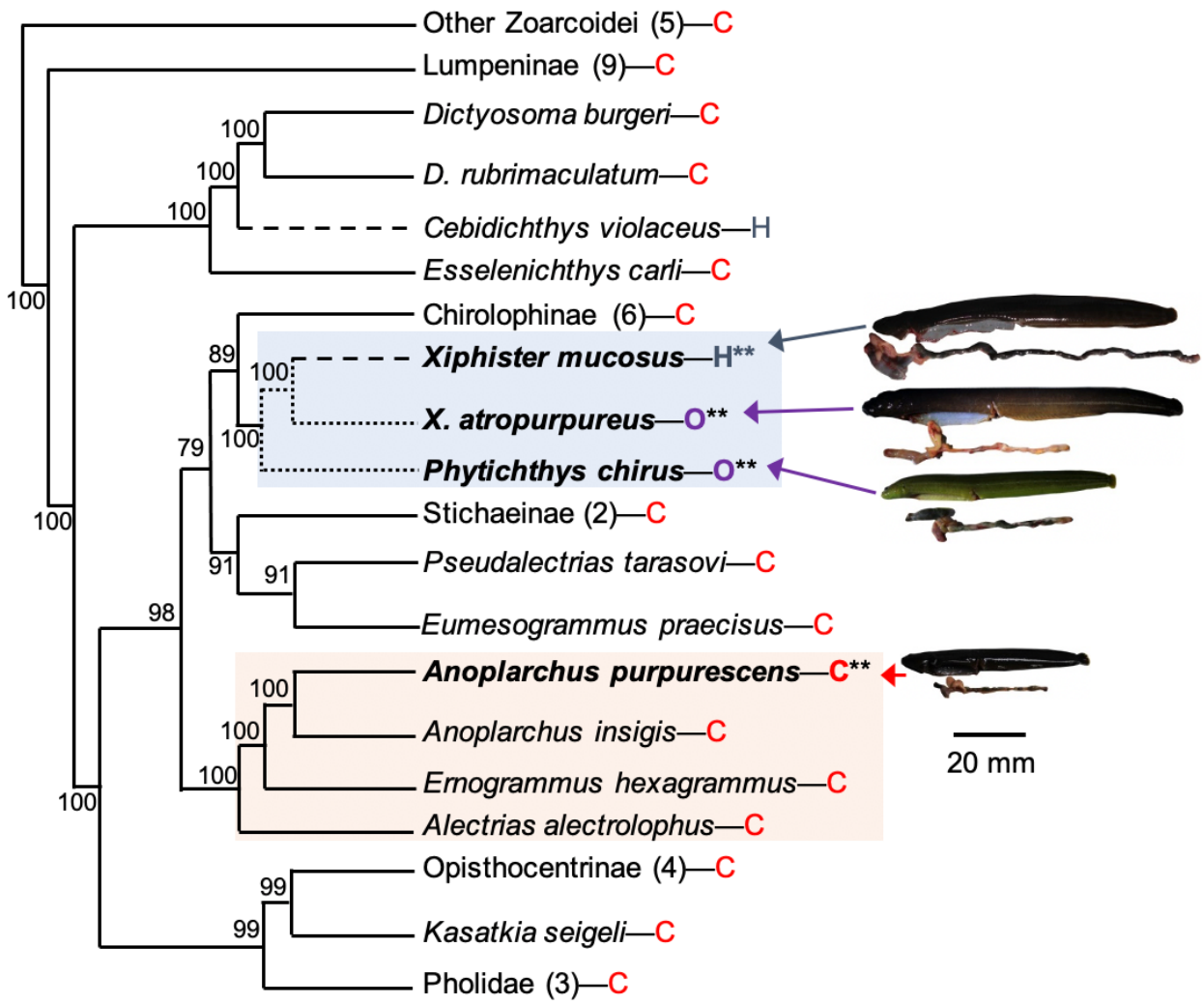

Figure 2.1 Phylogenetic relationships of the polyphyletic family Stichaeidae based on 2,100 bp of *cytb*, *16s*, and *tomo4c4* genes (Kim et al. 2014). Bayesian posterior probabilities are indicated on nodes. Studied taxa are bolded, and photos are shown with their digestive systems beneath their bodies. Note the differences in gut size. H=herbivory, O=omnivory, C=carnivory. Evolution of herbivory (— — — —) and omnivory (.....) are shown. Numbers in parentheses show number of taxa evaluated at that branch. Boxes highlight some of the alleged families or subfamilies within the polyphyletic family Stichaeidae, with Xiphisterinae (top), and Alectriinae (bottom) highlighted.

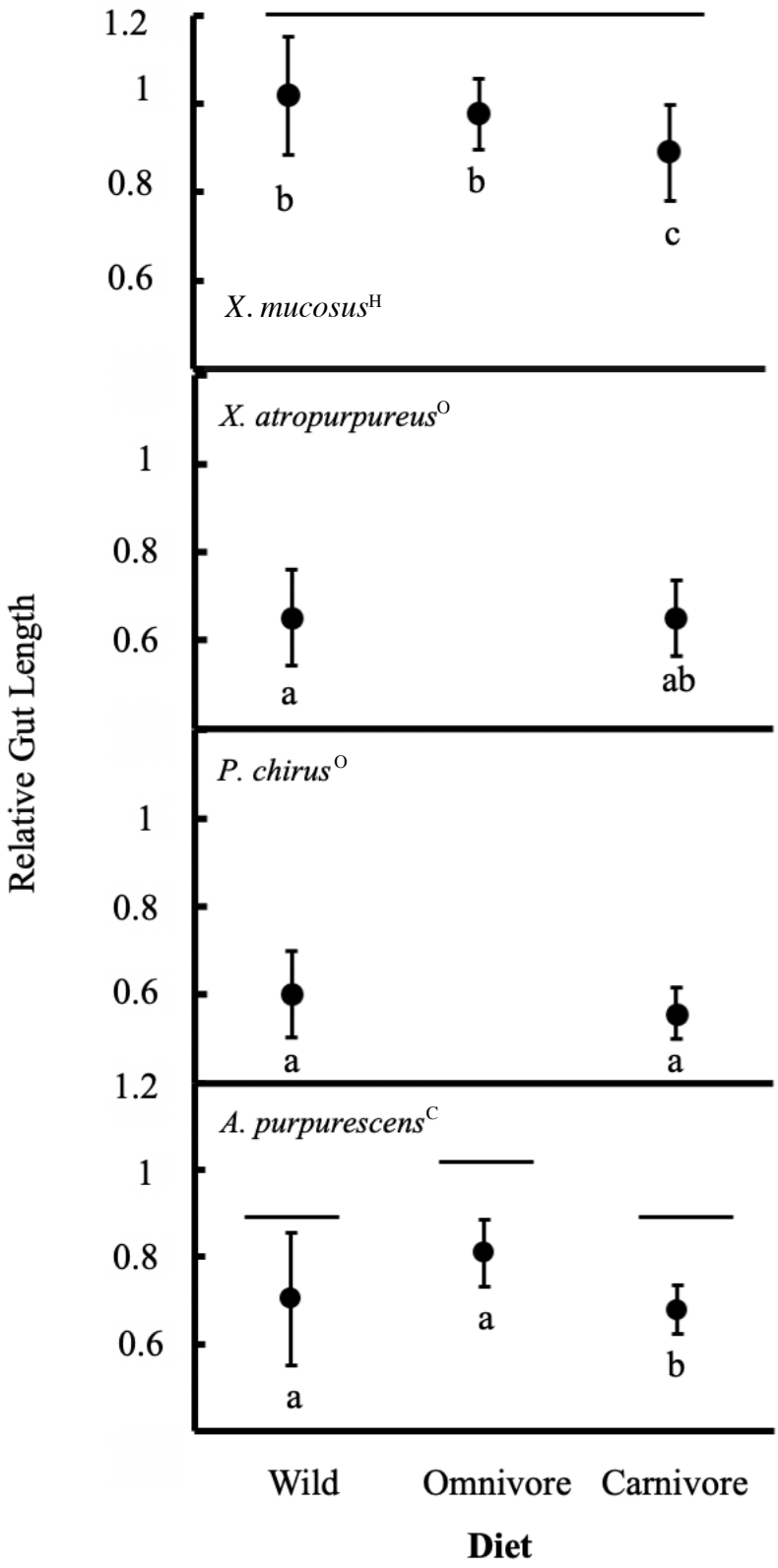

Figure 2.2 Relative gut length (gut length · standard length^{-1}) of wild-caught fishes, and those fed omnivore or carnivore diets in the laboratory. Top to Bottom: *X. mucosus* ^{H}, *X. atropurpureus*

^{O}, *P. chirus* ^{O}, and *A. purpurescens* ^{C}. Intraspecific comparisons of individuals on the different diets (along the x-axis) were made with ANCOVA (using body mass as a covariate; Supplemental Table S5), and symbols sharing a line of the same elevation are not significantly different (P>0.05) from each other. No intraspecific differences were found for *X. atropurpureus* ^{O} or *P. chirus* ^{O}, and hence, no lines are drawn. For a given dietary category (Wild, Omnivore, Carnivore), interspecific comparisons were made (vertically) with ANCOVA (with body mass as a covariate), and symbols sharing a letter are not significantly (P>0.05) different from each other.

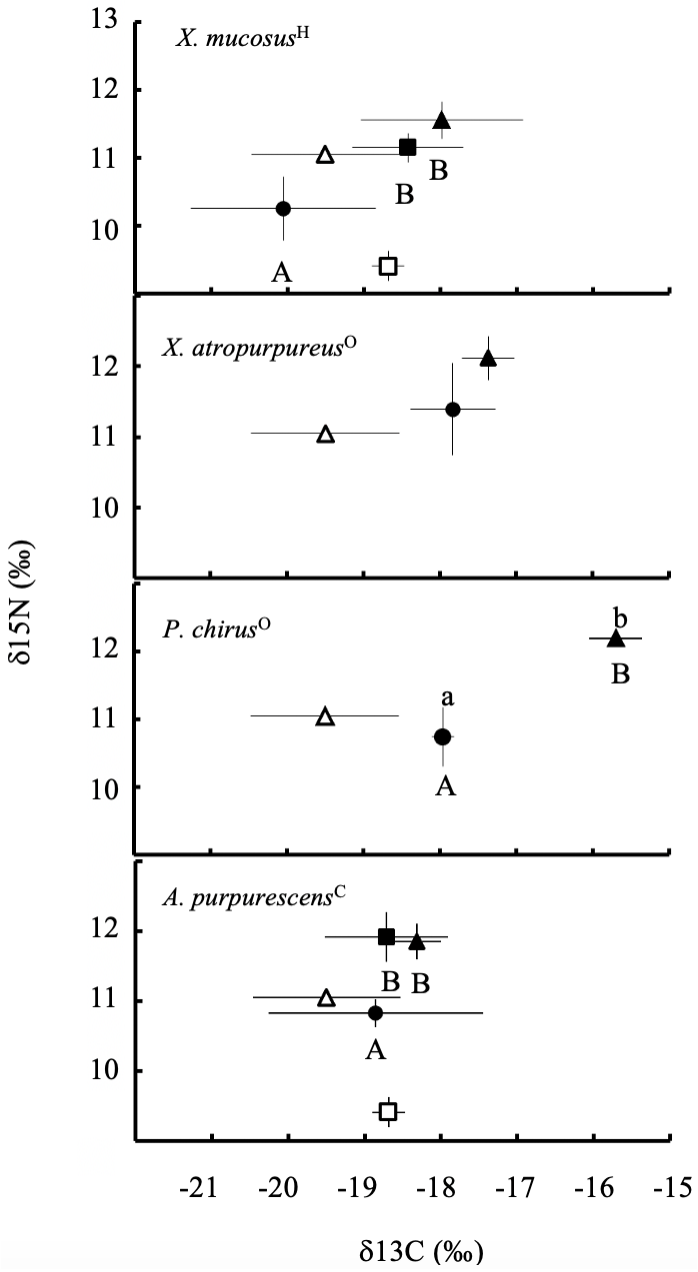

Figure 2.3 Carbon and nitrogen (‰) dual isotope plots of wild-caught fishes, and fishes fed omnivore and carnivore diets in the laboratory. Top to bottom: *X. mucosus*^{H}, *X. atropurpureus*^{O}, *P. chirus*^{O}, and *A. purpurescens*^{C}. Shapes indicate the following: open square: Lab Omnivore Diet; filled square: Lab Omnivore Fish; open triangle: Lab Carnivore Diet; filled triangle: Lab Carnivore Fish; filled circle: Wild fishes. Values are mean ± standard deviation. Intraspecific comparisons of the fish on the different diets were made with ANOVA for each species. Significant differences ($P<0.05$) for δ15N indicated with capital letters, whereas lower case letters indicate significant differences in δ13C values.

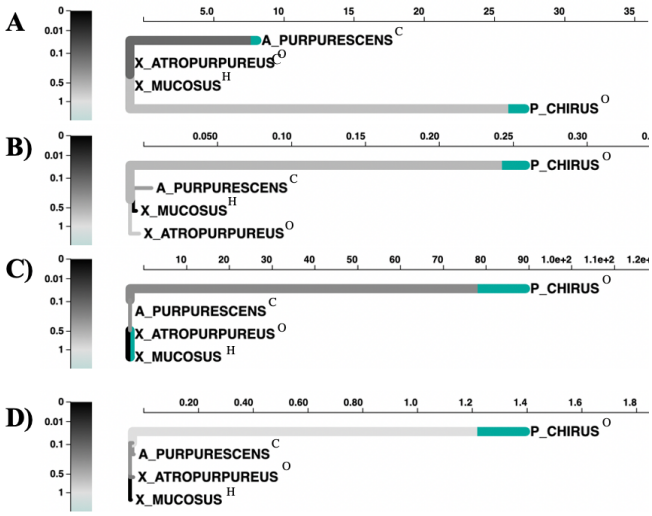

Figure 2.4: An adaptive branch-site random effects likelihood (aBSREL) test for episodic diversification phylogenetic tree constructed for various genes in the liver from four prickleback fish species: a) Glucose-6-Phosphate 1-Dehydrogenase (G6PD), b) fatty acid binding protein, c) Mitochondrial import inner membrane translocase subunit (TIM21), and d) endothelial lipase. ω is the ratio of nonsynonymous to synonymous substitutions. The color gradient represents the magnitude of the corresponding ω. Branches thicker than the other branches have a $p < 0.05$ (corrected for multiple comparisons) to reject the null hypothesis of all ω on that branch (neutral or negative selection only). A thick branch is considered to have experienced diversifying positive selection.

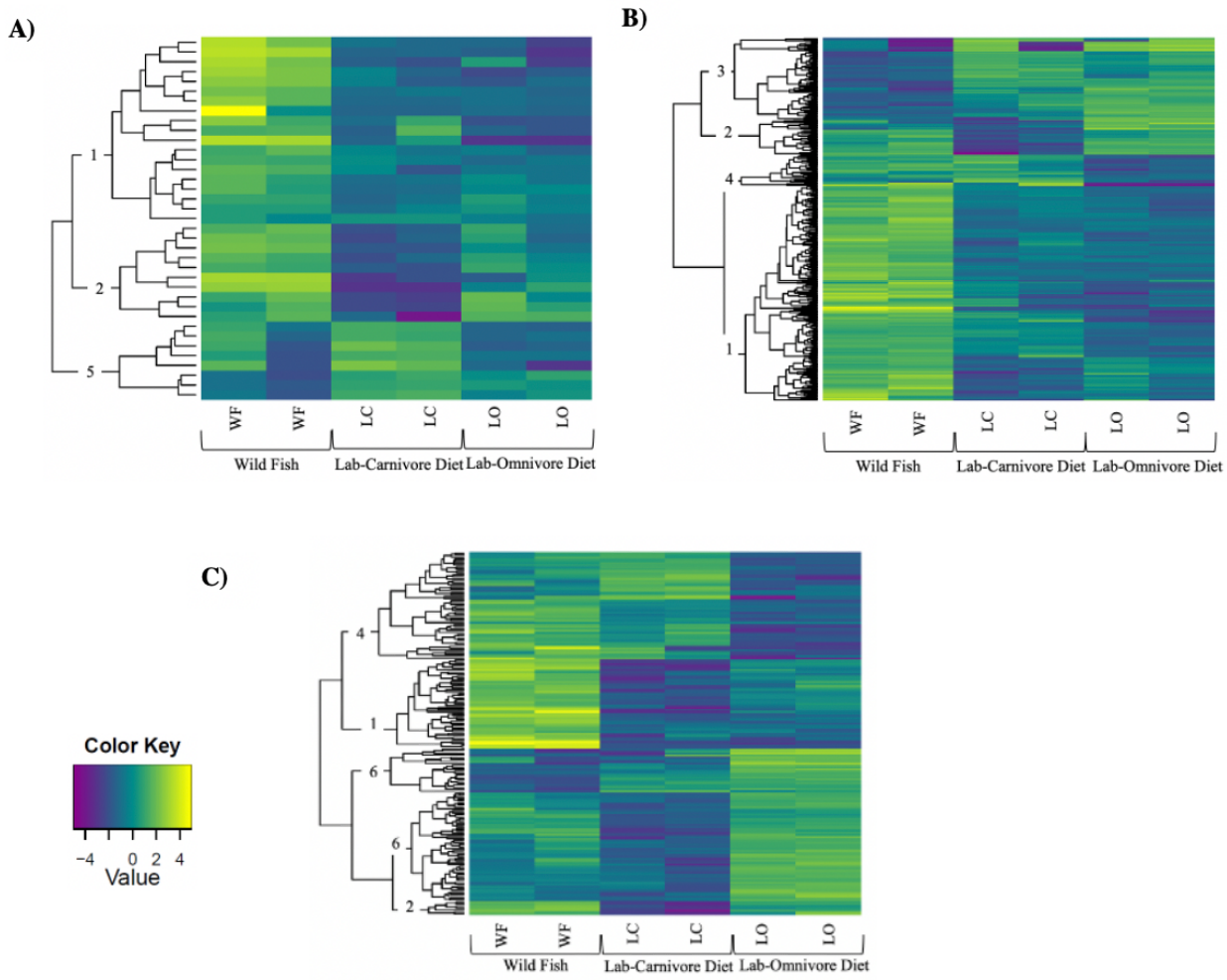

Figure 5 Differential gene expression depicted as heatmaps in different tissues of *X. mucosus*^{H}: a) Liver, b) Mid Intestine, and c) Pyloric Ceca. Yellow indicates elevated relative expression, whereas blue indicates low expression. Each row is a single gene, and genes are clustered in a dendrogram (on left of each heatmap) by similarity of expression patterns. The various clusters of genes are described in Table 4. Each column represents the gene expression in a single tissue from an individual fish, with WF = wild-caught fish, LO = fish fed an omnivore diet in the laboratory, and LC = fish fed a carnivore diet in the laboratory.

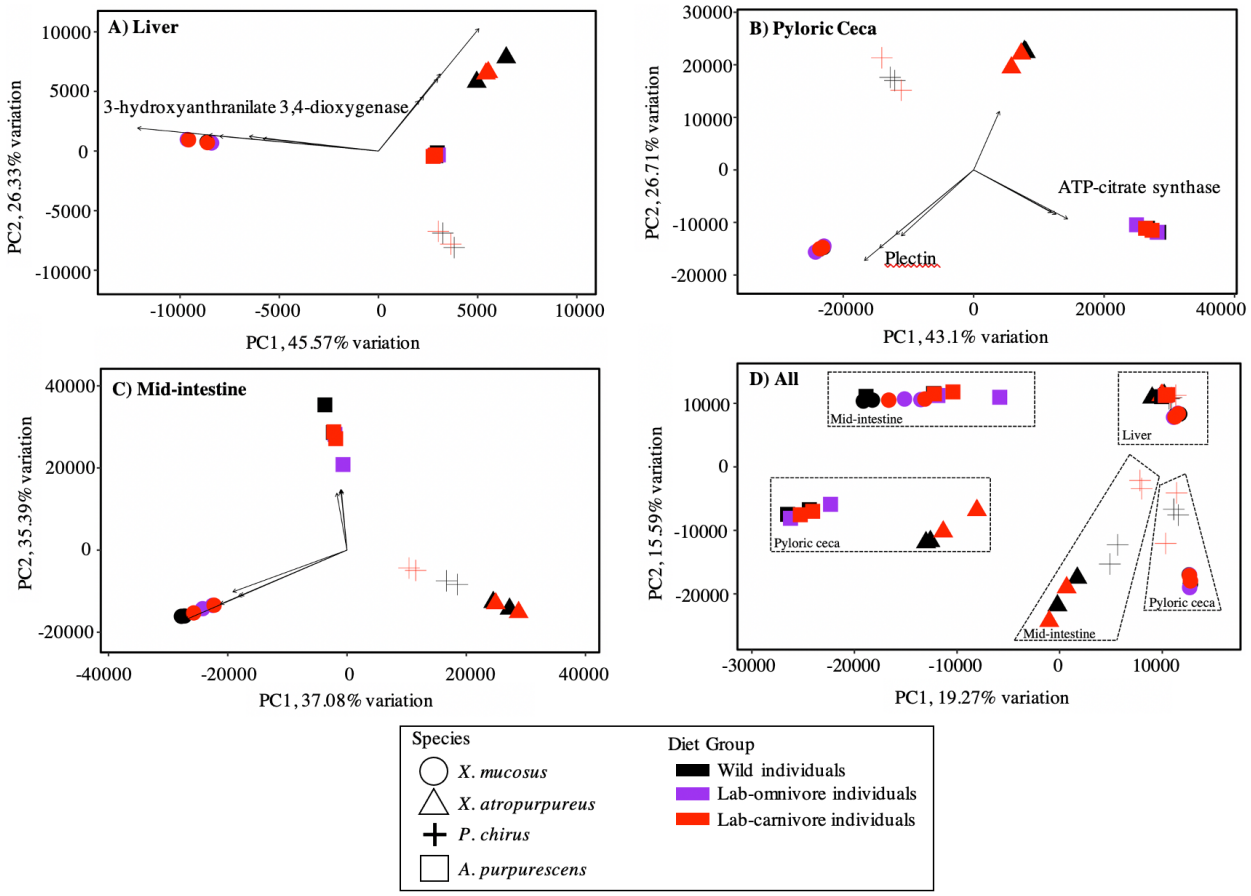

Figure 2.6. PCA plot of gene expression data from the four species and A) Liver, B) Pyloric ceca, C) Mid-intestine, and D) All tissues combined with all species and diet groups. Shapes represent species. Spheres depict *X. mucosus*^{H}, triangles depict *X. atropurpureus*^{O}, plus sign (+) depict *P. chirus*^{O}, and squares depict *A. purpurescens*^{C}. Colors represent diet, with wild individuals in black, lab-omnivore individuals in purple, and lab-carnivore individuals in red. Vectors in panels A-C indicate the 'weight' in different directions for the genes driving differences along each PC (fall within the top 5% of loadings range). The full gene list can be found in Supplementary Table S10 and the genes of interest that are related to digestion and metabolism are labeled on the graph.

Table 2.1 Predictions for wild-caught fishes				
Wild fishes	*Xiphister mucosus* (H)	*X. atropurpureus* (O)	*Phytichthys chirus* (O)	*Anoplarchus purpurescens* (C)
Relative Gut Length	Longest	Moderate	Moderate	Shortest
$\delta^{13}C$ signature	Less enriched	Moderately enriched	Moderately enriched	Highly enriched
$\delta^{15}N$ signature	Less enriched	Less enriched	Moderately enriched	High
Genes under positive selection	High in genes for carbohydrate degradation and carboxyl ester lipase	High in genes for carbohydrates and protein digestion and metabolism	High in genes for carbohydrate and protein digestion and metabolism	High in genes for protein metabolism
Note: H: Herbivore; O: Omnivore; C: Carnivore				

Table 2.2 Predictions for fishes fed different diets in the laboratory relative to Wild-caught fishes.		
Lab fishes	Laboratory Omnivore Diet	Laboratory Carnivore Diet
Relative Gut Length	Moderate	Smallest
Growth	Moderate	Largest
Metabolic Rate	Moderate	Highest
$\delta^{13}C$ signature	More enriched	More enriched
$\delta^{15}N$ signature	More enriched	More enriched
Relative Gene Expression	Elevated expression of genes involved in carbohydrate and protein digestion and metabolism	Elevated expression of genes involved in protein digestion and metabolism, gluconeogenesis, and lipid synthesis

Table 2.3 Ingredients and chemical composition of the omnivore and carnivore diets fed to prickleback fishes in the laboratory.		
Diets	**Omnivore Diet**	**Carnivore Diet**
Ingredients (g/100g)		
Mazzaella splendens	14.41	-
Porphyra sp.	14.42	-
Ulva lobata	14.42	-
Fish	43.25	86.5
Casein	2	2
Soybean Meal	2	2
Oil	6	6
Methyl cellulose	1.5	1.5
Vitamin Premix	1	1
Vitamin C	0.4	0.4
Mineral Premix	0.6	0.6
Chemical composition		
Protein (%)	45.40	68.80
Carbohydrate (%)	19.46	2.17
Lipid (%)	12.34	11.90
Calories (Cal)	263.8	342.0
Organic Matter (%)	81.40	89.14

Table 2.4 Orthologous gene pairs in different tissues of four closely-related prickleback fish species.

Liver				
	X. mucosus^{H}	*X. atropurpureus*^{O}	*P. chirus*^{O}	*A. purpurescens*^{C}
X. mucosus^{H}	-			
X. atropurpureus^{O}	13,124	-		
P. chirus^{O}	4,124	4,652	-	
A. purpurescens^{C}	5,311	5,963	4,113	-
Shared among all four species: 870				
Pyloric Ceca				
	X. mucosus^{H}	*X. atropurpureus*^{O}	*P. chirus*^{O}	*A. purpurescens*^{C}
X. mucosus^{H}	-			
X. atropurpureus^{O}	17,111	-		
P. chirus^{O}	13,546	16,511	-	
A. purpurescens^{C}	13,079	15,852	15,057	-
Shared among all four species: 3,787				
Mid-intestine				
	X. mucosus^{H}	*X. atropurpureus*^{O}	*P. chirus*^{O}	*A. purpurescens*^{C}
X. mucosus^{H}	-			
X. atropurpureus^{O}	16,166	-		
P. chirus^{O}	13,067	17,283	-	
A. purpurescens^{C}	12,573	16,600	16,543	-
Shared among all four species: 3,267				

Table 2.5 Differentially Expressed Genes Cluster Definitions as presented in the heat maps in Figure 4, and in Supplemental Figures S15-S23.

Species	Cluster	Wild	Lab-Carnivore Diet	Lab-Omnivore Diet
Xiphister mucosus[H] and *Anoplarchus purpurescens*[C]	1: elevated in wild fishes	High	Low	Low
	2 : wild-omnivore genes	High	Low	High
	3: elevated in the lab genes	Low	High	High
	4: wild-carnivore genes	High	High	Low
	5: carnivore genes	Low	High	Low
	6: omnivore genes	Low	Low	High
Xiphister atropurpureus[O] and *Phytichthys chirus*[O]	1: elevated in wild fish	High	Low	--
	2: elevated in LC fish	Low	High	--

PSG: Positively Selected Gene when comparing sequences (PAML and Datamonkey) among wild-caught fishes of the four prickleback species.

Table 2.6 Differentially Expressed Genes relevant to metabolism and digestion in all tissues for *Xiphister mucosus*[H]						
Tissue	Cluster	Gene	Function	W fish	LC fish	LO fish
Liver	1	Apolipoprotein	lipid metabolism	High (++++)	Low (++)	Low (++)
	2	Phosphoenolpyruvate carboxykinase	gluconeogenesis	High (+++++)	Low (+)	High (++++)
Pyloric ceca	1	Fatty acid synthase	fatty acid biosynthesis	High (+++++)	Low (+)	Low (+++)
	1	Endothelial lipase	lipid metabolic process	High (+++++)	Low (+)	Low (+++)
	1	72kDa type IV collagenase	collagen catabolism	High (+++++)	Low (+)	Low (+++)
	1	Cathepsin B, an endopeptidase	protease	High (+++++)	Low (+)	Low (+++)
	1	Lanosterol synthase	cholesterol biosynthesis	High (++++)	Low (+)	Low (++++)
	2	fatty acid synthase	fatty acid biosynthesis	High (++++)	Low (+)	High (++++)
	2	plectin	microtubules	High (++++)	Low (+)	High (++++)
	2	collagenase 3	collagen catabolism	High (++++)	Low (+)	High (++)
	2	gastrotropin	bile acid metabolism/lipid transport	High (+++++)	Low (+)	High (+)
	2	lanosterol 14-alpha demethylase	cholesterol biosynthesis	High (++++)	Low (+)	High (++++)

	2	3-hydroxy-3-methylglutaryl-coenzyme A reductase	cholesterol biosynthesis	High (++++)	Low (+)	High (++++)
Mid-intestine	1	Collagenase	Collagen catabolism	High (+++++)	Low (+)	Low (+)
	1	Chitinase	Chitin metabolism	High (+++++)	Low (+)	Low (+)
	1	Fatty acid synthase	Fatty acid biosynthesis	High (+++++)	Low (+)	Low (+)
	2	Lanosterol synthase	Cholesterol biosynthesis	High (++++)	Low (+)	High (++)
	3	Carboxypeptidase	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Carboxypeptidase A1	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Carboxypeptidase B	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Trypsin	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Trypsin 3	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Chymotrypsin-like elastase family member 1	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Endoplasmic reticulum aminopeptidase 1	Protein metabolism	Low (+)	High (++++)	High (++++)
	3	Mannosyl-oligosaccharide glucosidase	Glycolysis	Low (+)	High (++++)	High (++++)

	3	Neuropeptidase Y receptor 2	Feeding behavior	Low (+)	High (++++)	High (++++)
Gradient of expression is depicted by plus signs, in that one (+) is low expression to a roughly 5 fold increase (+++++).						

Chapter 3

Diet shifts affect gut and liver function and the distal intestine microbiome of an herbivorous fish

ABSTRACT

Advancing our understanding of factors influencing gut physiology and function is critical for comprehending ecological adaptations and potential impacts on ecosystem dynamics. Here, we investigated the effects of different diets, varying in protein content, on the physiology, host gene expression, and gut microbiome of *Cebidichthys violaceus*, a marine herbivorous fish. We integrated multiple parameters, including gut physiology, digestive enzyme activity, short-chain fatty acid concentrations, gut microbiome, and hindgut and liver transcriptomics to examine the response to dietary shifts. Fish successfully assimilated the laboratory diets, and as anticipated, there were diet-dependent shifts in SCFA levels and digestive enzyme activity levels. The host hindgut gene expression patterns closely resembled those of the gut microbial diversity patterns. Wild fish microbial communities were indeed different from laboratory-fed fish. However, contrary to our expectations, the fish fed an omnivore diet in the laboratory exhibited the highest similarity to wild fish, sharing a high abundance of taxa in the Bacteroidota and Bacillota (Families Ruminococcaceae and Rikenellaceae). In contrast, the laboratory-herbivore and laboratory-carnivore fish shared a high abundance of taxa from the Pseudomonodota (Families Burkholderiaceae and Oxalobacteraceae). Further, the hindgut and the liver displayed different

responses to dietary shifts in terms of the transcriptome. The hindgut had 519 differentially expressed genes, with wild fish exhibiting a high expression of genes associated with ion transport, lipid metabolism, and glucose metabolism. Conversely, the liver had 4650 differentially expressed genes, and wild fish highly expressed genes related to fatty acid synthesis and proteolysis. Our integrative study highlights the complex interactions between diet, gut physiology, gene expression and the hindgut microbiome in a marine herbivorous fish. These findings provide insight into how fish adapt to shifting resources, which helps inform efforts in marine resource management and the potential development of new aquaculture species.

INTRODUCTION

The digestive tract is central to an animal's survival, as it determines the absorption of essential nutrients for various processes, including (but not limited to) metabolism, movement, and reproduction (Karasov and Martínez del Rio 2007; Karasov and Douglas 2013). As the supplier of nutrients to the body, the gut must adjust to changes in diet composition and the quantity of digesta being processed, and exhibit changes along the entire digestive tract, from digestive tract size (Fuentes and Cancino 1990; German and Horn 2006; He et al. 2013; Leigh et al. 2018a) and digestive enzyme activities (German et al. 2004; German et al. 2010; Harpaz and Uni 1999; He et al. 2013) to gene expression (De Santis et al. 2015a; De Santis et al. 2015b; Gawlicka and Horn 2006; He et al. 2013; Kim et al. 2014; Król et al. 2016; Le et al. 2019; Parris et al. 2019; Wang et al. 2015) and nutrient transporter activity (Buddington et al. 1987; Day et al. 2014; Verri et al. 2017). In this vein, dietary specialization, ranging from detritivory and herbivory to omnivory and carnivory, is represented in most food webs, leading to predictable variations in gut size and function, although extreme specializations also exist (e.g., blood specialists, like

candiru catfishes) (Karasov and Martínez del Rio 2007; Karasov and Douglas 2013; German 2011; Breault 1991; Spotte 2002). For instance, animals consuming more plant material, which is generally lower in protein and higher in fiber content, have higher intake, and thus, have longer and larger guts to process more material per unit time (German and Horn 2006; German et al. 2015; Leigh et al. 2018a; Sibly 1981; Sibly and Calow 1986; Wagner et al. 2009; Davis et al. 2013).

Although fishes do possess pharyngeal teeth that can masticate ingested items, extensively in some species (e.g., (Clements et al. 2017; Horn 1989; Horn and Messer 1992), fishes generally don't chew food like many mammals do, and thus, fish digestion is primarily a chemical process (Clements 2006; German 2011). Therefore, digestive enzymes are the key agents of digestion in fishes, and the activities of these enzymes tend to vary among species with different diets. In this context, the Adaptive Modulation Hypothesis suggests that digestive enzyme activities should match ingested dietary substrate concentration to avoid the waste of enzymatic production for low-concentration substrates (Karasov and Martínez del Rio 2007; Karasov 1992). For example, animals that consume more starch-rich plant material show higher activity of amylase, a carbohydrase, while some carnivores with protein-rich diets exhibit elevated activities of aminopeptidase, a protease (e.g., (Axelsson et al. 2013; German et al. 2004; German et al. 2010; German et al. 2016; Heras et al. 2020; Le 2023; Kohl et al. 2011; Xie et al. 2018). There is some flexibility in enzymatic activities if fishes are fed different diets than what they consume naturally (German et al. 2004; German et al. 2010; Harpaz and Uni 1999; He et al. 2013), but certain aspects, like elevated amylase activity in plant-eating fishes, can remain constant, even when herbivorous fishes are fed a diet devoid of starch (e.g., (German et al. 2004)).

In addition to digestive enzymes produced by a fish, the intestinal microbiome of these animals also plays a large role in digestion and metabolism (Clements et al. 2014; Egerton et al. 2018; Llewellyn et al. 2014; Pardesi et al. 2022; Stevenson et al. 2022; Sullam et al. 2012; Escalas et al. 2021; Huang et al. 2020). The impacts of diet on the enteric microbiome of fishes are obvious, particularly for those species reliant on gastrointestinal fermentation to digest at least some aspects of their food (Mountfort et al. 2002; Egerton et al. 2018; Huang et al. 2020; Pardesi et al. 2022; Stevenson et al. 2022). For example, plant-eating fishes with moderate to high-levels of gastrointestinal fermentation localize these activities to the hindgut, where the enteric microbial community is the most diverse and active (Clements and Choat 1995; Clements et al. 2017; Mountfort et al. 2002; German et al. 2015; Pardesi et al. 2022; Pisaniello et al. 2022; Sparagon et al. 2022; Stevenson et al. 2022; Moran et al. 2005; Wu et al. 2020). The products of that fermentation, short chain fatty acids (SCFA) can be absorbed and used metabolically by the fish (Mountfort et al. 2002; Bergman 1990; Stevens and Hume 1998; den Besten et al. 2013; De Vadder et al. 2014; Burr 1998; Willmott et al. 2005). Foregut fermentation, like that in ruminant mammals, is unknown in fishes (Clements et al. 2014). But, how does the herbivorous fish gut respond to changing dietary resources (e.g., (German et al. 2004; Herrera et al. 2022)), particularly in response to environmental changes, or in the case of some herbivorous fishes, varying aquaculture diets (e.g., (Xie et al. 2018; Xie et al. 2019; Thépot et al. 2022). Although it is known that captive fishes have different, and perhaps less-diverse microbiomes than their wild counterparts, it isn't clear what the consequences of such an environmental change would be, nor how the enteric microbiome shifts if an herbivorous fish uses dietary resources it may not use naturally (Clements et al. 2014; Leigh et al. 2022; Fuentes and Cancino 1990; German and Horn

2006; He et al. 2013; Leigh et al. 2018a; German et al. 2004; Harpaz and Uni 1999; Fishelson et al. 1985; Montgomery and Pollak 1988; Nayak 2010; Roeselers et al. 2011; Clark et al. 2023).

Beyond the gut microbiome playing a key role in the host by influencing digestion and metabolism, the interplay between the gut microbiome and expression of genes within the host requires more study. There continues to be a weak understanding of the contribution of gut microbes to the host in the realm of metagenomic investigations and examining the gut microbiome with changes in expression of digestive genes (Karasov and Martínez del Rio 2007; Ley et al. 2008a; Ley et al. 2008b; Fuess et al. 2021; Richards et al. 2019; Blekhman et al. 2015; Muehlbauer et al. 2021). Certainly, diet can impact the gene expression of tissues involved in digestion and metabolism, such as the intestine and liver (e.g.,(De Santis et al. 2015a; Król et al. 2016; Jin et al. 2018; Betancor et al. 2018; Herrera et al. 2022; Le 2023)). There are dietary-related differences (e.g., gene copy number variation of digestive enzyme genes) on the genomic level in fishes with different diets that impact gene expression levels (Le 2023; Axelsson et al. 2013; German et al. 2016; Betancor et al. 2018; Heras et al. 2020; Herrera et al. 2022; Gout et al. 2010; Perry et al. 2007; Qian and Zhang 2014). But, how do these two elements—diet and microbiome—interact to influence fish host gut and liver gene expression, particularly for herbivorous fishes? Thus, examining the plasticity of the gut microbiome and host gene expression would be informative to understanding the influence of microbes on the host, and how the host and its microbiome may respond to perturbations, like a diet shift.

Here, we conducted an integrative study examining gut physiology, digestive enzyme activity, gastrointestinal fermentation (SCFA concentrations), gut microbial diversity, and transcriptomics to understand how an herbivorous fish responds to dietary shifts. *Cebidichthys violaceus* is a marine herbivorous prickleback fish (Family Stichaeidae) native to the west coast

of North America (Fig. 3.1). The fish consumes algae year-round (Horn et al. 1986)and is a true herbivore, efficiently digesting nutrients from algae and subsisting on algae alone (Horn et al. 1986; Fris and Horn 1993). This fish uses a yield-maximizing digestive strategy, showing relatively long gut transit times (generally >20 hours; (Fris and Horn 1993; Horn et al. 1995; Urquhart 1984), with microbial enzymatic activity and moderate levels of SCFA in its hindgut (German et al. 2015). Thus, unlike some rate maximizing herbivores in the same fish family (i.e., *Xiphister mucosus*) that have rapid transit of material through the alimentary canal and little gastrointestinal fermentation occurring in their hindguts, *C. violaceus* appears to be somewhat reliant on its enteric microbiome to aid in the digestive process (German et al. 2015). It also has elevated gene copy number of, and elevated intestinal activity of amylase and carboxyl ester lipase, showing its adaptation to its algal diet (Heras et al. 2020; Rankins 2023; Le 2023). The local delicacy of "Monkeyface eel" in the San Francisco Bay Area (Monkeyface eel becoming a star on dinner platters (sfgate.com)), combined with recent interest in pursuing aquaculture of this species (Monkeyface prickleback, the new face of aquaculture? | California Sea Grant (ucsd.edu)) has raised the possibility of culturing *C. violaceus* for human consumption (Heras et al. 2020). With the recent push to develop more sustainable aquaculture feeds that are more plant based, herbivorous fish aquaculture is gaining traction (Lozano-Muñoz et al. 2022; Jobling 2016; Xie et al. 2018). However, what can *C. violaceus* tolerate in terms of feeds: How does such an animal deal with larger than normal protein loads from more traditional fish-based feeds, and how flexible is the microbiome, gut physiology, and downstream metabolism to different nutrient loads in such a specialized fish? This investigation also pertains to the ecology of this animal and how it may respond to shifting resources as a function of global change.

Therefore, we brought *C. violaceus* into the laboratory and fed them herbivore, omnivore, and carnivore diets for six months to study how changing nutrient loads affected the fish. We examined gut size, digestive enzyme activities of the hindgut, gene expression patterns of the hindgut and liver, and hindgut microbial diversity, comparing the lab-fed fish with wild-caught individuals. In the laboratory fish, we also examined dietary stable isotopic incorporation to confirm that the fish were assimilating the assigned diets, and growth rates as a measure of whole animal function. Our predictions are outlined in Table 3.1. We expected gut length and mass to show plasticity in fish consuming different diets in the laboratory: fish on the higher protein diets (carnivore and omnivore diets) should have shorter and lighter guts compared to fish consuming the herbivore diet and wild-caught fish consuming their natural algal diet (Herrera et al. 2022; Farrell A.P. 2011; German and Horn 2006; Davis et al. 2013). We measured the activity levels of digestive enzymes that degrade carbohydrates (α-amylase, maltase, β-glucosidase, N-acetyl-β-D-glucosaminidase) proteins (trypsin, aminopeptidase) lipids (carboxyl ester lipase), and phosphate-containing compounds (alkaline phosphatase), and predicted that they would vary with the relevant nutrient content in the diet (Table 3.1). We expected the transcriptome and microbiome to shift with diet, with wild fish and fish fed an herbivore diet in the lab to be most similar (enteric microbial diversity would follow the pattern of wild>lab-herbivore>lab-omnivore>lab-carnivore; Table 3.1). (German et al. 2014; German et al. 2004; German et al. 2015; German and Horn 2006; Heras et al. 2020). In the liver transcriptome, we examined the metabolic pathways that changed in the face of dietary perturbations. Additionally, we compared the host hindgut transcriptome to the hindgut microbiome to provide insight into the unique interplay between the host, diet, and the gut microbiome.

Overall, by investigating how *C. violaceus* responds physiologically to different diets, we are examining what it takes for fish to deal with shifting resources, providing valuable insights for marine resource management and new potential aquaculture species development. Additionally, we aim to gain a deeper understanding of how the host and its microbiome respond to dietary perturbations, shedding light on their unique interactions and potential implications for host physiology and metabolism.

MATERIALS AND METHODS

Fish capture and feeding trial

Twenty six individuals of *Cebidichthys violaceus* were collected by hand and dipnet in September 2016 at low tide from the rocky intertidal habitat on the central California coast near Piedras Blancas (35.65ºN, 121.24ºW). Five of the fish were euthanized and dissected in the field to act as representatives of the wild condition. The remaining 21 fish were transported to the laboratory at University of California, Irvine, in air in 48-L coolers containing a small amount of the brown alga *Silvetia compressa* to provide cover and dampness. Bags of ice were suspended in the coolers to maintain a cool temperature. Upon arrival, the fish were transferred to a system of four 76-L cubicle plexiglass aquaria (six cubicles per aquarium, at ~13-L per cubicle with the only water exchange among cubicles occurring at the top) connected to a common recirculating system, including a sump, biological, particulate, activated carbon, UV filtration, protein skimmer, and chiller. Each fish was assigned to their own cubicle, which included a 12-cm section of 2.54-cm diameter pvc pipe in which the fish could hide (German et al. 2004). The system contained filtered seawater pumped from Newport Bay, CA, and fish were under a 12L:12D light cycle. The water temperature was maintained at 15ºC (the upper end of temperatures measured at the collection site; (German and Horn 2006; German et al. 2004) with

a coil chiller (Aqualogic, San Diego, CA, USA) for the duration of the experiment and the temperature and chemical conditions (pH, ammonia concentrations) of the tank system were monitored daily to confirm that they did not vary during the experimental period. Debris and feces were siphoned out daily, and 20% of the water changed weekly. A Tunze Osmolator (Penzberg, Germany) measured the water level in the sump and pumped in deionized water to replace water lost by evaporation, thus maintaining the salinity between 34-35‰. The fish were randomly assigned to one of three diets (Table 3.2, seven fish per diet; (Herrera et al. 2022)), a Lab-Herbivore (LH) diet, a Lab-Omnivore Diet (LO), or a Lab-Carnivore (LC) diet. Fish were fed the diets to satiation two-three times daily for three months to allow them to acclimate to the system. At the fourth month, each fish was anesthetized (0.1 g L^{-1} MS-222), measured and weighed, and returned to their tanks. Daily fecal collections for measurements of digestibility began at this point and the fish were fed in this manner for another six months.

At the conclusion of the experiment, each fish was euthanized with an overdose of MS-222 (1 g L^{-1} seawater), measured (SL ± 0.5 mm), weighed (body mass, BM ± 0.1 g;) and dissected on a sterilized cutting board kept on ice (4°C). The fish sizes are presented in Table 3.3. About 250 mg of epaxial muscle was taken with a razor blade and placed in a 1.5 mL centrifuge vial. Each digestive system was removed by cutting just anterior to the stomach and at the anus (Fig. 3.1). The guts were gently uncoiled, measured (gut length, GL), and the stomachs excised. The pyloric ceca and livers were excised with a razor blade and placed in their own centrifuge vials, whereas the intestine was divided into three sections of equal length, designated as the proximal, mid, or distal intestine (Fig. 3.1; (German et al. 2015)). Each section was emptied of their contents by pushing with the blunt side of a razorblade and the contents were frozen separately in individual centrifuge vials in liquid nitrogen. Subsamples (~100 mg) of each

intestinal tissue and the liver for transcriptomic analyses were rinsed with ice cold 25 mM Tris pH 7.5 buffer and immediately placed in 0.5-mL centrifuge vials containing RNAlater, and stored overnight at 4° C, and subsequently transferred to a -80° C freezer for storage until further processing (less than one week). The remaining tissues (stomach, pyloric ceca, intestinal sections) were frozen in liquid nitrogen for digestive enzyme activity assays and microbiome analyses; remaining liver and the epaxial muscle was frozen for stable isotopic analyses. Frozen tissue samples were stored at -80°C until analyzed. Gut lengths and body lengths were used to calculate relative gut length [RGL = gut length (mm) X standard length $(mm)^{-1}$], relative gut masses were calculated [RGM = gut mass (g) X body mass $(g)^{-1}$], as well as the distal intestine (DI) relative mass [DI relative mass = DI mass (g) X body mass $(g)^{-1}$] (German and Horn 2006). The same procedure was followed for fish dissected in the field.

Gut tissues or contents from distal intestine from individual fish were weighed (regional gut or content mass ± 0.001 g) and homogenized following German and Bittong (2009)(German and Bittong 2009). Intestinal contents and tissues were homogenized in 25 mM Tris-HCl, pH 7.5 (German et al. 2015). The supernatants of homogenates were collected and stored in small aliquots (100-200 µl) at –80ºC until just before use in spectrophotometric or fluorometric assays of digestive enzyme activities.

Gut Microbiome Sample Processing

The sample DNA was isolated from the distal intestine tissue and contents for both the laboratory-fed and wild-caught *C. violaceus* using the Zymobiomics DNA mini kit from Zymo Research. 16S rDNA amplicon PCR was performed targeting the V4 - V5 region (selected based on previous literature; (Walters et al. 2016; Caporaso et al. 2012)) using the Earth Microbiome Project primers (515F [barcoded] and 926R; (Walters et al. 2016; Caporaso et al. 2012)). The

libraries were sequenced at the UC Irvine Genomics Research and Technology Hub (GRTH) using a miseq v3 chemistry with a PE300 sequencing length. Sequencing resulted in 17.4 M reads passing filter (21% of that is phiX) with an overall >Q30 80.4%. An additional miseq run was conducted with additional samples that had low quality scores, and the ASV data was merged in QIIME2 (version 2022.8). The raw sequences were imported into QIIME2 (version 2022.8) using UCI's High Performance Community Computing Cluster (HPC3). After initial sample quality check (99% identity threshold), the paired-end sequences were quality filtered using the DADA2 pipeline in QIIME2, resulting in 1,573,237 merged paired-end reads. Taxonomic classification for Amplicon Sequence Variants (ASVs) was assigned using the Silva 138 99% OTUs from 515F/806R region of sequences (release 138;(Quast et al. 2013)). Analyses were conducted in both QIIME2 and the feature table was imported into R (Version 1.4.1103). Within R, we used Shannon alpha diversity and Tukeys HSD to determine significance. To examine beta diversity in R, we used Bray-Curtis distances to construct a non-metric multidimensional scaling plot (NMDS) and to determine significance, we conducted a PERMANOVA with 999 permutations, as well as a pairwise PERMANOVA using Benjamini-Hochberg p-adjusted values. To determine the which microbial taxa are driving differences in particular diet groups, we ran indicator species analysis which each diet group as different habitat or sites (De Cáceres et al. 2011). Additionally, we determined features with a high correlation (p value=0.005), and added feature vectors that explain variance in each NMDS plot (https://riffomonas.org/code_club/2022-04-11-biplot). To determine core microbial taxa that are shared amongst all fish host species samples, we utilized the core-features program in qiime2 to identify ASVs observed in 100% (fraction of 1.0) of all samples of fish host species.

Stable isotope analysis

To assess carbon and nitrogen assimilation from the diets, we measured $\delta^{13}C$ and $\delta^{15}N$ signatures of liver and muscle tissue from the fish, and of the herbivore, omnivore, and carnivore diets made in the laboratory. Liver, muscle, and each of the diets were dried overnight at 60°C, and ground into powder. Approximately 0.7 mg of individual liver, muscle, or diet samples were then transferred into individual 5 mm x 9 mm tin capsules (Costech Analytical Technologies). Samples were run through a Fissions NA 1500NC elemental analyzer interfaced to a ThermoFinnigan-DeltaPlus CF (Bremen, Germany) isotope ratio mass spectrometer in the Center for Isotope Tracers in Earth Science facility at UC Irvine. The delta calculations were performed as described previously in German and Miles (2010)(German and Miles 2010).

RNA isolation and transcriptomic analyses

Transcriptomic analyses were performed following Herrera et al. (2022)(Herrera et al. 2022). Briefly, Total RNA from distal intestine and liver tissue samples (20-50mg) from two individual fish representing each diet (and the wild fish) were isolated using TRIzol reagent (Thermo Fisher Scientific). Samples were quantified (ng/µl) using an RNA Nanodrop and RNA quality was determined by Bionalyzer (RNA Integrity > 7) at the UC Irvine GRTH. cDNA libraries were prepared using a TruSeq RNA sample prep kit (Illumina, San Diego, CA). Agencourt AMPure XP magnetic beads were used to re-purify the samples (Beckman Coulter Genomics, Danvers, MA). The cDNA pools were normalized to 10 nM and run as two paired-end 100 bp runs on a HiSeq 2500 (Illumina, San Diego, CA) by the UCI GRTH. All data generated were deposited into NIH Archive with accession number [].

Raw data files were filtered and trimmed with Trimmomatic v0.32 (Bolger et al. 2014) implemented in UCI's HPC3, in order to make certain that trailing bases have a phred score of a minimum of 30. Reads were then normalized to low systematic coverage to remove errors and

reduce data set size (Haas et al. 2013). A *de-novo* assembly using Trinity was conducted, using the RNA-seq by Expectation Maximization (RSEM) package to align RNA-Seq reads back to the Trinity transcripts (Grabherr et al. 2011). Relative expression levels of all genes expressed in tissue types of interest, which included genes involved in digestion (e.g., digestive enzymes, nutrient transporters, metabolic pathways), were standardized to constitutively expressed Ribosomal Protein L8 using FPKM ratios calculated with eXpress (Roberts and Pachter 2013). Then, relative gene expression levels were estimated using RSEM (Li and Dewey 2011), which allows for the identification of gene and isoform abundance. Therefore, the calculated gene expression can be directly used for comparing differences in gene expression among *C. violaceus* fed the different diets. Then, we calculated differences in the abundance of expression of each gene among all treatments using EdgeR with a FDR <0.001 and a dispersion value of 0.4. Heatmaps were generated and part of the Bioconductor package "edgeR" was used for differential expression analyses.

For clarity purposes, we broke down the heatmaps for distal-intestine and liver into clusters based on expression profile (e.g., Table 3.5). For the distal intestine, Cluster 1 consists of genes that are highly expressed in wild, LO and LC fish, and lowly expressed in LH fish. Cluster 2 consists of genes that are highly expressed in wild fish and lowly expressed in lab-fed fish. Cluster 3 consists of genes that are highly expressed in all lab-fed fish and lowly expressed in wild fish. And cluster 4 consists of genes that are highly expressed in wild, LH and LC fish and lowly expressed in LO fish. For the liver, cluster 1 consists of genes that are highly expressed in LC fish, and lowly expressed in wild, LH, and LO fish. Cluster 2 consists of genes that are highly expressed in wild fish and lowly expressed in laboratory-fed groups. Cluster 3 consists of genes that are highly expressed in wild, LH, and LO fish and lowly expressed in LC fish. Cluster

4 consists of genes that are highly expressed in wild and LO fish, and lowly expressed in LH and LC fish. Cluster 5 consists of genes that are highly expressed in wild and LH fish and lowly expressed in LO and LC fish. These clusters appear in the heatmaps for the distal intestine and liver respectively.

Assays of digestive enzyme activity

All assays were carried out at 15°C in duplicate or triplicate using a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer equipped with a monochromator (BioTek, Winooski, VT). All assay protocols generally followed methods detailed in German and Bittong (2009)(German and Bittong 2009), unless otherwise noted. All pH values listed for buffers were measured at room temperature (22°C), and all reagents were purchased from Sigma-Aldrich Chemical (St. Louis). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from *C. violaceus* (German et al. 2014; German et al. 2004). Each enzyme activity was measured in the distal intestine tissue of each individual fish, 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.

α-amylase activity was measured using 1% potato starch dissolved in 25 mM Tris-HCl containing 1 mM $CaCl_2$. The α-amylase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

Maltase activities were measured following Dahlqvist (Dahlqvist 1968), as described by German and Bittong (2009)(German and Bittong 2009). We used 112 mM maltose dissolved in 25 mM Tris, pH 7.5. The maltase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per minute) per gram wet weight of gut tissue.

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

Trypsin activity was assayed using a modified version of the method designed by Erlanger et al. (1961) (Erlanger et al. 1961). The substrate, 2 mM Nα-benzoyl-L-arginine-p-nitroanilide hydrochloride (BAPNA), was dissolved in 100 mM Tris-HCl buffer (pH 7.5). Trypsin activity was determined with a p-nitroaniline standard curve, and expressed in U (μmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

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

Lipase (carboxyl ester lipase) activity was assayed using 0.55 mM p-nitrophenyl myristate (in ethanol) in the presence of 5.2 mM sodium cholate dissolved in 25 mM Tris-HCl (pH 7.5). Lipase activity was determined with a p-nitrophenol standard curve, and expressed in U (μmol p-nitrophenol liberated per minute) per gram wet weight of gut tissue.

Gut fluid preparation and gastrointestinal fermentation

Measurements of symbiotic fermentation activity were performed as described in German et al. (2015)(German et al. 2015). Fermentation activity was indicated by relative concentrations of short-chain fatty acids (SCFA) in the fluid contents of the distal intestines of the fish at the time of death. Distal intestine gut content samples of the fish fed the different diets were weighed [gut content mass (GCM ± 0.001 g)], thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4°C) at 16,000 x *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) and centrifuged under refrigeration at 13,000 x *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 concentrations. Data on wild-caught *C. violaceus* were taken from German et al. (2015)(German et al. 2015).

Concentrations of SCFA in the gut fluid samples from each gut region were measured using gas chromatography (Leigh et al. 2021). 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.

Statistical analyses

Prior to all significance tests, Levene's and Bartlett's tests for equal variances were performed to ensure the appropriateness of the data for parametric analyses, and any datasets that did not meet the assumptions of ANOVA (including homoscedasticity) were transformed using a Box Cox Transformation. All tests were run using R (version 4.0.1). $\delta^{15}N$ and $\delta^{13}C$ signatures were compared among muscle or liver tissues of the fish fed the different diets with ANOVA followed by a Tukey's HSD with a family error rate of $P = 0.05$. Similarly, comparisons of short chain fatty acid (SCFA) concentrations from the distal intestines of the fish, and growth across the experiment, were made among the fish fed the different diets with ANOVA. Total SCFA concentrations were plotted as a function of branched-chain SCFA (isobutyrate and isovalerate) concentrations, which tends to reveal whether carbohydrates or amino acids are the substrates of fermentation (Clements et al. 2017; Choat and Clements 1998). Relative gut length, relative gut mass, and DI relative mass were compared with ANCOVA, using body mass as a covariate. Relative gut length showed body mass as a significant co-variate with a significant interaction term, and thus, these variables were compared with ANOVA.

RESULTS

Gut size and stable isotopic incorporation

All fish grew on the laboratory diets, putting on between 110 to over 1100% of their body mass across the six-month feeing trial (Table 3.3). The body masses of the fish at the beginning of the experiment ($F_{2,18} = 0.185$, $P = 0.832$), and at the completion of the feeding experiment ($F_{2,18} = 1.105$, $P = 0.353$), did not statistically differ among the fish eating the different diets (Table 3.3). Moreover, the percent body mass gained across the experiment did not significantly differ among the fish fed the different diets ($F_{2,18} = 0.972$, $P = 0.379$), largely due to the variability in the laboratory carnivore (LC) diet fish (Table 3.3). Relative gut length did not differ among the fish

fed the different diets, including wild-caught fish ($F_{3,23}$ = 0.635, P = 0.600), whereas relative gut mass (Diet: $F_{3,22}$ = 9.248, P<0.001; Body mass: $F_{1,21}$ = 0.215, P=0.647), and DI relative mass (Diet: $F_{3,22}$ = 8.507, P<0.001; Body mass: $F_{1,21}$ = 1.974, P=0.147) did show significant differences, with the wild fish (WF) and laboratory herbivore (LH) diet fish generally having heavier total relative gut masses and distal intestine relative masses than the LC diet fish; LO diet fish tended to be not different from either the LC diet or LH diet fish (Fig. 3.2). The stable isotopic data clearly showed that the fish fed the different diets assimilated those diets (Fig. 3.3). The LC fish had different muscle $\delta^{13}C$ signatures than the LO and LH fish, and from wild fish. The LO, LH, and wild fish had indistinguishable muscle $\delta^{13}C$ signatures, whereas the fish fed the LC and LO diets had significantly enriched muscle $\delta^{15}N$ values in comparison to the LH and wild fish (Fig. 3.3). The liver stable isotopic values showed varying tissue-diet discrimination, with the LC fish significantly more depleted in $\delta^{13}C$ (like the carnivore diet) than the LH fish, and the LO fish were indistinguishable from the fish fed the other diets (Fig. 3.3). The liver $\delta^{15}N$ signatures, although variable on the different diets, were not significantly different among the fish consuming the different diets.

Digestive enzyme activities and short chain fatty acid concentrations in the distal intestine

Amylase ($F_{3,23}$ = 7.382, P = 0.001), alkaline phosphatase ($F_{3,23}$ = 32.56, P < 0.001), and trypsin ($F_{3,23}$ = 3.286, P = 0.039) activities varied significantly among the fish fed the different diets and those from the wild (Fig. 3.4). For amylase, the LO and LH fish had significantly elevated amylolytic activities in comparison to the LC fish or WF. For alkaline phosphatase, the WF had lower activities than the lab-fed fishes, which didn't differ significantly from each other (Fig. 3.4). For trypsin, the LH and WF had higher activities of this enzyme than the LC fish, whereas the LO fish were not different from any of the other groups (Fig. 3.4). β-glucosidase activities

were significantly higher in WF than in the LC or LO fish, whereas the LH fish had β-glucosidase activities that were not different from any group (Appendix 1). For NAGase, WF had significantly lower activities than the lab-fed fish (Appendix 1). None of the other enzymes were different among the fish fed the different diets or WF. The WF and LO fish had total SCFA concentrations in their distal intestines that were not different from each other, and both were significantly higher than in those of the LC and LH fish (Table 3.4). When total SCFA concentrations are plotted as a function of the proportion of branch-chained SCFA (isobutyrate and isovalerate), the WF and LO fish clearly possessed less branch-chained SCFA than the LC and LH fish (Fig. 5).

Distal intestine and liver gene expression

Using RNA-seq methods, we observed the suites of genes that changed with different diet groups in the distal intestine and the liver. Relative expression levels of all genes expressed were analyzed, yet we only are reporting on pathways relevant to digestion and metabolism. Thus, if a cluster is not mentioned, the genes contained in that cluster were not relevant to digestion and nutrient metabolism, or it is an unannotated gene.

In the distal intestine, there are 519 differentially expressed genes (DEGs) between WF and laboratory-fed fish (Fig. 3.6), out of which 65.5% are annotated. The genes are clustered based on similar expression levels and those clusters are defined in Table 3.5. Cluster 2 (highly expressed in wild fish) contained genes for lipid transport, fatty acid biosynthesis, and glucose metabolism. Cluster 4 (highly expressed in WF, LH, and LC fish) contained genes for protein metabolism and calcium ion transport. Genes involved in protein glycosylation are highly correlated with WF and LC diet fish (Table 3.6; Fig. 3.6). A principle component analysis showed that the LH fish separated from the remaining fish in space (Fig. 3.7) and the genes

explaining these differences are related to carbohydrate metabolism or fish genomes more generally (Table 3.7).

In the liver, there are 4650 DEGs, out of which 63.6% are annotated. Cluster 2 (highly expressed in WF) contains genes for fatty acid synthesis and proteolysis (Tables 3.5, 3.8; Fig. 3.8). Cluster 3 (highly expressed in wild, LH, and LO fish) contained genes for lipid metabolism, fatty acid synthesis, proteolysis, glycolysis, carbohydrate metabolism, and lipid transport. Cluster 5 (highly expressed in wild and LH fish) contained genes for gluconeogenesis, lipid metabolism, proteolysis and carbohydrate metabolism. Genes involved in lipid transport and inhibiting protease are highly correlated with LO fish (Table 3.8; Fig. 3.8). The principle components analysis showed how fish on the diets had different liver gene expression patterns, with genes involved in mediating the down-regulation of growth factor signaling highly correlated with LH fish (Table 3.9, Fig. 3.9).

Distal intestine tissue and contents microbiome

Among all wild fish, there are 24 bacterial taxa that are present in all individuals, which form the "core microbiome" and are highly abundant in wild fish compared to laboratory-fed fish (Table 3.10). These taxa are from the Families Rikenellaceae, Lachnospiraceae, Ruminococcaceae, Oscillospiraceae, Erysipelatoclostridiaceae, and Peptococcaceae. With a combined analysis of intestinal tissue and gut content, the top ten most abundant taxa in all individuals are from the orders Clostridiales, Bacteroidales, Erysipelotrichales, Bacillales, Vibrionales, Burkholderiales, Alteromonadales, Rhizobiales, Lactobacillales, many of which are also indicator species for specific diet groups (Figure 3.10 and Table 3.11). Regardless of sample type (intestinal tissue or gut contents), individuals from their respective diet groups are grouping together in the NMDS plot (Figure 3.11), with significant differences between wild and

laboratory fed fishes and amongst each group of laboratory-fed fishes (MANOVA, P<0.001) (Figure 3.11). There were no significant differences in the Shannon's alpha diversity index among different diet groups (Figure 3.12, ANOVA: $F_{3,30}$=1.49; P=0.239).

For the distal intestine tissue, there were no significant differences in the Shannon's alpha diversity index among wild and laboratory-fed fish (Appendix 2, ANOVA: $F_{3,13}$=2.11; P=0.149). In terms of beta diversity in the distal intestine tissue based on bray-curtis distances, wild fish are significantly different from laboratory-fed fish, and LO fish are significantly different from both LC and LH fish (MANOVA, P<0.001). Through examining the features that are significantly correlated with each diet group, there are taxa from the family Moraxellaceae, Oxalobacteraceae, Bacillaceae, and Chromobacteriaceae are highly correlated with LH and LC fish and are indicator species for LC fish. Taxa from the family Oxalobacteraceae are highly correlated with and are indicator species for LH and LC fish. Taxa from the family Vibrionaceae explain variation in wild and LO fish (Appendix 3).

For shannon's alpha diversity within the distal intestine contents, wild and LC fish were significantly different from each other, yet no other statistical differences were observed (Appendix 2right, ANOVA: $F_{3,13}$=3.52, P=0.046). In terms of beta diversity based on bray-curtis distances, wild fish are significantly different from laboratory-fed fish, and LO fish are significantly different from both LC and LH fish (MANOVA: P<0.001). Taxa highly correlated with wild fish include the Family Rikenellaceae, Butyricicoccaceae, Ruminococcaceae, and Erysipelatoclostridiaceae (Appendix 3). The Family Lachnospiraceae is an indicator species for wild fish. Taxa highly correlated with LO fish include the Family Rhizobiaceae and Chromobacteriaceae. Taxa highly correlated with LH and LC fish include the Family Rhodobacteraceae, Burkholderiaceae and Lachnospiraceae (Appendix 3). For a full list of

indicator species for each diet group, see Table 3.11. The most abundant taxa in the gut contents of all diet groups include from the Family Ruminococcaceae, Rikenellaceae, Erysipelatoclostridiaceae, Bacillaceae, Lachnospiraceae, Butyricicoccaceae, and Vibrionaceae (Appendix 4).

DISCUSSION

In this study, we investigated how *C. violaceus*, a marine herbivorous fish, responds to different diets with varying protein content in the laboratory. We observed notable shifts in host physiology (including digestive enzyme activities), gene expression, and the hindgut microbiome in response to these dietary changes. As expected, SCFA and digestive enzyme activity levels changed depending on the diet consumed, and fish assimilated their assigned diets. Our initial expectation was that wild fish and fish fed an herbivore diet in the laboratory would display more similarities with each other. For gut mass, including distal intestine relative mass, we did see similarities among these groups of fish, but for microbiome analyses, we found that wild fish exhibited similarities with fish fed an omnivore diet in the lab. Specifically, WF and LO diet fish had more similar hindgut transcriptome and microbiome profiles and higher SCFA levels compared to LH and LC fish. Despite this unexpected pattern in the hindgut, WF and LH diet fish shared similar metabolic pathways in the liver transcriptome, and had higher expression of genes that play roles in gluconeogenesis, protein metabolism, carbohydrate metabolism, and lipid metabolism (Table 3.8; Fig. 3.8). For many digestive enzymes, the laboratory diets had positive impacts on activity levels relative to wild fish, except for the microbially-derived β-glucosidase, the activities of which were highest in LH and wild fish. Each of these findings will be discussed below.

The interplay between gene expression, the microbiome, and SCFAs

In our study, we aimed to examine the interplay between genes involved in digestion and metabolism in the host hindgut transcriptome and the hindgut bacterial diversity. Contrary to our expectations, wild *C. violaceus* and LO diet fish share the most similar gene expression and gut microbiome profiles. Given that the gut microbiome plays a critical role in maintaining host digestive functions, immune function, and overall health (McFall-Ngai et al. 2013), the similarities in microbiome and host transcriptome is intriguing, as host gene expression can be directly influenced by the gut microbiome in stickleback fish (Fuess et al. 2021), mice (Richards et al. 2019), and humans (Blekhman et al. 2015; Muehlbauer et al. 2021). Moreover, host genetics, particularly genes coding for proteins of the immune system, also have a direct influence on enteric microbial diversity (Nagarajan et al. 2023; Fietz et al. 2018; Davenport 2016; Blekhman et al. 2015), which can help explain inter-individual variation within a species.

Here, we found that genes involved in lipid and glucose metabolism were highly expressed in the hindgut of wild *C. violaceus* (Table 3.6), consistent with SCFAs, such as acetate and butyrate, inducing expression of these pathways in other animals (Birkeland et al. 2023; He et al. 2020; Kimura et al. 2020). The bacterial families Rikenellaceae, Ruminococcaceae, and Lachnospiraceae, which contain species that are known to produce SCFAs like acetate and butyrate, were highly abundant in WF and LO fish (Fig. 3.10) coinciding with higher SCFA levels these two groups (Table 3.4; Fig. 3.5) (Clements and Choat 1997; Mountfort et al. 2002; Nicholson et al. 2012; Pardesi et al. 2022; Stevenson et al. 2022). Wild *C. violaceus* also has high activity of the microbially derived enzyme β-glucosidase, which degrades cellobiose, in their DI o which may be an indicator of microbial activity in the hindgut (Appendix 1; (German et al. 2015)). LH fish had double the β-glucosidase activity of the other laboratory-fed fish, but these activities were not statistically different among the fish fed the laboratory diets (Appendix

1). Further, WF and LH fish had significantly larger relative gut mass and DI relative mass (Fig. 3.2) compared to LC fish, which is at least indicative of higher intake, and potentially for more microbial activity in this gut region (Scheppach 1994; Sibly 1981; German et al. 2015). Thus, we see an interplay between SCFAs and the gut microbiome on the expression of the genes involved in lipid metabolism in the hindgut of *C. violaceus*, which highlighted the potential microbial contributions to nutrient utilization. Why the WF and LO fish shared more similarity could have to do with the protein type or content available to these fish. Although *C. violaceus* is herbivorous, it does consume up to 2% animal matter in nature (Horn et al. 1986), and the amount and type of protein in their natural diet (largely from Amphipods; (German and Horn 2006) may more resemble the LO diet in this study, but this is speculation in terms of how that would impact enteric microbial diversity. Diet content, including protein type, can impact the enteric microbiome (Escalas et al. 2022; Thépot et al. 2022).

Interestingly, we found the liver responded more dynamically to dietary changes compared to the hindgut. There were more differentially expressed genes in the liver (4650 DEGs; Fig. 3.8) compared to the distal intestine (519 DEGs; Fig. 3.6). Similar to our findings in the hindgut, genes involved in lipid metabolism, including carboxyl ester lipase (cel), were highly expressed in wild and LH fish compared to LO and LC fish and may reflect more of the ketotic metabolism that comes with SCFA absorption and metabolic utilization in *C. violaceus* (Willmott et al. 2005; Karasov and Martínez del Rio 2007; Heras et al. 2020; Bergman 1990; Sahuri-Arisoylu et al. 2016; den Besten et al. 2013; Zhou et al. 2021)). Indeed, SCFA absorption can impact host energy homeostasis (Byrne et al. 2015; De Vadder et al. 2014) potentially affecting the gut-microbiome-liver axis (Burr 1998; Willmott et al. 2005; Stevens and Hume 1998; Mountfort et al. 2002; Bergman 1990; den Besten et al. 2013). Other than SCFAs, many

microbial metabolites can impact the host in a number of different tissues, including the brain (Soty et al. 2017; Koh et al. 2016; Mithieux 2018; De Vadder et al. 2014). It is worth mentioning that the liver transcriptome of other fish species seems to be less susceptible to dietary changes (i.e., fewer DEGs than observed in *C. violaceus*), but the most responsive pathway is lipid metabolism (De Santis et al. 2015a; Bernal et al. 2019; Merkin et al. 2012; Betancor et al. 2018). Species-specific patterns of liver gene expression are the norm, and we found that *C. violaceus* has the ability to modify metabolic pathways in the liver in response to dietary changes, more so than other prickleback fishes (Herrera et al. 2022). These findings suggest far more flexibility in nutrient metabolism than strict dietary specialization in *C. violaceus*.

The influence of diet on gene expression, gut microbiome, and digestive enzyme activity

Genes involved in protein metabolism were upregulated in the DI and liver of WF and LH diet fish, and the DI of the LC diet fish (Table 3.6, 3.8; Figs. 3.6, 3.8). Moreover, the LH diet fish and WF had elevated trypsin activities in their DI (Fig. 3.4). Thus, there are elements of utilizing protein when it is abundant (LC diet fish), or scavenging protein when it is limiting (LH diet fish and WF), since the algae consumed by *C. violaceus* is low in protein (Table 3.2; (Neighbors and Horn 1991; Murray et al. 2003)). Interestingly, *Podarcis siculus*, a lizard that recently evolved a lower-protein, omnivorous diet in specific populations, showed similar patterns of elevated trypsin in its hindgut (Wehrle et al. 2020), coinciding with protein-scavenging microbes, like those in the family Peptostreptococcaceae, as part of their microbiome (Lemieux-Labonté et al. 2022). Coincidentally, Peptococcaceae are indicator taxa for WF *C. violaceus* (Fig. 3.11)(Karasov and Martínez del Rio 2007; Karasov 1992).

In terms of carbohydrate digestion and metabolism, we did find some support for the Adaptive Modulation Hypothesis as the lab-formulated diets do vary in carbohydrate content

(Karasov and Martínez del Rio 2007; Karasov 1992). In the hindgut, we found the wild fish highly expressed genes involved in glucose metabolism. And in the liver, we found that wild, LH, and LO fish upregulated genes involved in carbohydrate metabolism, glycolysis and gluconeogenesis. In terms of digestive enzyme activity, we found that as fishes consumed more carbohydrates in the laboratory diets (LH and LO diet fish), they exhibited higher levels of amylase activity in their hindgut, even though WF had lower amylolytic activity in their DI; WF of *C. violaceus* have elevated amylase activity in their foregut sections (German et al. 2016; German et al. 2015). Thus, *C. violaceus* has some flexibility in their ability to digest and process more carbohydrates as those carbohydrates increase in the diet. These amylase activities were measured in the distal intestine specifically, and similarly, herbivorous rabbitfish (*Siganus canaliculatus*) fed algal diets had more elevated amylolytic activity in their distal intestines than individuals of this species fed fish-based diets (Xie et al. 2018). Likewise, zebrafish (Danio rerio) had more elevated intestinal amylase activities with more starch in the diet (Leigh et al. 2018a). Hence, some plasticity of amylase activity may be the norm, even in the distal intestine.

In general, wild fish exhibited significantly different hindgut microbiomes compared to laboratory-fed fish, suggesting an environmental influence on the fish gut microbiome due to the transition from the wild to laboratory setting. This is similar to the impacts of captivity on other animal gut microbiomes (i.e. (Frankel et al. 2019; McKenzie et al. 2017; Guo et al. 2019; Thépot et al.)). Yet, even among laboratory-fed fish, we found differences between fish fed different diets, in particular LO fish were significantly different from LC and LH fish. This could be explained by the function of the hindgut in digestion. The hindgut is where most microbial diversity and microbially-mediated digestive function is localized and is more supportive of

anaerobe (facultative or obligate) growth because it has a lower oxygen concentration compared to the rest of the gut (Stevens and Hume 1995).

LH and LC fish seemed to potentially struggle with the different laboratory-diets as indicated by the presence of potential pathogenic taxa from the Family Burkholderiaceae, which were highly correlated with these groups and not with WF or LO diet fish (Lipuma et al. 2011). The individual variability in growth rate data within the LC diet fish further suggests that some fish did not tolerate this diet well. For example, the lowest growth rates for the whole experiment were in several LC diet fish, and in those exact individuals, *Shigella* were indicator species, suggesting potential for disease (Duan et al. 2021; Eeckhaut et al. 2016; Fan et al. 2017; Tighe et al. 2022) in *C. violaceus* consuming a fish-based diet.

Members of the bacterial family Oxalobacteraceae that are known to degrade oxalate were abundant and indicator species in LH and LC diet fish. Oxalate can be found in some red and green algal species (Pueschel 2001; Pueschel 2007; Pueschel 2019), but would also be produced in fishes eating higher protein diets (Whittamore 2020; Robijn et al. 2011). Fishes appear to be reliant on enteric secretion of oxalate to eventually excrete this compound into the environment as a waste product (Whittamore 2020). In fact, Whittamore (2020) hypothesized that fishes may harbor oxalate-degrading bacteria similar to some mammals, to maintain gradients of oxalate into the gut environment(Whittamore 2020). For instance, in mice fed excess oxalate producing compounds, the presence of *Oxalobacter* sp. in the intestine does precisely this: the *Oxalobacter* degrades oxalate secreted into the gut, thus maintaining gradients of oxalate secretion into the enteric environment (Hatch et al. 2006; Miller Aaron et al. 2014; Miller et al. 2017). Our data support this, as the groups consuming the most oxalate in dried, ground algae (LH diet fish), or producing more oxalate due to excessive amounts of fish protein in the

diet (LC diet fish) have members of Oxalobacteraceae as indicator species in their guts. The entire metabolism of oxalate requires more attention in fishes in general (Whittamore 2020), since fishes excrete so much of it via the gut, and particularly as we alter fish diets in aquaculture settings. In addition to these microbial taxa changing in abundance with different diets, there are some similarities in the gut microbiome between wild *C. violaceus* and other herbivorous fishes that are reliant on gastrointestinal fermentation, including taxa from the Families Erysipelatoclostridiaceae and Comamonadaceae (Stevenson et al. 2022).

Thus, we found similarities in the microbiome of *C. violaceus* with other marine herbivorous fish and other herbivorous vertebrates and found dietary-induced changes in the hindgut microbiome. Surprisingly, regardless of sample type (i.e. hindgut gut intestinal tissue or hindgut gut contents), the patterns of dietary-induced shifts in the microbiome remained the same, which is interesting given previous studies that have found differences between tissues and contents (Stevenson et al. 2022; Rankins 2023; Nielsen et al. 2017). Overall, our study provides valuable insights into the intricate interplay between host gene expression and the composition of the hindgut microbiome in response to dietary changes.

We used stable isotope analyses to show that *C. violaceus* was assimilating the diets assigned to them in the laboratory (Fig. 3.3). Liver tissue has a shorter turnover rate for carbon of approximately 28 days and is a common tissue used to track dietary history of fishes using $\delta^{13}C$ values (Guelinckx et al. 2007b). Fish fed different diets in the laboratory had $\delta^{15}N$ and $\delta^{13}C$ signatures that were within the expected tissue-diet discrimination factor for each assigned diet, which indicates assimilation of the laboratory diet in the liver of these fish (Guelinckx et al. 2007b; German and Miles 2010; Matley et al. 2016). Unlike the rapid turnover of liver tissue, muscle tissue has a longer turnover rate of about three months (Guelinckx et al. 2007b), thus

providing a longer-term dietary signal. The muscle isotopic signatures showed how similar, in terms of $\delta^{13}C$ and $\delta^{15}N$ signatures, the LH diet fish were to WF consuming their natural algal diet, and how different the LO and LC diet fish were from the LH fish; there was little overlap. The one study of wild-caught pricklebacks shows the lower trophic standing of *C. violaceus* (Saba 2004), but here, we showed that their stable isotopic signature can be altered with higher protein diets

CONCLUSION

In this study, we investigated the effects of dietary changes in a marine herbivorous prickleback fish. Our results highlight the remarkable flexibility of the fish digestive and metabolic systems, which enables them to adjust to changing dietary conditions in their environment. We found that depending on the nutritional composition of the assigned diet, there are genes upregulated to metabolize different food components. This ability is particularly important in the face of changing environments whether it be in an aquaculture setting, or due to shifting resource availability due to global change, and challenges what we think of as "dietary specialization". We found that the fish were capable of assimilating different diets, leading to variations in performance, gut structure, gut function, microbial diversity, and SCFA levels. One of the most dramatic findings is that the gene expression of the liver is far more flexible in *C. violaceus* than in many other fishes, including other pricklebacks (Herrera et al. 2022). Why the LO diet fish possessed enteric microbial diversity and DI transcriptomic profiles similar to WF in *C. violaceus* requires further study, and perhaps hints that animal protein plays a larger role in meeting their nitrogen needs than previously considered. Our findings contribute to a deeper understanding of how marine herbivorous fish acclimate to dietary changes and pave the way for

future investigations into the broader implications of these interactions in the context of ecological and evolutionary dynamics.

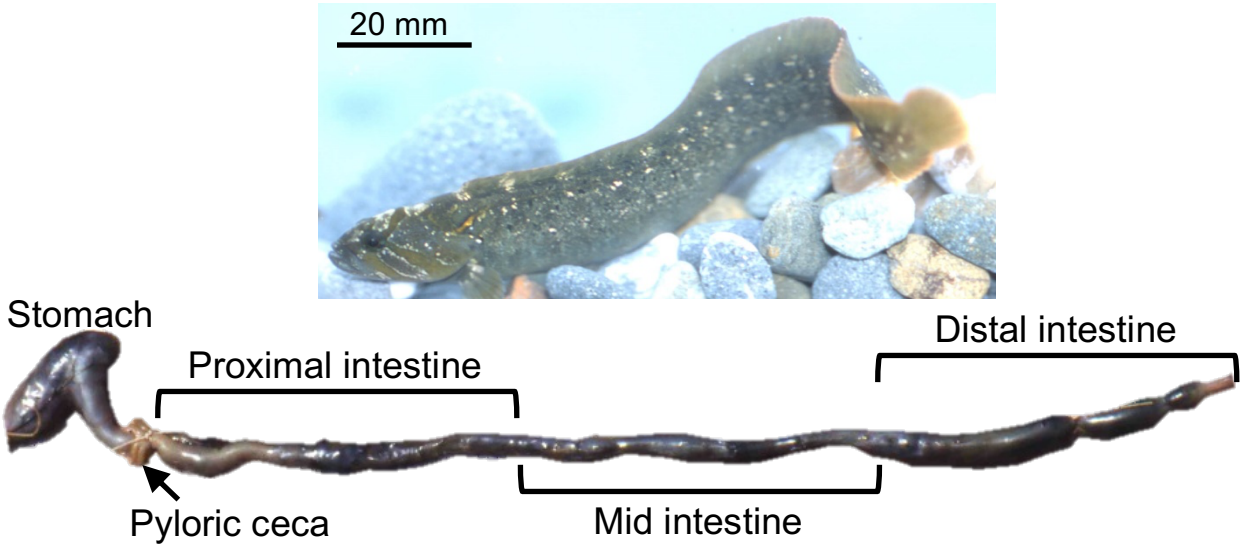

Figure 3.1 *Cebidichthys violaceus* and its digestive system showing the different parts of the gut. For this study, we focused on measuring digestive enzyme activities, short chain fatty acid (SCFA) concentrations, tissue transcriptomics, and microbial diversity of the distal intestine in fish fed carnivore, omnivore, and herbivore diets in the laboratory. We also examined the transcriptomics of the liver in these same fish. Photo by Michael H. Horn.

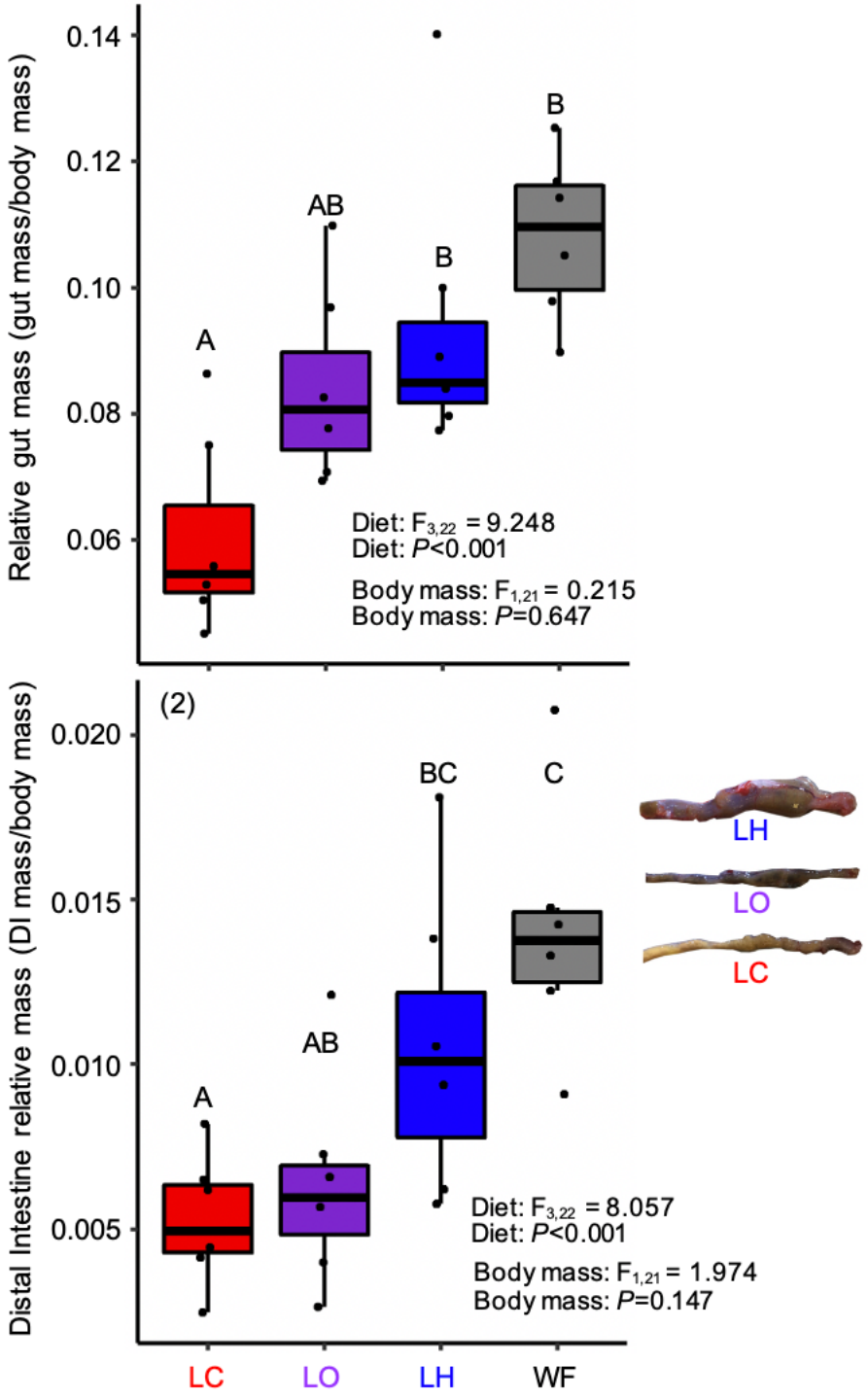

Figure 3.2 Box and whisker plots of (1) the relative gut mass (gut mass/body mass) and (2) distal intestine (DI) relative mass (DI mass/body mass) in *Cebidichthys violaceus* from the wild (WF), or after consuming different diets in the laboratory for six months. LC=lab carnivore diet, LO=lab omnivore diet, and LH=lab herbivore diet. ANCOVA detected significant differences in relative gut mass and distal intestine relative mass among the different treatments, whereas body mass was not a significant co-variable (statistics on graphs). Those boxes within a graph not

sharing a capital letter are statistically significantly different. Representative photos of the final 60 mm of the distal intestine from the laboratory-fed fish are shown next to the DI relative mass graph for comparison among the fish on the different diets in the laboratory.

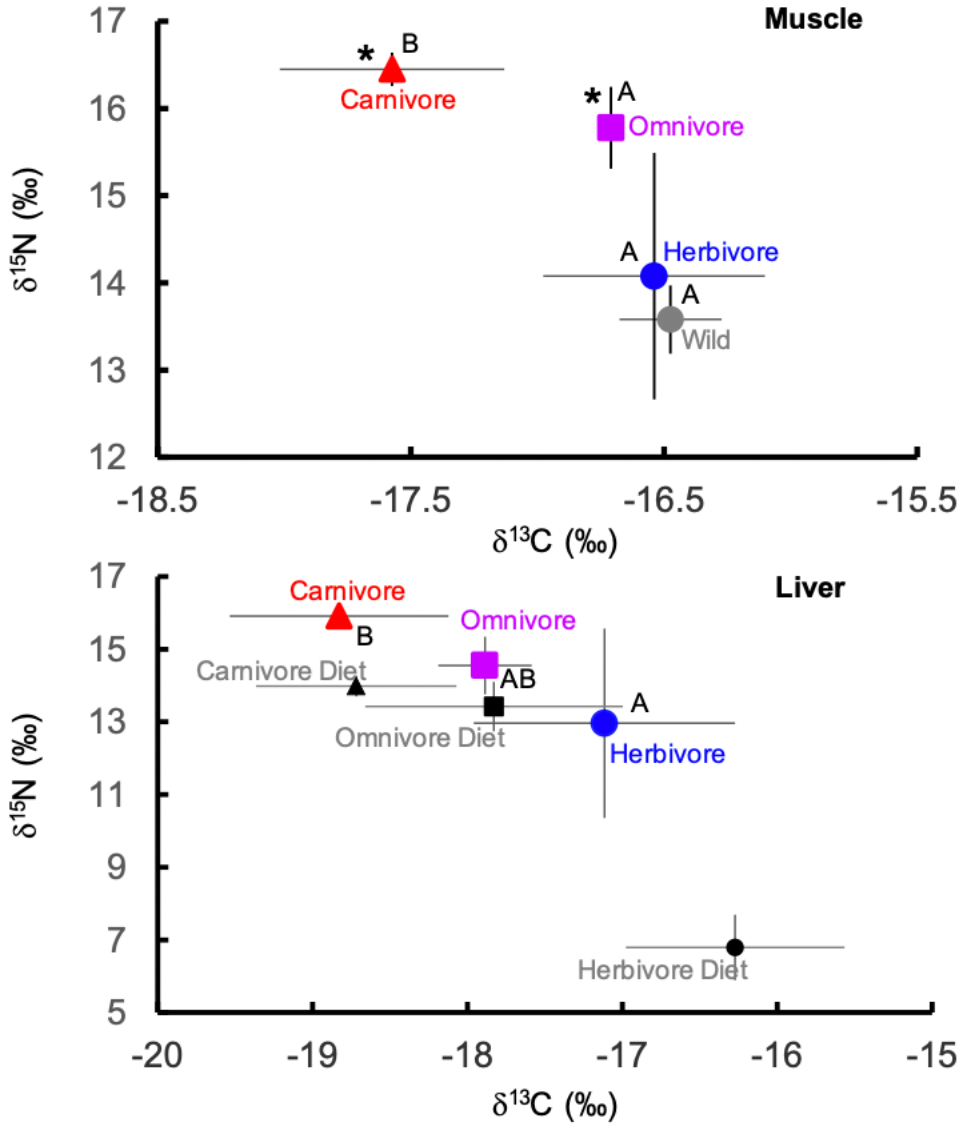

Figure 3.3. Carbon and nitrogen (‰) dual plot showing muscle tissue (top) and liver tissue (bottom) of *Cebidichthys violaceus* collected directly from the wild, or fed herbivore, omnivore, or carnivore diets in the laboratory over six months. The top plot contrasts muscle isotopic signatures of the laboratory-fed fish with wild-caught fish, whereas the bottom plot shows the isotopic signatures of fish livers relative to the diets the fish were consuming in the laboratory. Values are mean ± standard deviation on both axes. N=3 for all laboratory-fed fish, whereas N=5

for wild fish and each of the experimental diets. Asterisks indicate significant differences in $\delta^{15}N$ values, and letters for $\delta^{13}C$ values (ANOVA, symbols sharing an asterisk or letter are not different from each other).

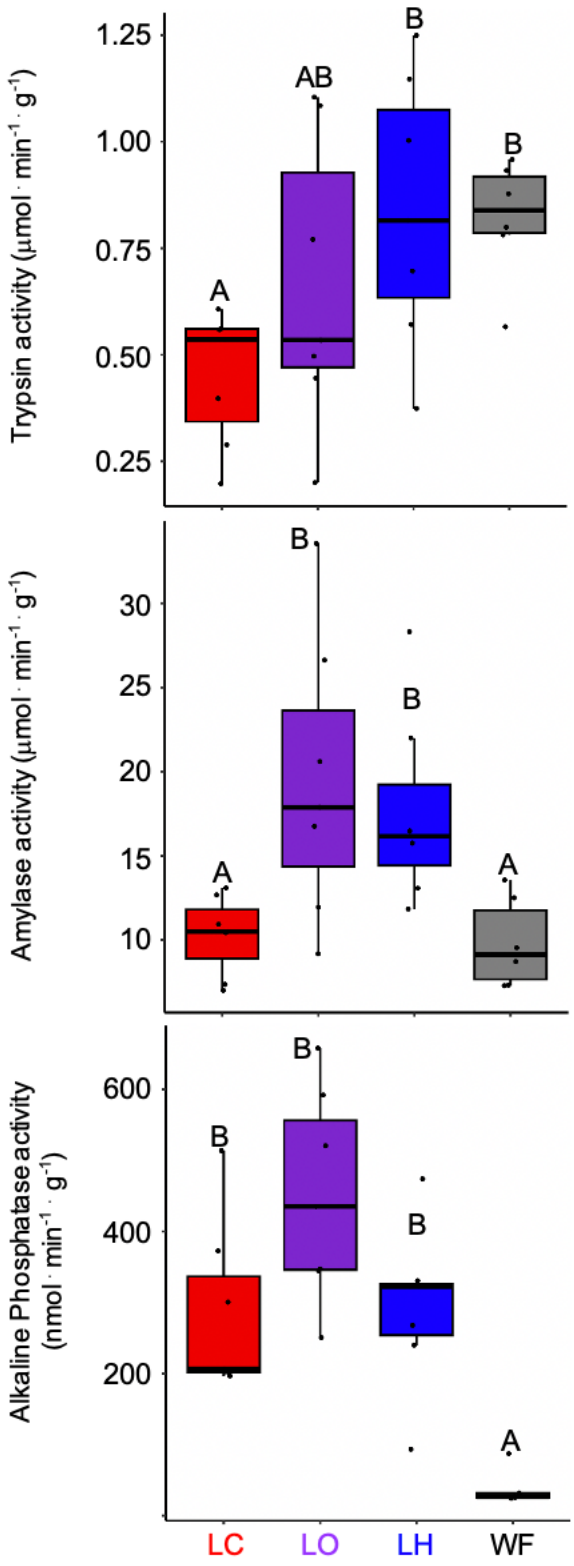

Figure 3.4. Box and whisker plots of trypsin (top), amylase (middle), and alkaline phosphatase (bottom) activities in the distal intestines of *C. violaceus* from the wild (WF), or after consuming different diets in the laboratory for six months. LC=lab carnivore diet, LO=lab omnivore diet,

and LH=lab herbivore diet. Values for each enzyme were compared among the fish fed the different diets and the wild-caught fish with ANOVA followed by a Tukey's Honest Significant Difference with a family error rate of $P = 0.05$. Data on wild-caught fish from German et al. (2015), except for alkaline phosphatase, which were measured on recently captured fish. Trypsin activities ($F_{3,23} = 3.29$, $P = 0.039$), amylase activities ($F_{3,23} = 7.38$, $P = 0.001$) and alkaline phosphatase activities ($F_{3,23} = 35.56$, $P < 0.001$) activities showed differences among the fish fed the different diets. Those values sharing a letter for a particular enzyme are not significantly different.

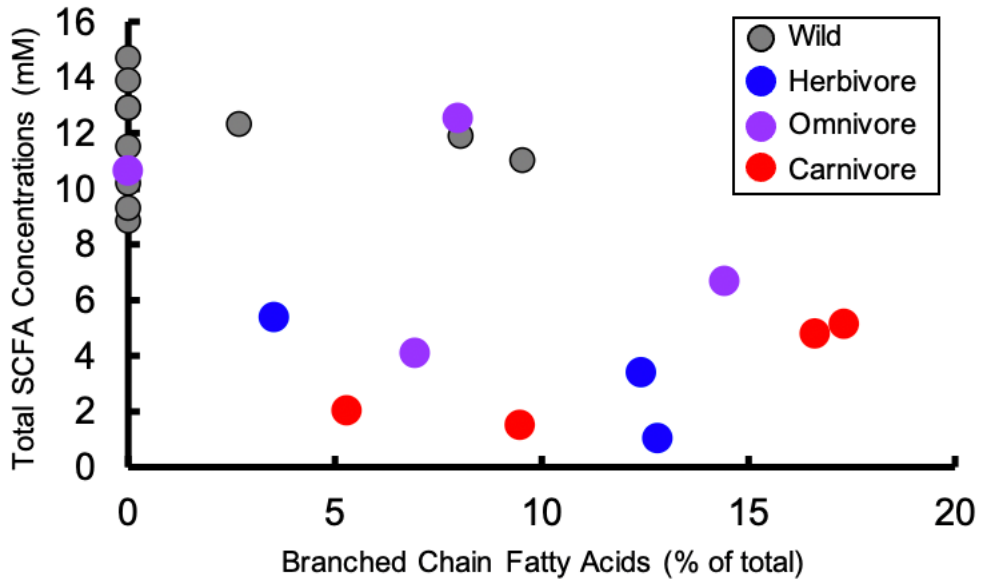

Figure 3.5. Total short-chain fatty acid (SCFA) in distal intestine vs. mean branched-chain fatty acids (isobutyrate and isovalerate) as a percentage of total SCFA in *Cebidichthys violaceus* captured from the wild, or fed herbivorous, omnivorous, or carnivorous diets in the laboratory for six months. Each point is an individual fish.

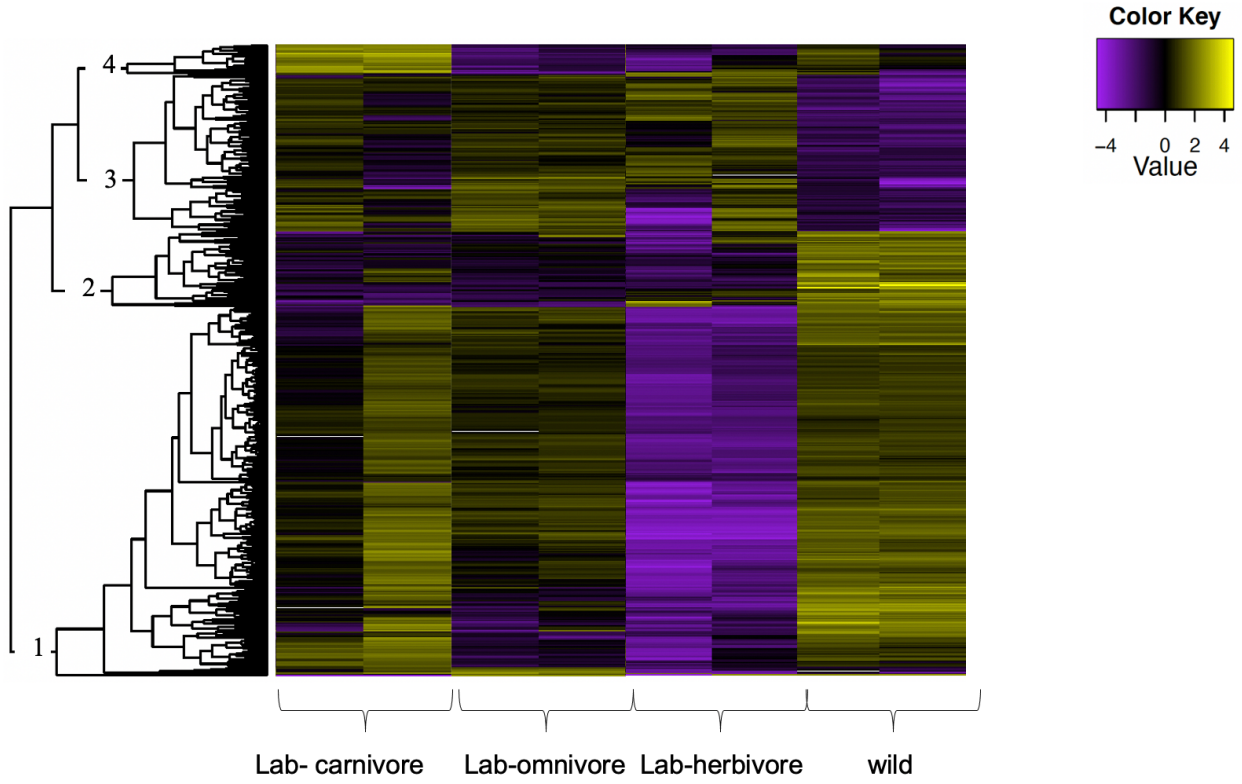

Figure 3.6. Differential gene expression depicted as a heatmap in the distal-intestine of *C. violaceus* either from the wild, or after consuming different diets in the laboratory for six months. Yellow indicates elevated relative expression, whereas purple indicates low expression. Each row is a single gene, and genes are clustered in a dendrogram (on left of each heatmap) by similarity of expression patterns (see Table 4.4). There are 519 differentially expressed genes. Each column represents the gene expression in an individual fish.

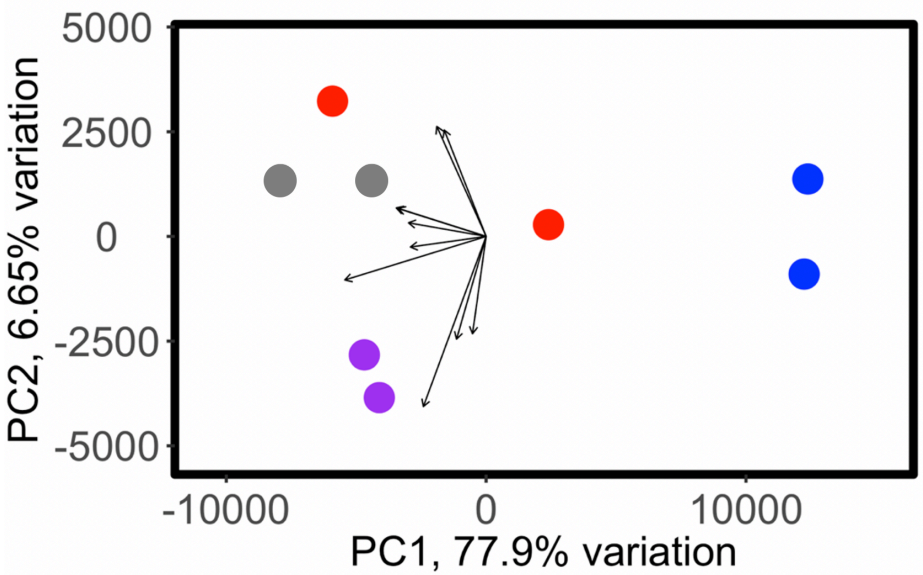

Figure 3.7. Principle Components Analysis plot of gene expression data of distal intestine of *C. violaceus* either from the wild, or after consuming different diets in the laboratory for six months. Colors represent diet, with wild individuals in black, lab-herbivore individuals in blue, lab-omnivore individuals in purple, and lab-carnivore individuals in red. Vectors indicate the 'weight' in different directions for the genes driving differences along each PC (fall within the top 5% of loadings range). The gene list can be found in Table 6.

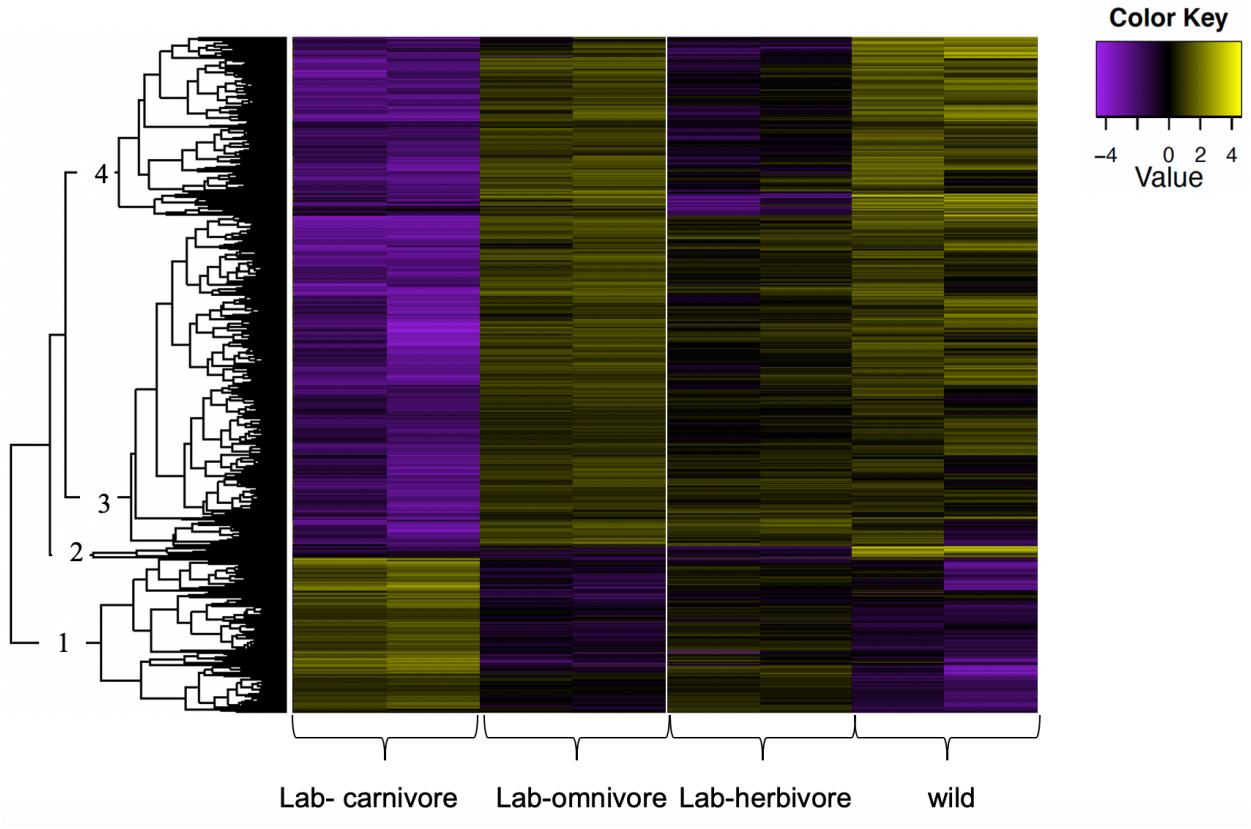

Figure 3.8. Differential gene expression depicted as a heatmap in the liver of *C. violaceus* either from the wild, or after consuming different diets in the laboratory for six months. There are 4650 DEGs, out of which 63.6% are annotated. Yellow indicates elevated relative expression, whereas purple indicates low expression. Each row is a single gene, and genes are clustered in a dendrogram (on left of each heatmap) by similarity of expression patterns (Table 3.4). Each column represents the gene expression in an individual fish.

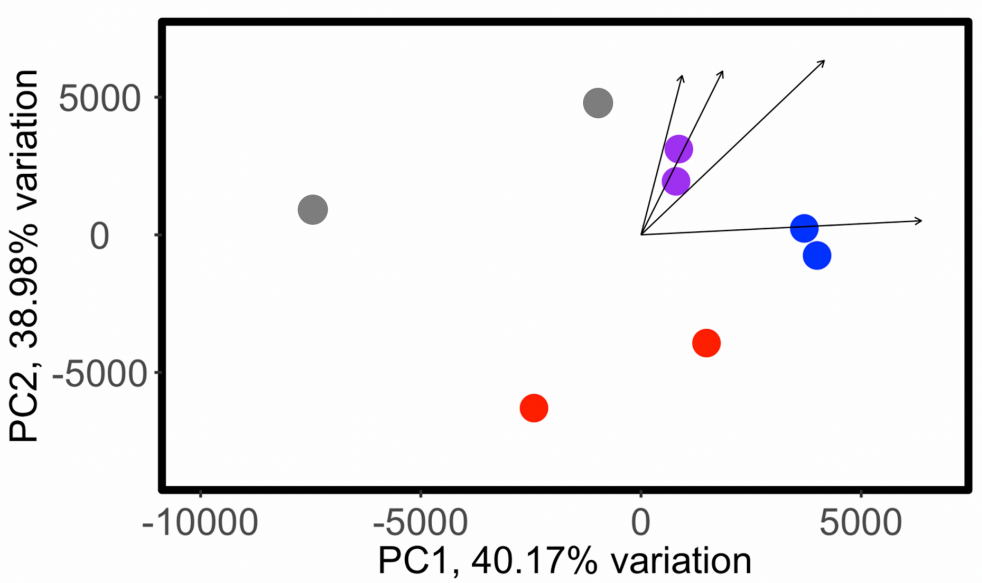

Figure 3.9. Principle Components Analysis plot of gene expression data of liver of *C. violaceus* either from the wild, or after consuming different diets in the laboratory for six months. Colors represent diet, with wild individuals in black, lab-herbivore individuals in blue, lab-omnivore individuals in purple, and lab-carnivore individuals in red. Vectors indicate the 'weight' in different directions for the genes driving differences along each PC (fall within the top 5% of loadings range). The gene list can be found in Table 8.

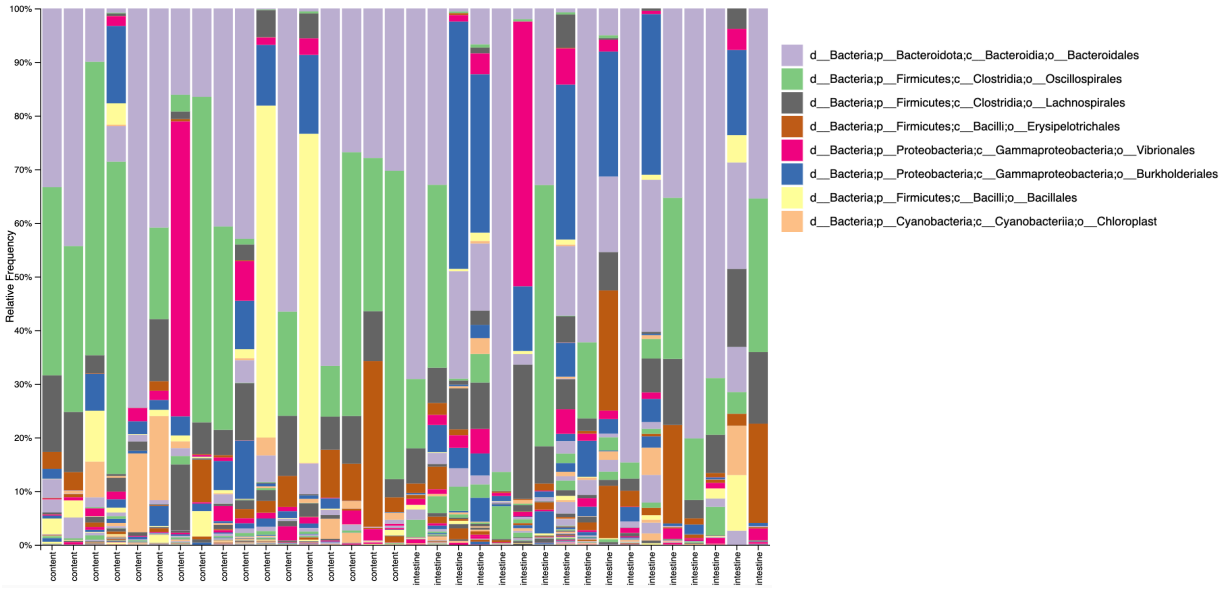

Figure 3.10. Stacked bar plot of relative frequency of microbial communities at the Order level. The key depicts the top ten abundant microbial Orders.

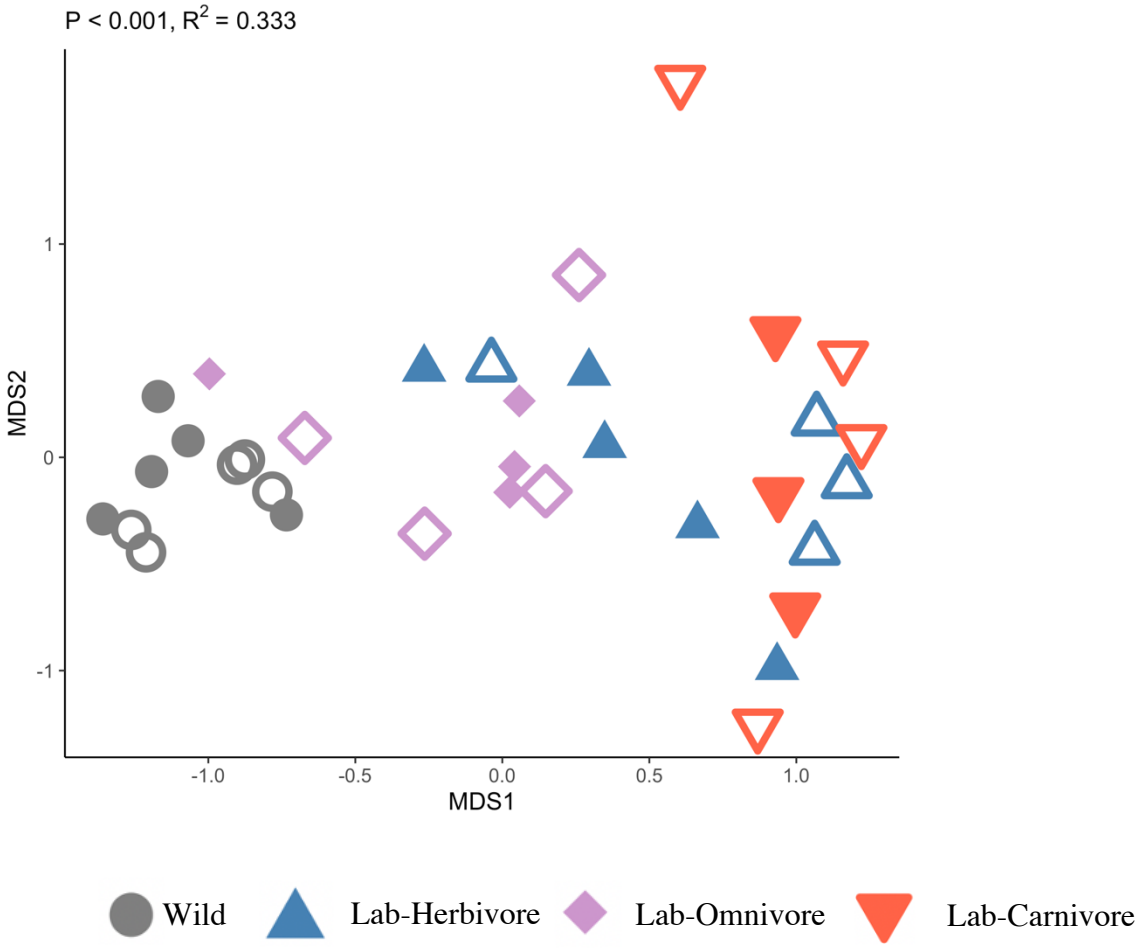

Figure 3.11 NMDS plot of *C. violaceus* gut intestinal tissue and gut contents based on Bray-Curtis distances

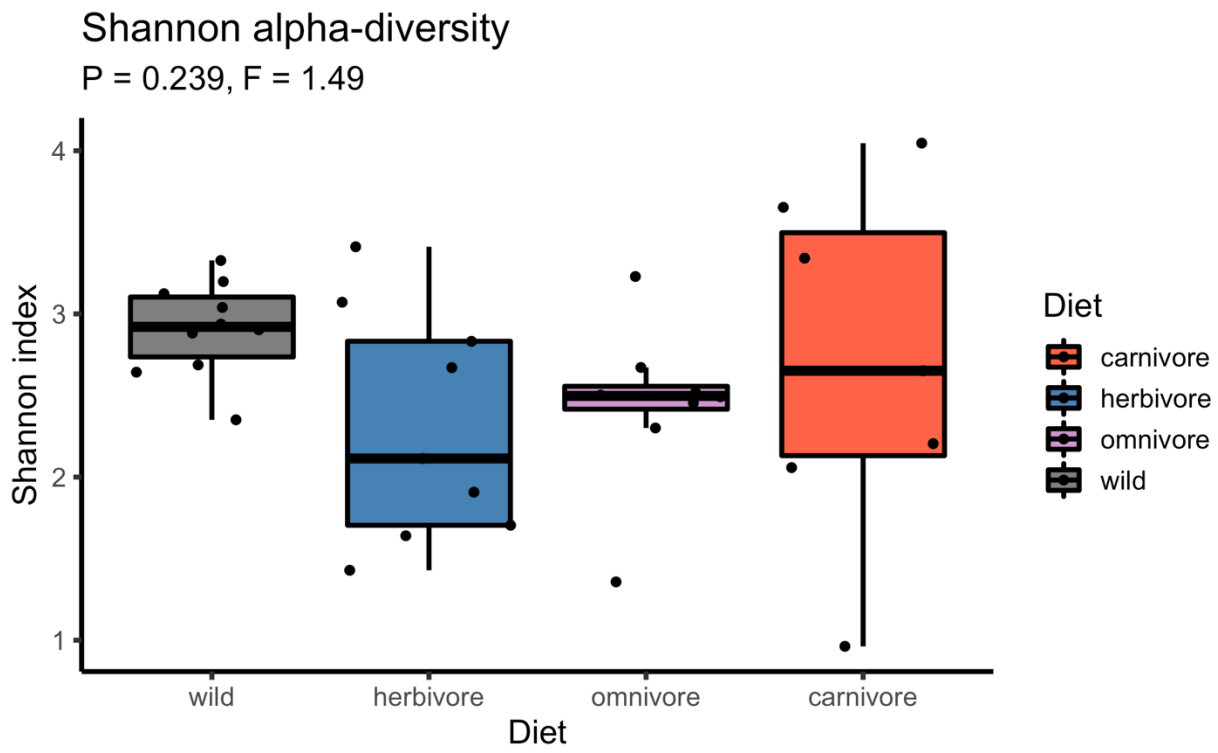

Figure 3.12 Shannons alpha diversity comparing wild fish with fish fed different diets in the laboratory

Table 3.1. Predictions for morphological and performance outcomes of *Cebidichthys violaceus* after consuming different diets in the laboratory.

	Lab-Carnivore	Lab-Omnivore	Lab-Herbivore	Wild fish
Gut Length	Shortest	Moderate	Long	Long
Relative Gut Mass	Lightest	Moderate	Heaviest	Heaviest
Growth Rate	Fastest	Moderate	Moderate	N/A
Stable Isotope ($\delta^{13}C$ and $\delta^{15}N$ signatures)	$\delta^{15}N$: More enriched $\delta^{13}C$: match carnivore diet	$\delta^{15}N$: Moderately enriched $\delta^{13}C$: match omnivore diet	$\delta^{15}N$: Less enriched $\delta^{13}C$: match herbivore diet	$\delta^{15}N$: Less enriched $\delta^{13}C$: more like herbivore diet
Digestive Enzymes	High activity for protein degrading enzymes	High activity for carbohydrate and protein degrading enzymes	High activity for carbohydrate degrading enzymes	High activity for carbohydrate degrading enzymes
Gut Microbiome	Unique microbiome	Unique microbiome	Similar microbiome to wild fish	Similar microbiome to lab-herbivore fish
Gene Expression of Hindgut and Liver	High expression of genes involved in protein digestion and metabolism	High expression of genes involved in carbohydrate and protein digestion and metabolism	High expression of genes involved in carbohydrate digestion and metabolism and carboxyl ester lipase	High expression of genes involved in carbohydrate digestion and metabolism and carboxy ester lipase

Table 3.2. Ingredients and chemical composition of the herbivore, omnivore, and carnivore diets fed to *C. violaceus* in the laboratory.*

Ingredient (g/100g)	**Herbivore**	**Omnivore**	**Carnivore**
Mazzaella splendens	29.50	14.41	0.00
Porphyra sp.	27.00	14.42	0.00
Ulva lobata	30.00	14.42	0.00
Fish	0.00	43.25	86.50
Casein	2.00	2.00	2.00
Soybean Meal	2.00	2.00	2.00
Oil	6.00	6.00	6.00
Methyl cellulose	1.50	1.50	1.50
Vitamin Premix	1.00	1.00	1.00
Vitamin C	0.40	0.40	0.40
Mineral Premix	0.60	0.60	0.60
Chemical analyses			
Protein (%)	22.81	45.40	68.80
Carbohydrate (%)	36.82	19.46	2.17
Lipid (%)	7.66	12.34	11.90
Kilojoules	606.26	1103.74	1430.90
Organic Matter (%)	72.08	81.40	89.14

* The omnivore and carnivore diets are identical to those described in Herrera et al. (2022).

Table 3.3. Standard length (SL), body mass (BM) of, and weight gained by *Cebidichthys violaceus* at the beginning and end of the six month feeding experiment with different diets.

Diet	Beginning SL (mm)	End SL (mm)	Beginning BM (g)	End BM (g)	% Weight Gain
Carnivore	111.86 ± 24.80	164.71 ± 20.34	9.18 ± 5.65	29.40 ± 15.97	403.33 (109.88-1132.48)
Omnivore	105.29 ± 24.07	152.14 ± 23.88	7.56 ± 4.81	26.09 ± 13.67	436.85 (145.53-860.88)
Herbivore	105.57 ± 31.33	139.57 ± 26.20	9.37 ± 7.51	18.87 ± 10.46	249.69 (133.32-438.37)

For SL and BM, values are mean ± standard deviation. For % Weight Gain, values are mean (total range of data in parentheses). Beginning BM ($F_{2,18}$ = 0.185, P = 0.832), Ending BM ($F_{2,18}$ = 1.105, P = 0.353), and % Weight gain ($F_{2,18}$ = 0.972, P = 0.379) were not different among the diets.

Table 3.4. Total Short Chain Fatty Acid (SCFA) concentrations (mM), and the ratios of Acetate:Propionate:Butyrate in the distal intestines of *C. violaceus* fed different diets in the laboratory for six months, and in those captured from the wild.

SCFA	Carnivore diet	Omnivore diet	Herbivore diet	Wild*	
Total	3.36 ± 1.86^{A}	8.49 ± 3.82^{B}	3.27 ± 2.18^{A}	11.68 ± 1.78^{B}	$F_{3,19}$=19.87, P<0.001
Ratios	64:11:8	61:21:8	61:16:10	53:32:11	

Values are mean ± standard deviation. SCFA concentrations were compared among the fish on the different diets and the wild fish with ANOVA, and those values sharing a letter are not statistically significantly different. * Data from wild fish from German et al. (2015).

Table 3.5. Differentially Expressed Genes Cluster Definitions as presented in the heat maps

Tissue	Cluster	Lab-Carnivore Diet	Lab-Omnivore Diet	Lab-Herbivore	Wild fish
Liver	1: elevated in lab carnivore genes	High	Low	Low	Low
	2 : elevated in wild genes	Low	Low	Low	High
	3: wild-lab herbivore-lab omnivore genes	Low	High	High	High
	4: wild-lab omnivore genes	Low	High	Low	High
	5: wild-herbivore genes	Low	Low	High	High
Distal-intestine	1:elevated in wild, lab omnivore, and lab carnivore	High	high	low	High
	2: High in wild, low in lab fish	low	low	Low	high
	3: Low in wild, high in lab	High	High	High	Low
	4: Low in omnivore fish, high in wild, herbivore and carnivore fish	High	Low	High	High

Table 3.6. Differentially Expressed Genes relevant to metabolism and digestion in the distal intestines of *C. violaceus* either from the wild or fed different diets in the laboratory for six months.

Cluster	Gene	Function	Lab-carnivore	Lab-omnivore	Lab-herbivore	Wild fish
2	Low-density lipoprotein receptor	Lipid transport	++	+	++	++++
2	Acetyl-CoA carboxylase 1	Fatty acid biosynthesis	+++	+	++	++++
2	UDP-glucose:glycoprotein glucosyltransferase 1	protein glycosylation	+	+	+	++++
2	Spermatogenesis-associated serine-rich protein 2	RNA binding	++	+	+	+++
2	Insulin receptor substrate 2	Glucose metabolic process	+	+	+	+++
2	Solute carrier family 23 member 2	response to oxidative stress;ascorbic acid transport	+	+	+	+++
2	Low-density lipoprotein receptor-related protein 2	lipoprotein transport;negative regulation of endopeptidase activity;vitamin metabolic process	++	+	+	++++
3	Intelectin	lipopolysaccharide binding	++	++	+++++	+
3	Retinoid-binding protein 7	Retinol binding	+++++	+++++	+++++	++
4	Monoacylglycerol lipase	acylglycerol catabolic process	+++	+	+++	++++
4	Leucyl-cystinyl aminopeptidase	protein catabolic process	+++	+	+++++	+++++
4	ADP-dependent glucokinase	glycolytic process	+++++	+	+++++	+++++
4	Glycogen debranching enzyme	glycogen biosynthetic process	+++	+	+++	+++
4	Solute carrier family 12 member 4	calcium ion homeostasis	+++	+	++++	+++++

4	Plasma membrane calcium-transporting ATPase 1	calcium ion export	++	+	++++	++++
4	Carboxypeptidase D	peptide metabolic process	+++++	+	+++++	++++ +

Table 3.7. Annotated gene identification of the distal intestine for those genes associated with the vectors of the Principle Component Analysis in Figure 3.7.

Genes associated with carnivores and wild fish (towards top left of the PCA plot)	domain-containing protein 3-like
	Pholis chromosome 5
	chitin synthase
	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
	pholis chromosome 4
	transcriptional repressor
Genes associated with omnivores (towards left and bottom left of the PCA plot)	actin-binding repeat-containing protein 1-like
	pholis chromosome 23
	pholis chromosome 12
	zinc transporter

Table 3.8. Differentially Expressed Genes relevant to metabolism and digestion in the livers of *C. violaceus* either from the wild or fed different diets in the laboratory for six months.

Cluster	Gene	Function	Lab Carnivore	Lab Omnivore	Lab Herbivore	Wild fish
2	Fatty acid synthase	Fatty acid synthesis	++	+	++	+++++
2	Glucose-6-phosphate 1-dehydrogenase	produces NADPH for fatty acid and nucleic acid synthesis	+	+	++	+++++
2	Lipoyl synthase	lipoic acid metabolism	+	+	++	++++
2	Lanosterol synthase	cholesterol biosynthesis	+	+	+	+++
2	Acetyl-CoA carboxylase 1	Fatty acid synthesis	+	+	++	++++
2	Lanosterol synthase	cholesterol biosynthesis	+	+	++	+++++
2	Phospholipase D2	Fatty acid synthesis	+	++	+++	++
2	Cytosolic carboxypeptidase 1	Proteolysis	+	+	++	++++
3	Apolipoprotein B-100	Lipid metabolism	+	+++++	+++++	+++++
3	Glucosidase 2 subunit beta	Glycolysis	+	+++	+++	+++

3	Glucose-6-phosphate 1-dehydrogenase	produces NADPH for fatty acid and nucleic acid synthesis	+++	+++++	+++++	+++++
3	Phosphate-regulating neutral endopeptidase	Fatty acid biosynthesis	+	+++++	+++++	+++++
3	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Glucose metabolism	+	++++	+++++	+++++
3	UDP-glucose:glycoprotein glucosyltransferase 1	protein glycosylation	+	++	++	++
3	Signal peptide peptidase-like 2B	Proteolysis	+	++	++++	+++++
3	Glucosidase 2 subunit beta	Glycolysis	+	+++++	+++++	++
3	Lysosomal alpha-glucosidase	Glycolysis	+	++	+++++	+++++
3	Long-chain fatty acid transport protein 1	Lipid transport	++	++++	+++++	+++++

3	Glucosamine 6-phosphate N-acetyltransferase	protein and lipid glycosylation	+	++	++++	++
3	UDP-glucose:glycoprotein glucosyltransferase 1	protein glycosylation	+	++++	+++++	++++
3	Phospholipase D1	Phopholipid catabolism	+	++	++++	++
3	Alpha-N-acetylgalactosaminidase	Carbohydrate catabolism	+	++	+++++	+++++
3	Glucosylceramidase	Beta-glucoside catabolism	++	+++++	+++++	+++++
3	Endoplasmic reticulum aminopeptidase 2	Proteolysis	++	+++++	+++++	+++++
5	Carboxyester lipase (CEL)	Lipid metabolism	+	+	+++++	+++++
5	Phosphatidate phosphatase LPIN2	Lipid metabolism	+	+	+++++	+++++
5	Phosphoenolpyruvate carboxykinase [GTP],	Gluconeogensis	+	+	+++++	+++++

	mitochondrial Lysosomal acid lipase/cholesteryl ester hydrolase	Lipid metabolism	+	++	++++	+++++
5	Phospholipid phosphatase 2	Lipid metabolism	+	++	++++	+++++
5	Leucyl-cystinyl aminopeptidase	Proteolysis	+	+	++	++
5	Calcium-independent phospholipase A2-gamma	Lipid metabolism	+	+	++	+++
5	Carboxy ester lipase (CEL)	Lipid metabolism	+	+	+++++	+++++
5	Aminopeptidase Ey	Proteolysis	+	++	+++++	+++++
5	Protein-glucosylgalactosyl hydroxylysine glucosidase	Carbohydrate metabolism	+	+	++	++

Table 3.9. Annotated gene identification of the liver for those genes associated with the vectors of the Principle Component Analysis in Figure 3.9.

Genes associated with lab-omnivores (towards top right of the PCA plot)	inter-alpha-trypsin inhibitor heavy chain 3 (itih3), mRNA Function: Protease inhibitor
	helicase with zinc finger 2 (helz2), mRNA Function: RNA binding and transcription regulation
	apolipoprotein B-100-like Function: Lipid transport
Genes associated with lab-herbivores (towards right of the PCA plot)	sterile alpha motif domain-containing protein 9-like Function: mediates down-regulation of growth factor signaling

Table 3.10 Core feature microbial taxa in wild *C. violaceus* fishes present in 100% of samples

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes

d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum

d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum; s__Gadus_morhua

d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae

d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Massilia

d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium

d__Bacteria; p__Bacillota; c__Clostridia; o__Peptococcales; f__Peptococcaceae; g__uncultured; s__metagenome

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus; s__Butyricicoccus_pullicaecorum

d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae

d__Bacteria; p__Bacillota; c__Bacilli; o__Erysipelotrichales; f__Erysipelatoclostridiaceae; g__Erysipelatoclostridium; s__Erysipelotrichaceae_bacterium

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__Alistipes_indistinctus

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__Alistipes_finegoldii

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Angelakisella; s__uncultured_bacterium

d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae

d__Bacteria; p__Bacteroidota; c__Bacteroidia

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium

d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium

Table 3.11 Indicator Species Analysis for Intestine and Contents	
Diet	**Taxa Indicator Species for Intestine**
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Tannerellaceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__Alistipes_indistinctus
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__Alistipes_finegoldii
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Pygmaiobacter; s__Pygmaiobacter_massiliensis
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae
herbivore	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__Flavobacterium_terrigena
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum
omnivore	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales
omnivore	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae
carnivore	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Propionibacteriales; f__Propionibacteriaceae; g__Cutibacterium
carnivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Alkanindiges
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Anoxybacillus

carnivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Chromobacteriaceae; g__Vogesella
carnivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Enterobacterales; f__Enterobacteriaceae; g__Escherichia-Shigella
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Massilia
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Massilia
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Massilia
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Burkholderiaceae; g__Polynucleobacter; s__Polynucleobacter_cosmopolitanus
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Caulobacterales; f__Caulobacteraceae; g__Brevundimonas
omnivore &wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus; s__Butyricicoccus_pullicaecorum
Diet	**Taxa Indicator Species for Contents**
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Tannerellaceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__Alistipes_indistinctus
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes
wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Clostridia_UCG-014; f__Clostridia_UCG-014; g__Clostridia_UCG-014

wild	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__Pseudahrensia
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
wild	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Rikenella; s__uncultured_bacterium
omnivore &wild	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum
omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae; g__NK4A214_group
herbivore &omnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
carnivore &herbivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Paracoccus
carnivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingorhabdus; s__Sphingorhabdus_rigui
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Lactobacillales; f__Listeriaceae; g__Brochothrix
carnivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Comamonadaceae
carnivore	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Chromobacteriaceae; g__Vogesella
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus
carnivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhodobiaceae; g__Amorphus; s__Amorphus_suaedae
carnivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum; s__Methylobacterium_variabile
carnivore	d__Bacteria; p__Bacteroidota; c__Bacteroidia; o__Flavobacteriales; f__Flavobacteriaceae
carnivore	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Caulobacterales; f__Caulobacteraceae
carnivore	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Epulopiscium
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Staphylococcales; f__Staphylococcaceae; g__Macrococcus
carnivore	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus

Chapter 4

Digestive physiology and individual variation impact the hindgut microbiome of prickleback fishes (Stichaeidae) with different diets

ABSTRACT

The gut microbiome is important in health, physiology and immunology of the host. However, it remains unclear which factors play the largest role in shaping gut microbial communities. With closely-related, sympatric species that vary in diet, prickleback fishes (Family Stichaeidae) are a unique system in which to examine the gut microbiome in the context of their evolutionary relationships, biogeography and diet. We used 16S rRNA sequencing to examine the hindgut microbial compositions of prickleback fish species collected from various locations along the California coast and to examine how the gut microbiome can shift with dietary perturbations in the laboratory. We expected the gut microbiome to mirror host identity regardless of different geographical locations. If diet is a strong influencer of microbial diversity, then those fish consuming the same diet in the laboratory should converge on a similar microbiome. We found that host species identity and individual variation play large roles in affecting the enteric microbial diversity of closely-related, sympatric fish species with different natural diets. Even when they were fed different foods in the laboratory, there was still considerable overlap in the microbiomes of fishes with the exception of one species: *Cebidichthys violaceus*, which is reliant on gastrointestinal fermentation to digest its herbivorous diet. Our study shows some of the factors that influence enteric microbial diversity and the gut's ability to shift in microbial composition.

INTRODUCTION

The gut microbiome plays a crucial role in host physiology and immunology, exchanging signals with organ systems to maintain digestive and immune functions, and overall health (Huttenhower et al. 2012; Thaiss et al. 2016; Nicholson et al. 2012; Nicholson et al. 2005; Moran et al. 2019; McFall-Ngai et al. 2013). The gut microbiota affects a range of host physiological traits and influences the digestive processes and assimilation of nutrients in the intestine (Sullam et al. 2012; Clements et al. 2014; Egerton et al. 2018; Llewellyn et al. 2014; Pardesi et al. 2022; Stevenson et al. 2022; Bäckhed et al. 2004; Nicholson et al. 2005; Nicholson et al. 2012; Lee and Mazmanian 2010). Changes in both internal and external factors of the host, such as host genetics or species identity (Lutz et al. 2019; Trevelline et al. 2020; Brooks et al. 2016; Ingala et al. 2018; Phillips et al. 2012; Nishida and Ochman 2018; Amato et al. 2019; Moeller et al. 2014; Ley et al. 2008a), host development or spatiotemporal variables (Hroncova et al. 2015; Oren et al. 2017; Kolodny et al. 2019; Li et al. 2017; Kundu et al. 2017), diet (David et al. 2014; Miyake et al. 2015; Wastyk et al. 2021; Flint et al. 2017; Rothschild et al. 2018; Oliver et al. 2021; Baxter et al. 2019; Venkataraman et al. 2016; Sawicki et al. 2017; Maier et al. 2017; McDonald et al. 2018; Muegge et al. 2011; Colman et al. 2012), and geographical location (Linnenbrink et al. 2013; Hroncova et al. 2015; Lankau et al. 2012; Shin et al. 2016; Rehman et al. 2016; Sudakaran et al. 2012; Suzuki et al. 2019; Godoy-Vitorino et al. 2012; Goertz et al. 2019) can influence changes in the microbial composition of an animal's microbiome. However, we still do not understand which of these factors play the largest role in shaping gut microbial communities.

The concept that an animal's genetics and phylogenetic history can influence the diversity of microbes on and in their bodies is known as Phylosymbiosis. Within

Phylosymbiosis, the similarity of microbial communities among various animals mirrors the phylogenetic relationships of the animals themselves, where more closely-related host species, regardless of diet, have more similar microbiomes (Brooks et al. 2016; Clements et al. 2014; Ley et al. 2008a; Kohl et al. 2018b; Dunaj et al. 2020; Guzman and Vilcinskas 2020; Weinstein et al. 2021). A common phylosymbiotic example is the Giant Panda. Although the Giant Panda consumes an herbivorous diet, its gut microbiome resembles that of carnivores in the same family (Ursidae; (Ley et al. 2008a)), and, from a metabolomic perspective, panda digestive physiology has more similarity with other ursids than with distantly related, specialized herbivores (e.g., ruminants or horses) (Nie et al. 2019). In pikas (Lagomorpha) across different North American geographical locations, differences in the gut microbiome were congruent more with host phylogenetic relationships and species identity than changes in geographical location (Kohl et al. 2018b; Wang et al. 2022; Weinstein et al. 2021).

Apart from host evolutionary history, it is clear that diet has a major impact on enteric microbial community membership (Escalas et al. 2021; Miyake et al. 2015; David et al. 2014; Wastyk et al. 2021; Flint et al. 2017; Rothschild et al. 2018; Oliver et al. 2021; Baxter et al. 2019; Venkataraman et al. 2016; Sawicki et al. 2017; Maier et al. 2017; McDonald et al. 2018; Muegge et al. 2011; Colman et al. 2012; Bäckhed et al. 2004; Nicholson et al. 2005; Nicholson et al. 2012; Lee and Mazmanian 2010). For most vertebrate animals, the hindgut is where most microbial diversity and microbial digestive function is localized, with a handful of clades (e.g., ruminants, kangaroos, hoatzins, some rodents) showing foregut compartments with rich microbial activity (Stevens and Hume 1995; Van Soest 1994). The hindgut is more supportive of microbial growth that may be involved in fermentative digestion because it has a lower oxygen concentration, and less plug-flow transport (Lentle and Janssen 2008), usually due to a different

morphology, in comparison to the stomach or proximal intestine (Stevens and Hume 1995). Thus, the hindgut provides the right environment for microbial proliferation and fermentative metabolism. As a biproduct of fermentation, many microbes produce short chain fatty acids (SCFA, like acetate, propionate, or butyrate) that can then be absorbed and used metabolically by the host animal (Stevens and Hume 1998; Bergman 1990). Although most organisms appear to harbor microbes in their guts (Moran et al. 2019), many herbivorous vertebrates have attracted attention because they may require microbial assistance to digest and assimilate plant components (e.g., cellulose, mannitol), a process which endogenous, or host-produced, digestive enzymes are unable to achieve (Mountfort et al. 2002; Burr 1998; Leigh et al. 2018b; White et al. 2010; Van Soest 1994). In many cases, the host animal can obtain some oxidizable substrates from plant structural or fibrous carbohydrates in the form of SCFA after the enteric microbes have digested these "recalcitrant" carbohydrates and produced their fermentative waste products (Van Soest 1994; Bergman 1990; Clements and Choat 1995; Mountfort et al. 2002; Stevens and Hume 1998).

Overall, the gut microbiomes of fishes are less investigated than those of terrestrial vertebrates. Through the utilization of 16S rRNA sequencing, patterns within the fish gut microbiome are becoming clearer. The relationship between diet and the gut microbiome has started to be revealed in some fish species, and there are associated gut microbiota that play a role in nutritional provisioning, metabolic homeostasis and immune defense (Burns et al. 2016; Levraud et al. 2022; Stevenson et al. 2022; Gómez and Balcázar 2008; Sullam et al. 2012). Additionally, it appears that host species identity seems to influence the gut microbiome more than environment or geographical location(Sullam et al. 2012). For instance, there appears to be a core gut microbiome in anadromous fishes, such as salmon, that undergo freshwater to

seawater habitat transitions(Rudi et al. 2018). In marine fishes, there are gut microbes that seems to be more similar to that of terrestrial vertebrates compared to the microbiome of the immediate surrounding environment (Scott et al. 2020; Sullam et al. 2012). Some common microbial taxa in fish gut microbiomes include the phyla Bacillota and Bacteroidota, which are also common in terrestrial vertebrates (Lozupone et al. 2012). However, Pseudomonodota are most prominent microbial phyla found in the fish gastrointestinal tract (Ghanbari et al. 2015; Rombout et al. 2011; Clements et al. 2016; Sullam et al. 2012; Miyake et al. 2015; Smriga et al. 2010), particularly in the guts of carnivorous fishes (Sullam et al. 2012; Egerton et al. 2018; Llewellyn et al. 2014; Stevenson et al. 2022; Liu et al. 2022; Rankins 2023; Leigh et al. 2021). The fish gut microbiome has mostly been examined in the context of ecology (Clements et al. 2009; Clements et al. 2014) or for the purpose of nutritional manipulation in aquaculture settings (as reviewed in (Egerton et al. 2018)). However, there is a gap in our understanding of the gut microbiome of wild fish that exhibit dietary diversity and the ability of overall fish physiology and the gut microbiome to shift with dietary perturbations (Escalas et al. 2021).

Prickleback fishes (Family Stichaeidae) are an intriguing system in which to examine the gut microbiome in the context of their evolutionary relationships, ecosystem and diet (Fig. 4.1). Several prickleback fish species live sympatrically in rocky intertidal habitats along the west coast of North America, and many of them have different diets, including sister-taxa with different diets: *Xiphister mucosus* is herbivorous, whereas *X. atropurpureus* is omnivorous (German et al. 2016; German et al. 2014; German and Horn 2006; German et al. 2004; Heras et al. 2020; Herrera et al. 2022; Kim et al. 2014). Therefore, prickleback fishes provide an ideal system for examining fish gut microbiomes in the contexts of phylosymbiosis, diet, and biogeography.

In this study, we used 16S rRNA sequencing to examine the hindgut microbial compositions of prickleback fish species. This study had two main objectives. *Objective One: How does geography or species identity influence the gut microbiome in prickleback fishes with different diets?* For this first objective of our study, we compared the gut microbiome of *Cebidichthys violaceus* (herbivore), *Xiphister mucosus* (herbivore), *X. atropurpureus* (omnivore) and *A. purpurescens* (carnivore) collected from various locations along the California coast (USA) spanning three degrees of latitude. We focused on two parts of the stichaeid phylogeny: the Cebidichthyinae, which features the evolution of herbivory in *Cebidichthys violaceus*, and the Xiphisterinae, which features the evolution of herbivory in *Xiphister mucosus*, and omnivory in *X. atropurpureus* (Kim et al. 2014). The carnivorous species, *Anoplarchus purpurescens*, is representative of the basal stichaeid dietary condition (Fig. 4.1). We expect that the gut microbiome will mirror host identity regardless of different geographical locations, with more closely-related species, such as the *Xiphister* sister-taxa, to have more similar microbial communities, vs. diet being a stronger determinant of gut microbiomes (i.e., *C. violaceus* and *X. mucosus* being more similar to each other). This prediction is partially formed by the fact that despite having nearly identical diets among the two herbivorous prickleback species (Horn et al. 1986), *C. violaceus* appears to have more microbial involvement in the digestive process—higher SCFA concentrations and β-glucosidase activities in its distal intestine, plus relatively long retention of material in the gut (Fris and Horn 1993; Urquhart 1984)—than *X. mucosus* (German et al. 2015). The prickleback fish populations from the different northern and southern locations along the California coast are likely different based on these fishes' low larval dispersal, making these species highly unlikely to have wide dispersal of genes beyond relatively small biogeographic areas (Hickerson and Cunningham 2005; Wourms and Evans 1974). Thus,

based on Phylosymbiosis and the influence of host genetics on the gut microbiome, we expect there will be a core microbiome among the same species regardless of geographical location, while there may be rarer members of the enteric microbial communities that may change due to different habitats and environments (Rudi et al. 2018; Rankins). Therefore, we will test the strength of Phylosymbiosis across biogeographical locations in fishes with different diets.

Objective Two: How do dietary changes influence the fish gut microbiome? For the second objective of our study, we examined how dietary perturbations can influence the gut microbiome of prickleback fishes collected from the waters off San Juan Island, WA (USA): *Xiphister mucosus* (herbivore), *X. atropurpureus* (omnivore), *P. chirus* (omnivore), and *A. purpurescens* (carnivore) (Fig. 4.1). We started by comparing the hindgut microbiomes of wild individuals of these sympatric species with different natural diets (Rankins). In support of Phylosymbiosis, we expected to find that more closely related species (e.g. the *Xiphister* taxa) would have more similar enteric microbial communities when collected from a single location. Then, to observe how dietary shifts impact the intestinal microbiome of these four fish species, we brought them into the laboratory and fed them omnivore and carnivore diets for four weeks (Herrera et al. 2022). If diet is a strong influencer of microbial diversity, then those fish consuming the same diet in the laboratory should converge on a similar microbiome. However, if Phylosymbiosis holds true, those more closely related fish species should maintain more similar enteric microbiomes even when faced with dietary perturbations in the laboratory. In addition to investigating hindgut microbial diversity, we also observed microbiome function by measuring SCFA concentrations and the activities of N-acetyl-β-D-glucosaminidase (NAGase) and β-glucosidase in the hindguts of the wild fishes, and *A. purpurescens* individuals fed the different diets in the laboratory. NAGase digests components of chitin, which can come from crustacean

exoskeletons or fungal cell walls, whereas β-glucosidase digests components of cellulose digestive products that would come from algal cell wall digestion. Considering that fish hindgut microbes can degrade carbohydrates that the fish do not (Stevenson et al. 2022), we expect SCFA levels and β-glucosidase activity to be correlated with algal concentration in their diet (herbivore > omnivore > carnivore). Because *P. chirus* and *A. purpurescens* naturally consume more chitin in the form of crustaceans, NAGase would be elevated in the opposite direction (carnivore>omnivore>herbivore; (Rankins 2023; German et al. 2015)). How SCFA and enzymatic activities within *A. purpurescens* are altered by the laboratory diets will depend on how the communities shift in response to the dietary perturbations.

Our main goal is to test Phylosymbiosis in the face of biogeography and laboratory dietary shifts to help answer the question of what impacts gut microbial diversity more: species identity, biogeography, or proximate or historical diet? Our use of closely-related, sympatric animals with different diets, allows us to uniquely test these variables and their impacts on the gut microbiome. Our study helps inform biomedical science by advancing our understanding of the different factors that influence the gut microbiome and expands our understanding of the gut's ability to shift in microbial composition due to genetic, dietary, or environmental differences.

MATERIALS AND METHODS

Objective One: How does geography or species identity influence the gut microbiome in prickleback fishes with different diets?

Wild Fish capture in California and Washington State

Juveniles of *Xiphister mucosus* (herbivore), *Cebidichthys violaceus* (herbivore), *X. atropurpureus* (omnivore), and *A. purpurescens* (carnivore) (105 individuals total) were

collected by hand and dipnet in May, June, and July 2021 at low tide from rocky intertidal habitats across the California coast (Vandenberg 34.7°N 120.62°W; San Luis Lighthouse 35.15ºN, 120.76ºW; Piedras Blancas 35.65ºN, 121.24ºW; Santa Cruz 37.24°N 122.41°W; Bodega Bay 38.3°N 123.05°W).

All wild fish were euthanized with an overdose of tricaine methanesulfonate (MS-222 in 1 gL^{-1} seawater), tricaine methanesulfonate (MS-222 in 1 g L^{-1} seawater), measured [standard length (mm)], weighed (g), and dissected on a cutting board kept on ice (4° C) within 4 hours of collection. The digestive system of each fish was removed by cutting at the esophagus and at the anus. The gut was removed, uncoiled, and the liver, stomach, and pyloric ceca were excised. The intestine was divided into three sections of equal length and the sections were designated as the proximal, mid, or distal intestine. The contents of the stomach and each intestinal section were emptied into their own vials. The intestinal tissue was rinsed with ice cold Tris HCl pH 7.5 to ensure all contents were removed. Samples were frozen in liquid nitrogen and subsequently transferred to a -80 freezer for storage until further microbiome processing as described in "*Gut microbiome analysis for all samples"*.

Objective Two: How do dietary changes influence the fish gut microbiome?

Wild fish capture and feeding experiment

In June 2016, wild-caught *X. mucosus*, *X. atropurpureus*, *P. chirus*, and *A. purpurescens* were collected from Deadman's Bay, San Juan Island, Washington, USA (48.510° N, 123.140° W; Herrera et al., 2022). Fifteen juveniles of each species were transported live in seawater to Friday Harbor Laboratories (Friday Harbor, WA) where they were placed in wet table aquaria with flow through seawater (held at approximately 13° C, the ambient temperature of Friday Harbor water that summer) to be used in a feeding experiment. The remaining individuals of

each species (at least 11 of each species), were euthanized to represent the wild-caught fish. Fish were euthanized, dissected, and tissues harvested as described for "*Objective One: How does geography or species identity influence the gut microbiome in prickleback fishes with different diets?*".

The remaining 15 individuals of *X. mucosus, X. atropurpureus, P. chirus,* and *A. purpurescens* were individually placed in cubicles (approximately 1.5-L in volume) within wet table flow-through aquaria and used for a feeding experiment. Each individual fish was anesthetized (0.1 g L^{-1} MS-222), measured and weighed, and assigned to a carnivore, abbreviated as LC (Lab Carnivore), or omnivore diet, abbreviated as LO (Lab Omnivore), at the start of the experiment. All individuals of *X. atropurpureus* and *P. chirus* were fed the LC diet, as none would consume the LO diet in the laboratory. The fishes were acclimated to laboratory conditions and the formulated diet for two weeks. Feeding trial and diet formulations were conducted following the identical methods as described in (Herrera et al. 2022). Diets were made using thalli of the algal species *Ulva lobata* (Chlorophyta), *Mazzaella splendens* (Rhodophyta), and *Porphyra* sp. (Rhodophyta), all of which are common in the diets of *X. mucosus, X. atropurpureus, P. chirus* (Horn et al. 1986; German et al. 2004; German et al. 2014; German et al. 2015), and fillets of several flatfish species, as described in Herrera et al. (2022) (Herrera et al. 2022). Vitamin and mineral premixes as well as fish oil, casein, soybean meal, and methyl cellulose (as a binder) were part of the diets. The omnivore and carnivore diets contained 45.4% and 68.8% protein, 19.46% and 2.17% carbohydrate, 12.34% and 11.90% lipid, and 263.8 and 342 Calories (per gram), respectively (Herrera et al. 2022). Fish were fed their respective diets two-three times daily to satiation for four weeks. Feces were collected just before each feeding and the debris in each tank was siphoned out after each feeding.

At the conclusion of the feeding trials (four weeks on the prescribed diets), fish were euthanized, measured, weighed, and dissected as described above under "*Objective One: How does geography or species identity influence the gut microbiome in prickleback fishes with different diets?*".

Gut fluid preparation and gastrointestinal fermentation

Measurements of symbiotic fermentation activity were performed as described in German et al. (2015)(German et al. 2015). Fermentation activity was indicated by relative concentrations of short-chain fatty acids (SCFA) in the fluid contents of the distal intestines of the wild-caught fish of "*Objective Two How do dietary changes influence the fish gut microbiome?*" at the time of death. Distal intestine gut content samples of the wild fish were weighed [gut content mass (GCM ± 0.001 g)], thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4°C) at 16,000 x *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) and centrifuged under refrigeration at 13,000 x *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 concentrations.

Concentrations of SCFA in the gut fluid samples from the hindgut gut region were measured using gas chromatography (Leigh et al. 2021). 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.

Hindgut Digestive enzyme activity

Alkaline phosphatase, β-glucosidase, and N-acetyl-β-D-glucosaminidase (NAG) activities were measured in wild fishes and *A. purpurescens* fish fed different laboratory diets following German et al. (2011) (German 2011), using 200 μM solutions of the substrates 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-β-D-glucoside, and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively, dissolved in 25 mM Tris-HCl (pH 7.5). Briefly, 90 μL of substrate were combined with 10 μL of homogenate in a black microplate and incubated for 30 minutes at 12.5°C. 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.

Statistical analyses

Prior to all significance tests, Levene's and Bartlett's tests for equal variances were performed to ensure the appropriateness of the data for parametric analyses, and any datasets that did not meet the assumptions of ANOVA (including homoscedasticity) were transformed using a Box Cox Transformation. All tests were run using R (version 4.0.1). Comparisons of short chain fatty acid (SCFA) concentrations from the distal intestines of the fish were made among the fish fed the different diets with ANOVA. Total SCFA concentrations were plotted as a function of branched-chain SCFA (isobutyrate and isovalerate) concentrations, which tends to reveal

whether carbohydrates or amino acids are the substrates of fermentation (Clements et al. 2017; Choat and Clements 1998).

Gut microbiome analysis for all samples

The sample DNA was isolated from the distal intestine tissue and contents for all five species using the Zymobiomics DNA mini kit from Zymo Research. 16S rDNA amplicon PCR was performed targeting the V4 - V5 region (selected based on previous literature) (Walters et al. 2016; Caporaso et al. 2012)using the Earth Microbiome Project primers (515F [barcoded] and 926R; (Walters et al. 2016; Caporaso et al. 2012). Using miseq v3 chemistry (PE300 sequencing length), the libraries were sequenced at the UC Irvine Genomics Research and Technology Hub (GRTH). This resulted in 17.4 M reads passing filter (21% of that is phiX) with an overall >Q30 80.4% for samples from Washington collected in 2016. Due to low quality scores of some samples, an additional miseq run was performed to re-sequence some samples, and the ASV data from both runs were merged in QIIME2 (version 2022.8). For the California study, there were 13.4 M reads passing filter.The raw sequences were imported into QIIME2 (version 2022.8) using UCI's High Performance Community Computing Cluster (HPC3). After initial sample quality check (99% identity threshold), the paired-end sequences were quality filtered using the DADA2 pipeline in QIIME2, resulting in 1,573,237 merged paired-end reads for the Objective Two study and 3,759,423 paired-end reads for the Objective One study. Taxonomic classification for Amplicon Sequence Variants (ASVs) was assigned using the Silva 138 99% operational taxonomic unit from 515F/806R region of sequences (release 138) (Quast et al. 2013). Analyses were conducted in both QIIME2 and R (Version 1.4.1103). We used ANOVA followed by Tukeys HSD to determine whether there were significant differences in α-diversity (Shannon alpha diversity) of the microbial communities in the fish species. Bray-Curtis

dissimilarity matrices were used to generate non-metric multidimensional scaling plots for microbial communities in the tissues and intestinal contents of the various fish species. PERMANOVA with 999 permutations, as well as a pairwise PERMANOVA with Benjamini-Hochberg p-adjusted values, were used to test for differences in microbial community β-diversity among the fish species and by location. Based on Bray-Curtis dissimilarity matrices, we made a dendrogram using the vegan package and "vegdist" in R based on ASV and host species to determine if microbial similarity would reflect host genetics (Jari Oksanen and McGlinn 2022). To determine what microbial taxa were driving differences among the fish host species, we ran indicator species analysis, which shows which microbial taxa are uniquely associated to particular fish host species (De Cáceres et al. 2012). Furthermore, to observe what microbial taxa drove the spatial distributions in the non-metric multidimensional scaling plots, we added vectors to the plots to show those microbial features with a significantly high correlation (p value=0.005) to specific fish species (https://riffomonas.org/code_club/2022-04-11-biplot). To determine core microbial taxa that are shared amongst all fish host species samples, we utilized the core-features command in qiime2 to identify ASVs observed in 100% (fraction of 1.0) of all samples of fish host species. These ASVs were identical among all samples.

RESULTS

Objective One: How does geography or species identity influence the gut microbiome in fishes along the California coast?

Comparing four host species across all California coast sites

As visualized in our constructed dendrogram based on Bray-Curtis distances, we found that while all individuals of *C. violaceus* have similar gut microbiomes, the other species were scattered in terms of their microbial similarity, and were not generally grouping by diet or

genetics (Fig. 4.2). *Cebidichthys violaceus* had 10 individuals group as each other's most similar neighbor in terms of Bray-Curtis Dissimilarity of their distal intestine microbiomes, whereas the only other species with more than two individuals grouping next to each other was *X. mucosus* with five individuals (Fig. 4.2). Otherwise, the microbial communities showed little host species microbial communities among the four host species, and *C. violaceus* was significantly different from both *X. mucosus* and *X. atropurpureus*, but not *A. purpurescens*, which wasn't different from any species (Fig. 4.3, ANOVA by Species: $F_{3,52}$=3.099; P=0.0346). Shannon's alpha diversity did not significantly differ based on the geographical location from which fish were collected (ANOVA by Location: $F_{5,48}$=0.57; P=0.723). In terms of beta diversity based on Bray-Curtis distances visualized in a non-metric multidimensional scaling plot, *C. violaceus* clustered significantly apart from *X. mucosus, X. atropurpureus*, and *A. purpurescens* as well as *X. atropurpureus* and *A. purpurescens* also grouped significantly apart from each other (Fig. 4.4, PERMANOVA: Species $F_{3,52}$=4.86, R^2=0.218, P=0.001; Location $F_{5,50}$=1.688, R^2=0.144, P=0.002; Species:Location interaction P=0.278). There were no pairwise comparisons by geographical location that group significantly apart from each other, although the southern location of San Simeon and the northern location of Santa Cruz were almost significantly different (Tukeys HSD P=0.063). And Vandenberg was almost significantly different from San Simeon (Tukeys HSD, P=0.07), San Luis Lighthouse (Tukeys HSD, P=0.063), and Bodega (Tukeys HSD, P=0.063), but not Santa Cruz (Tukeys HSD, P=0.22)

Microbial taxa in the families Beijerinckiaceae (Genus Methylobacterium-Methylorubrum), Peptostreptococcaceae, Nocardiaceae, and Anaplasmataceae (Table 4.1) drove some of the vertical distribution of the NMMDS (Fig. 4.4). The indicator species for *C. violaceus*

included bacterial species from the Families Lachnospiraceae, Butyricicoccaceae, Ruminococcaceae, and Oscillospiraceae (Table 4.2).

Comparison of host species from one Southern California location

When comparing the four host species from a single geographical location of San Simeon, California, there were significant differences in the Shannon's alpha diversity between *C. violaceus* and both *Xiphister* taxa (*X. mucosus* and *X. atropurpureus*), but there were no other species comparisons that significantly different from each other (ANOVA: $F_{3,26}$=6.85; P=0.0155). For beta diversity based on Bray-Curtis distances visualized in a non-metric multidimensional scaling plot, *Cebidichthys violaceus* clustered significantly apart from *X. mucosus, X. atropurpureus*, and *A. purpurescens* (PERMANOVA: Species $F_{3,26}$=5.42, R^2=0.384, P=0.001). *X. mucosus* and *A. purpurescens* also grouped significantly apart from each other, whereas *X. atropurpureus* was not different from either species (Appendix 1).

Uniqueness of C. violaceus gut microbiome across all California coast sites

When comparing *C. violaceus* across all sites, there were significant differences in the Shannon's alpha diversity index (ANOVA: $F_{3,29}$=3.13; P=0.041) and in beta-diversity (Bray-Curtis distances) (PERMANOVA: $F_{3,29}$=1.65, R^2=0.145, P=0.037), with most of the variation in alpha diversity within fish from Vandenberg (Fig. 4.5). However, there were no significant differences in pairwise comparisons based for alpha and beta diversity, but Vandenberg and San Simeon were almost significantly different from each other in terms of alpha diversity (P=0.10) and before the Benjamini-Hochberg adjustment (P=0.084), but not after the p value is adjusted (P=0.23)

Objective Two: How does diet influence the fish gut microbiome?

There was individual variation among the intestinal tissue gut microbial communities of wild prickleback fishes collected from Washington State (Fig. 4.6, P=0.119, R^2=0.206, PERMANOVA). Although, *X. mucosus* and *A. purpurecens* didn't overlap, they were not significantly different from one another (P=0.192, pairwise PERMANOVA).

Levels of gastrointestinal fermentation varied significantly amongst the wild species, with the herbivorous *X. mucosus* having a significantly higher level of SCFAs than both omnivorous species, *X. atropurpureus* and *P. chirus*, but not higher than *A. purpurescens*, which wasn't different from any species (Table 4.3, $p<0.05$, $F_{3,10}$=7.87, ANOVA). Additionally, gastrointestinal fermentation did not vary significantly between LO diet fish of *X. mucosus* and *A. purpurescens*. SCFA levels across wild, lab omnivore, and lab carnivore *A. purpurescens* did not significantly differ from each other (Table 4.3, P=0.373, $F_{2,7}$=1.14, ANOVA).

Phytichthys chirus possessed significantly higher NAGase activity in its distal intestine than *X. mucosus* had in theirs; none of the other species were different from one another for NAGase activity (Fig. 4.7, Table 4.4 P=0.039, $F_{3,25}$=3.244, ANOVA). There was not a lot of measurable activity for β -glucosidase among the different species, and thus, there were no statistical differences in the activity of this enzyme (Fig. 4.7, Table 4.4, P=0.253, $F_{3,27}$=1.441, ANOVA). Alkaline Phosphatase activity did not differ significantly among wild fish (Fig. 4.7, Table 4.4, P =0.253, $F_{3,27}$=1.441, ANOVA). Among wild and laboratory-fed *A. purpurescens*, there are no significant differences in Alkaline Phosphatase activity, NAGase activity, or β -glucosidase (Table 4.4).

In terms of the feeding trial, the beta diversity of the distal intestine microbial communities were different among the WF and LO diet fish of *X. mucosus* (Fig. 4.8, P =0.006, R^2=0.526, PERMANOVA). The LC diet fish of *X. mucosus* couldn't be included statistically

because only one individual amplified in the sequencing efforts (Fig. 4.8). The beta diversity of LC diet *A. purpurescens* were significantly different from LO *A. purpurescens* (Fig. 4.8, P =0.009, R^2=0.248, PERMANOVA). When comparing all LO fish, the beta diversity of *X. mucosus* and *A. purpurescens* are not significantly different from each other (P =0.062, PERMANOVA). When comparing all LC fish, there are no significant differences in beta diversity between any species (P =0.12, PERMANOVA).

DISCUSSION

The interactions between animal hosts and their gut microbes impact the overall physiology and health of the host (Nicholson et al. 2012; McFall-Ngai et al. 2013), yet the nuances of how different factors, such as host evolutionary history, geographical location, and diet, can influence the gut microbiome still need to be resolved. In our study, we aimed to understand the determinants of gut microbial community structure in the context of host ecology and evolution. Based on predictions of phylosymbiosis, we expected that there would be a strong relationship between host genetics and microbial communities (Stevens and Hume 1995; Stevens and Hume 1998; Kohl et al. 2018b; Brooks et al. 2016; Ley et al. 2008a). We found that the gut microbiome changed based on individual variation and species identity within closely-related, sympatric fishes, rather than just diet or geographical location being the main determining factors. Individual variation and species identification are known to shape the gut microbiome and our results support the fact that there are species-specific microbial community patterns (Benson et al. 2010; Kohl et al. 2018b).

Objective One: How does geography or species identity influence the gut microbiome in fishes along the California coast?

In prickleback fishes, we found that species identity, and likely digestive strategy, influence the gut microbiome, regardless of geographical location (Fig. 4.2). Consistent with Phylosymbiosis, the prickleback gut microbial communities were influenced by species identity, with *C. violaceus* and *X. mucosus* showing blocks of similarity in microbial community among conspecifics (Phillips et al. 2012; Brooks et al. 2016; Rojas et al. 2021). The microbial composition of host fish species in terms of both indicator species analysis and taxa that are significantly driving the variation in beta-diversity conveys a strong signal of species identity, at least for *C. violaceus* and *X. mucosus*. However, *X. atropurpureus* and *A. purpurescens* showed broad diversity in their enteric microbial community that seemed to correlate little with geography or host species identification (Figs. 4.2, 4.3).

In particular, *C. violaceus* stands out from the rest of the host fish species, and has different microbial taxa that may play a role as fermenters in their hindgut, which is consistent with a digestive physiology that is at least somewhat reliant on enteric microbes (Herrera dissertation Chapter 3)(German et al. 2015; Sibly 1981). For instance, *C. violaceus* contained many indicator species from the bacterial families Lachnospiraceae, Butyricicoccaceae, and Ruminococcaceae that are known to engage in fermentative digestion (Table 4.2) (Clements and Choat 1997; Mountfort et al. 2002; Nicholson et al. 2012; Pardesi et al. 2022; Stevenson et al. 2022). The uniqueness of the gut microbiome of *C. violaceus* and its ability to shift in the face of dietary perturbations is explored in more detail in dissertation Chapter 3. On the other hand, the other herbivorous species *X. mucosus* seems to be utilizing a different digestive strategy that is characterized by high-intake, rapid movement of material through the gut, low levels of microbial fermentation, low abundance of fermenting microbes, and perhaps more protein/amino acid nutrient scavenging by microbes such as those in the Peptostreptococcaceae (Costello et al.

2010; Fan et al. 2017; Arnaud et al. 2020; German et al. 2015; Sibly 1981). Abundant members of the Peptostreptococcaceae and the depletion of Akkermansia, as found in *X. mucosus*, are consistent with obesity and gut dysbiosis in mammals (Amabebe et al. 2020), and in animals scavenging for nutrients while consuming lower-quality diets (Lemieux-Labonté et al. 2022; Wehrle et al. 2020). Perhaps an altered metabolism in *X. mucosus* is advantageous in the different digestive strategy this species uses to consume a low-protein, herbivorous diet. Moreover, the differences in microbial communities between *X. mucosus* and *C. violaceus*, despite these species eating nearly identical foods, highlights how different digestive strategies can favor completely different microbial communities. Thus, consideration of digestive physiology should be taken into account when examining a fish's microbiome (German et al. 2015).

Overall, it is fascinating that host genetic factors seem to dominate over differences in geographical location, and matches with previous studies in mammals, such as primates, that found the gut microbiota is shaped by genetics and species identity rather than geography or dietary niche (Weinstein et al. 2021; Amato et al. 2016; Ley et al. 2008a).

Objective Two: How does dietary changes influence the fish gut microbiome?

Diet plays a crucial role in the structure of gut microbial communities and the diversity of gut microbiota can shift with changes in host diet (David et al. 2014; Wastyk et al. 2021; Flint et al. 2017; Rothschild et al. 2018; Oliver et al. 2021; Baxter et al. 2019; Venkataraman et al. 2016; Sawicki et al. 2017; Maier et al. 2017; McDonald et al. 2018; Muegge et al. 2011; Colman et al. 2012). In our study, we determined if there is a dietary effect on the gut microbiome by comparing wild-caught species with different diets, and then examining the microbiomes of those same species fed either omnivore or carnivore diets over a four-week feeding experiment

(German et al. 2004; Herrera et al. 2022). Interestingly, we found such variation in wild-caught fish, that no significant differences were detected in their microbial diversity (Fig. 4.6). However, wild *X. mucosus* and individuals of this species fed an omnivore diet in the lab group apart from each other, showing a dietary effect on the microbiome within a species. Similarly, *A. purpurescens* fish fed a carnivore diet are significantly different from *A. purpurescens* fed an omnivore diet in the laboratory (Fig. 4.8). However, when comparing for instance all individuals fed omnivore or carnivore diets in the laboratory, respectfully, to each other, there is again, like the wild-caught fishes, enough variation that there are no statistically significant differences in microbial beta diversity. Captive mammals tend to converge on a similar microbiome that is different from what they show in nature (Ley et al. 2008a; Brooks et al. 2016; Kohl et al. 2018a; Franzenburg et al. 2013), but it is possible that the microbiomes of these wild sympatric prickleback fishes are variable enough that the variation itself overwhelms the dietary signal on the gut microbiome when making inter-specific comparisons.

Among our studied fish species, *X. mucosus* and *A. purpurescens* are the most distantly related and have the most divergent natural diets (Fig. 4.1). Although not statistically different in our comparisons (Fig. 4.6), perhaps reflecting our relatively low sample sizes for the microbiome analyses, the microbiomes of these two species do not overlap in large ways. A lack of statistical difference in their microbiomes was maintained when consuming the same diets in the laboratory, despite differences in growth rate, and gut size, in the laboratory-fed fish of the two species (Thépot et al. 2022; Herrera et al. 2022). Levels of gastrointestinal fermentation did vary amongst the wild-caught individuals of these species, with *X. mucosus* (herbivore) having a significantly higher level of SCFAs than both omnivorous species, *X. atropurpureus* and *P. chirus*, but not *A. purpurescens*. It needs to be acknowledged, however, that the concentrations

of SCFA in the pricklebacks from Washington are low, particularly for herbivorous fishes that are more reliant on microbial fermentation, and suggests that none of these fish species are reliant on gastrointestinal fermentation to meet their energetic needs from their diets (Clements et al. 2017; Clements and Choat 1995). The activity level of N-acetyl-β-D-glucosaminidase (NAGase), a brush border enzyme that breaks down components of crustaceans, is statistically significantly different, and more than double in the distal intestines of *P. chirus* in comparison to *X. mucosus*. These findings have been consistent over time, and likely reflect the crustacean-rich diet of *P. chirus* (German et al. 2015; Rankins).

CONCLUSION

This study provided insight into how host genetics, environment, and diet influence the gut microbiome of closely-related prickleback fishes that live in the same rocky intertidal habitat, yet vary in diet. We found that digestive physiology, individual variation, and species identification affect microbial diversity patterns of the hindgut. This suggests that generalizations about the gut microbiome cannot be made based on diet alone, since the digestive strategy of the animal, and whether they have digestive physiology conducive to microbial activity, must be taken into account (e.g., (Clements et al. 2017)). *Cebidichthys violaceus* is known to have the hallmarks of microbial digestion: a hindgut that is expandable, particularly when eating an algal-rich diet (see Dissertation Chapter 3), elevated levels of SCFA (at least relative to other prickleback fishes; (German et al. 2015)), and microbial taxa that are from lineages that are known to contain microbes that engage in fermentative digestion. The herbivorous *X. mucosus* shows none of these things, and therefore, despite dietary similarity with *C. violaceus*, these two fish species should not be expected to have similar microbiomes, and alas, they do not. Instead, we see that host species identity and individual variation play large roles in affecting the enteric

microbial diversity of closely-related, sympatric fish species with different natural diets. Even when they are brought into the laboratory and fed different foods, there is still considerable overlap in the microbiomes of fishes other than *C. violaceus*. Including more distantly related species could lend more support for Phylosymbiosis more broadly, suggesting that one could see phylogenetic signals in an animal's enteric microbiome dependent on the scale across which comparisons are being made. Therefore, there may be limits to Phylosymbiossis depending on what species are being compared, and whether they are sympatric. Our study helps inform biomedical science by advancing our understanding of factors that influence the gut microbiome and the gut's ability to shift in microbial composition, and certainly provides insight into how fish gut microbiomes can change in laboratory or aquaculture settings.

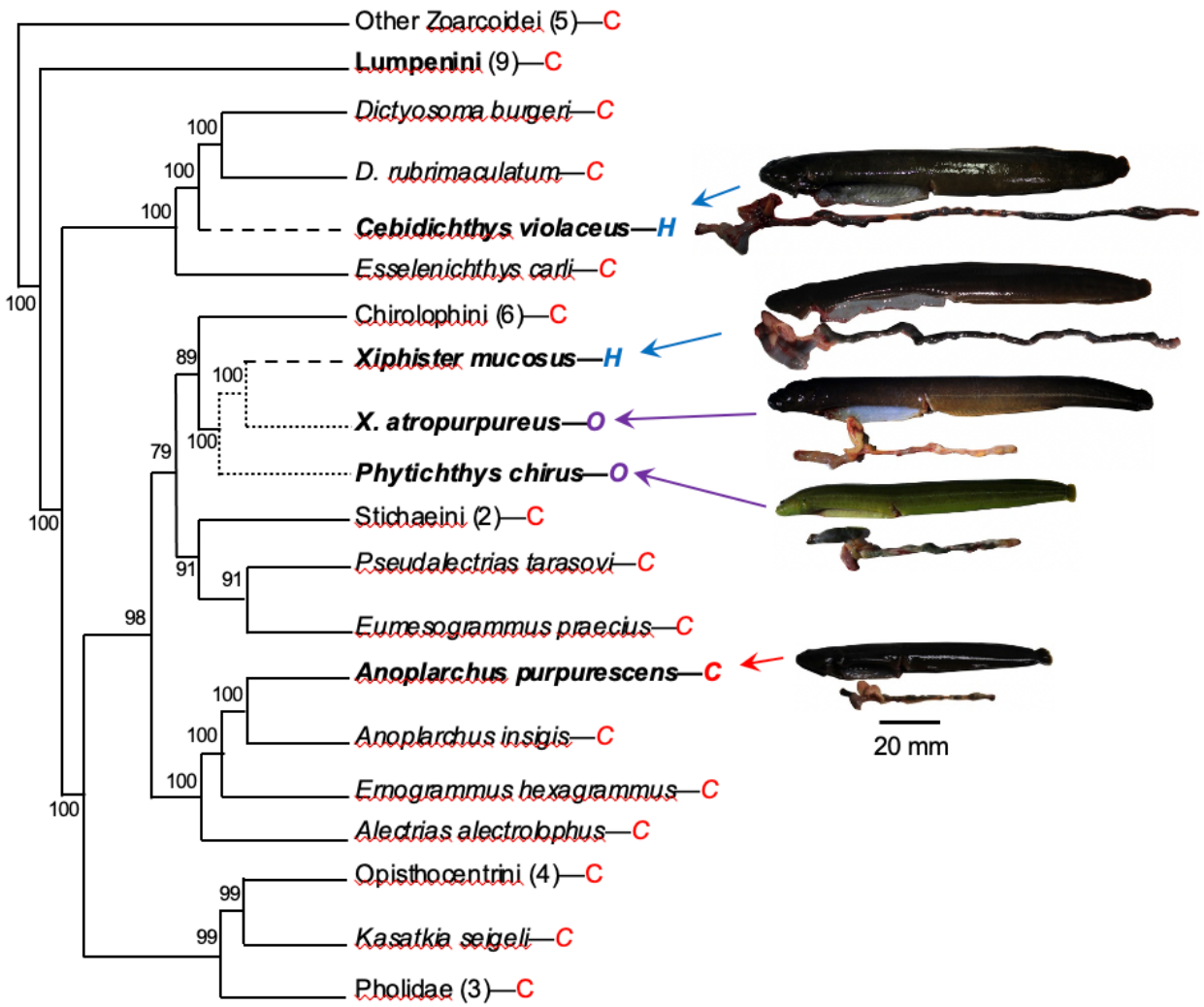

Figure 4.1 Phylogenetic relationships of the polyphyletic family Stichaeidae based on 2,100 bp of *cytb*, *16s*, and *tomo4c4* genes (Kim et al. 2014). Bayesian posterior probabilities are indicated on nodes. Studied taxa are bolded, and photos are shown with their digestive systems beneath their bodies. Note the differences in gut size. **H**=herbivory, **O**=omnivory, **C**=carnivory. Evolution of herbivory (— — — —) and omnivory (.....) are shown. Numbers in parentheses show number of taxa evaluated at that branch.

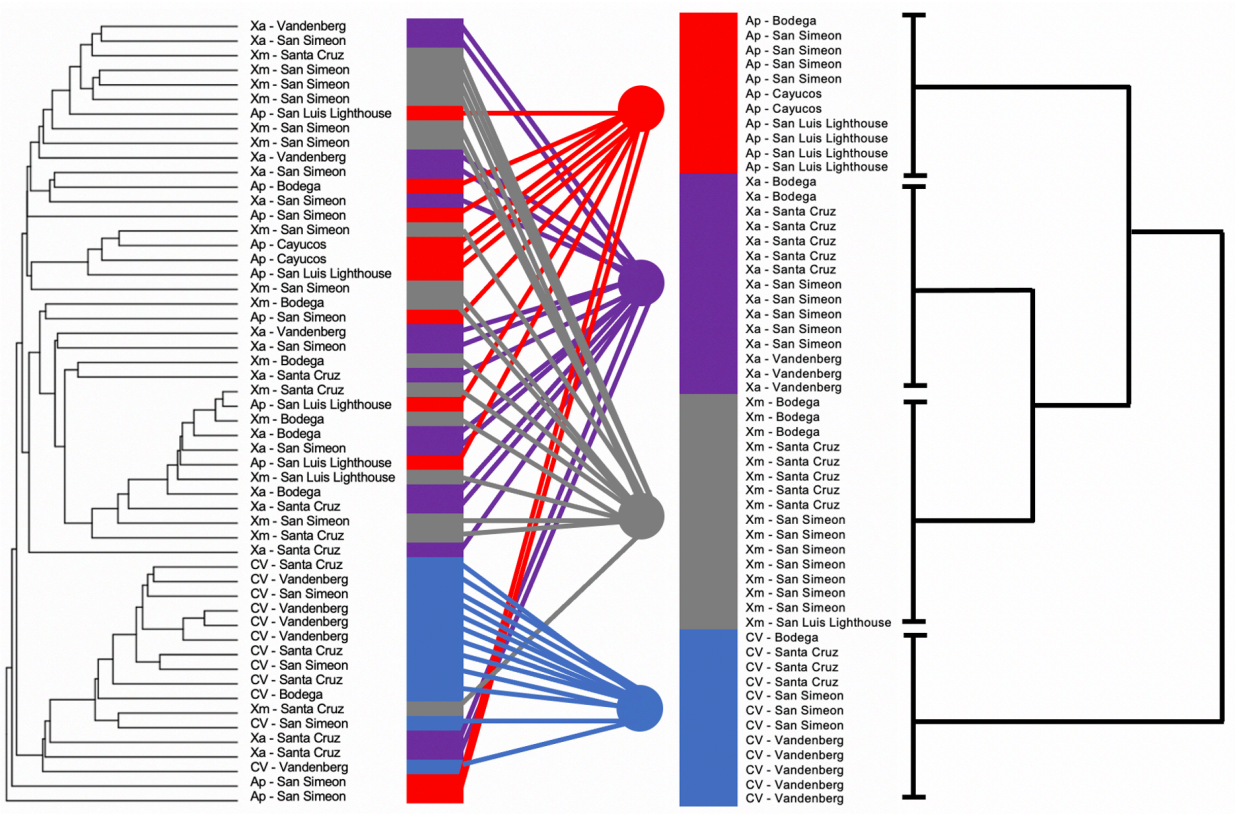

Figure 4.2 Dendrogram based on bray curtis distances among wild fishes from Objective One.

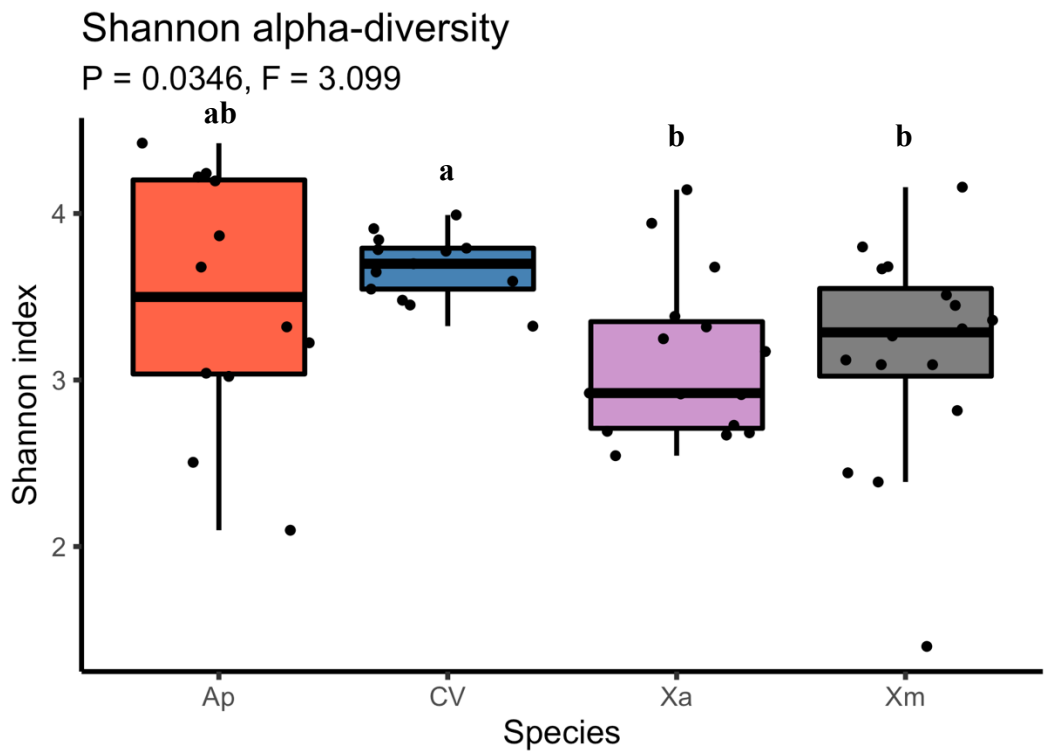

Figure 4.3 Shannon's alpha diversity index among the four host species for Objective One: Ap: *A. purpurescens*; Cv: *Cebidichthys violaceus*; Xa: *X. atropurpureus*; and Xm: *Xiphister mucosus* (ANOVA: $F_{3,52}$=3.099; P=0.0346).

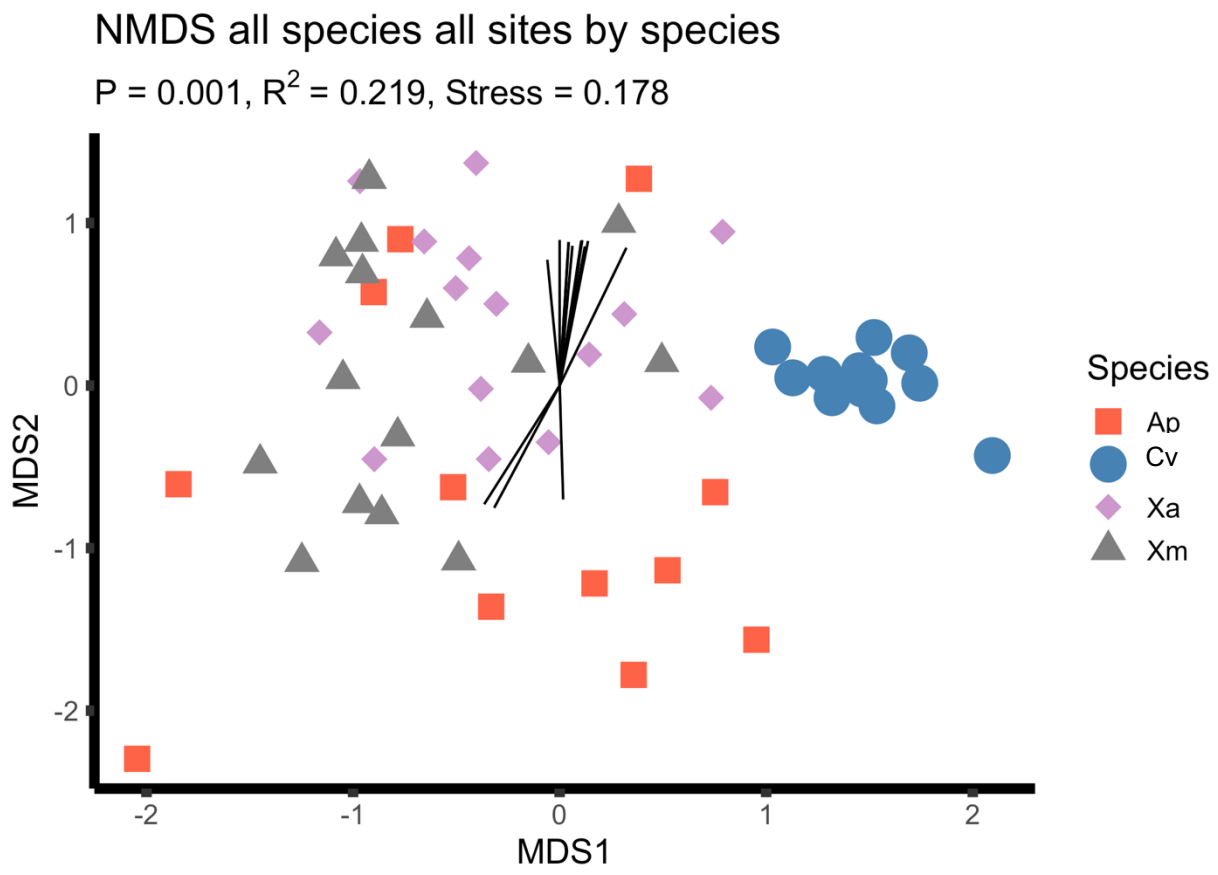

Figure 4.4 Beta Diversity of all host species across all sites (PERMANOVA: Species $F_{3,52}$=4.86, R^2=0.218, P=0.001; Location $F_{5,50}$=1.688, R^2=0.144, P=0.002; Species:Location interaction P=0.278). Ap: *A. purpurescens*; Cv: *Cebidichthys violaceus*; Xa: *X. atropurpureus*; and Xm: *Xiphister mucosus.* Vectors indicate taxa that are significantly driving the variation in the NMDS plot.

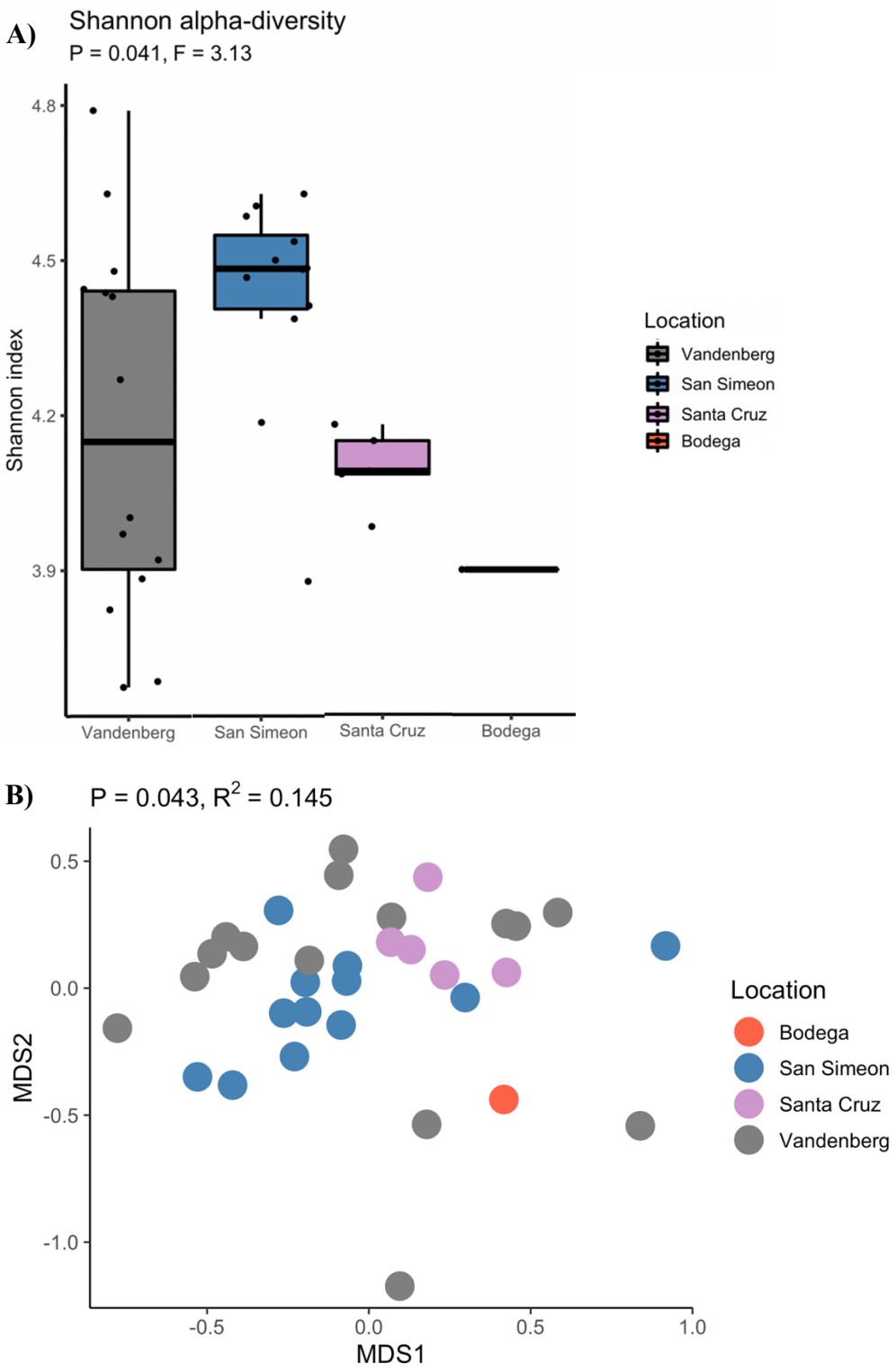

Figure 4.5 Uniqueness of *C. violaceus* gut microbiome across all California coast sites A) Alpha Diversity based on Shannon Index. B) Beta diversity based on bray Curtis distances.

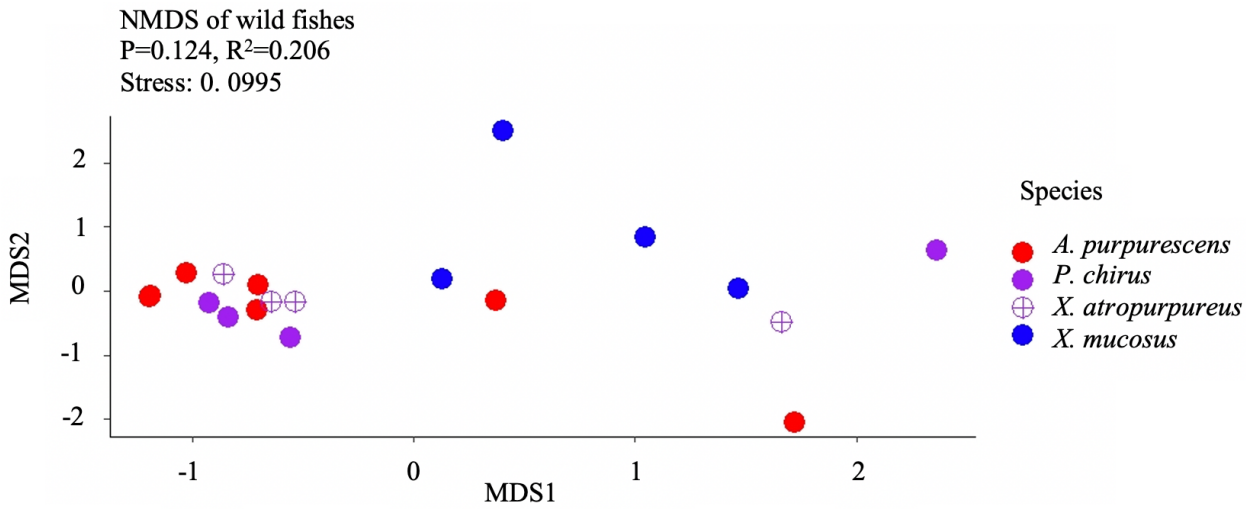

Figure 4.6 Microbial beta diversity shows no host affinity (*P* =0.119, R^2=0.206, PERMANOVA (Adonis function)) in wild prickleback fishes' hindgut tissue, where only 21% of the variation is explained by species identity. Blue spheres are *X. mucosus*, purple rings are *X. atropurpureus*, purple spheres are *P. chirus*, and red spheres are *A. purpurescens*. Tests are based on Bray-Curtis dissimilarity distances and 999 permutations.

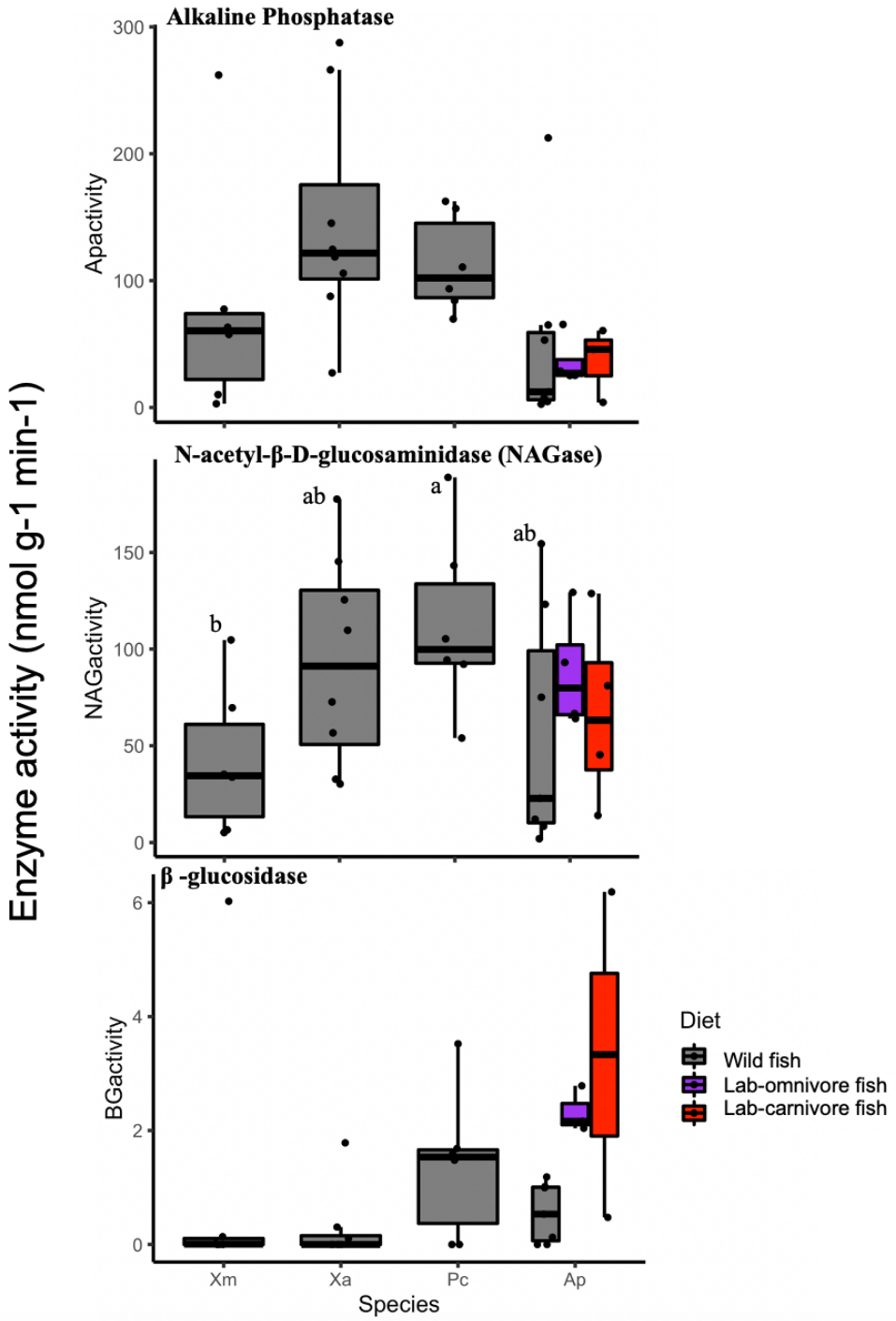

Figure 4.7 Enzyme activity of wild prickleback fishes: top to bottom: Alkaline Phosphatase, N-acetyl-β-D-glucosaminidase (NAG), and β -glucosidase (BG). Ap: *A. purpurescens*; Cv: *Cebidichthys violaceus*; Xa: *X. atropurpureus*; and Xm: *Xiphister mucosus*. Note that we do not have enzymatic activity data for the lab-fed fishes for the species other than *A. purpurescens*

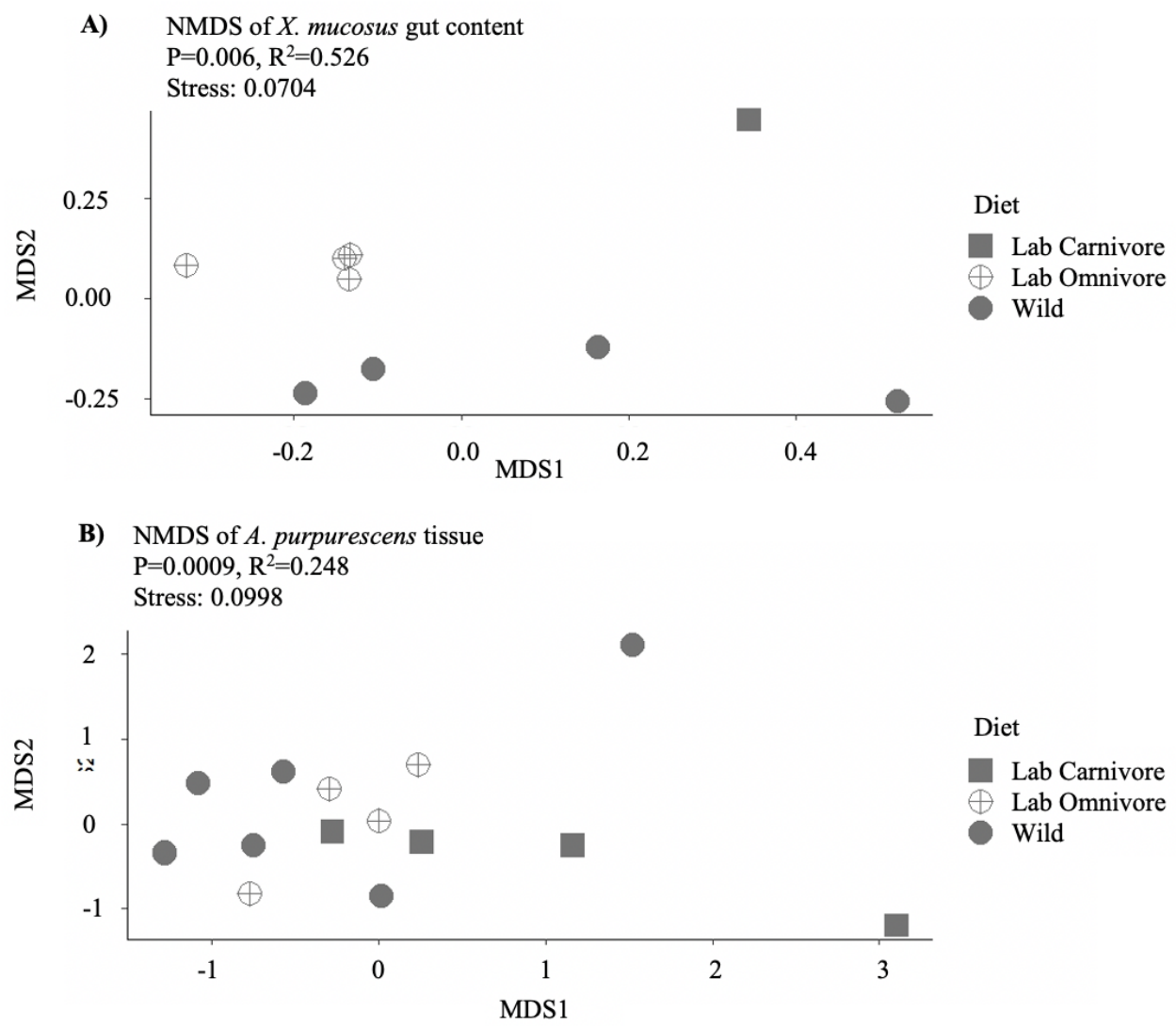

Figure 4.8 Beta Diversity FHL Feeding Trial A) Microbial beta diversity shows gut microbiomes significantly group by diet (*P* =0.006, R^2=0.526, PERMANOVA (Adonis function)) in *X. mucosus* prickleback fish hindgut content, where 53% of the variation is explained by diet. Spheres are wild *X. mucosus* fish, rings are *X. mucosus* fish fed an omnivore diet in the laboratory, squares are *X. mucosus* fish fed a carnivore diet in the laboratory. Wild fish and fish fed an omnivore diet in the laboratory are significantly different from each other, showing a dietary effect on the microbiome (P=0.034 or padjust=0.102, pairwise PERMANOVA (Adonis function)). Tests are based on Bray-Curtis dissimilarity distances and 999 permutations. B) Microbial beta diversity shows gut microbiomes group significantly by diet (*P* =0.009, R^2=0.248, PERMANOVA (Adonis function)) in *A. purpurescens* prickleback fish hindgut intestine wall, where 25% of the variation is explained by diet. Spheres are wild *A. purpurescens* fish, rings are *A. purpurescens* fish fed an omnivore diet in the laboratory, squares are *A. purpurescens* fish fed a carnivore diet in the laboratory. Wild *A. purpurescens* fish are significantly different from fish fed an omnivore diet in the laboratory and fish fed a carnivore diet in the laboratory, showing a dietary effect on the microbiome in the same environment (wild

vs carnivore p=0.016 or padjust=0.048; wild vs omnivore p=0.041 or padjust=0.0615, pairwise PERMANOVA (Adonis function)). Tests are based on Bray-Curtis dissimilarity distances and 999 permutations.

Table 4.1 Vectors driving differences among all wild prickleback fishes from Objective One in Figure 4

Direction	**ASV ID**	**Taxa**
up	1dfc5ac106262f11982f0845903cc27f	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	2c0508a9b5c72b0e32418cd2e87981fa	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	2e0cf846aa51f0c31de0d631a9f4218e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	3816cea2cc3f5d4fab29e1f236687d59	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	6e3c0e6a90cf6eb7dbf90c145386cc13	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	70ab9452844f6d800a8eb8b338eac32e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	8497274cdae4e5aa8d9e5d3a6dad0697	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	a3656121366f967546f9495d0ab0b03e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	ac0c2a4f7a81a6bed041183298979133	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
up	e0825a99b4a65dc764d65db42acd931e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum

up	fcddb0a8e579802bddfbc3aa44195306	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
down	b5f7ad7affbbc3f42fd647cf46d7fccb	d__Bacteria; p__Bacillota; c__Clostridia; o__Peptostreptococcales-Tissierellales; f__Peptostreptococcaceae; g__Tepidibacter; s__uncultured_Gram-positive
down	7544ebc12439b731bf777b2fe1158bea	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
down	e5a3766dbb8953c3ce6cff9ba2d084ea	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rickettsiales; f__Anaplasmataceae; g__Neorickettsia; s__uncultured_bacterium

Table 4.2 Indicator Species by Host Species for Objective One		
Species	Indicator ASV	Taxa
AP	029fe3a55892c067750186338c791a37	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Marivita
AP	0448c2191df1ef9db6ecc60c67852117	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Halocynthiibacter
AP	11e18912e898cc20f288f257b1c930d0	d__Bacteria; p__Bacillota; c__Clostridia; o__Clostridiales; f__Clostridiaceae; g__Clostridium_sensu_stricto_1
AP	244dbcf26ac1ffd30df1f5975505cd09	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__Pseudahrensia
AP	368842bf59a1b74f294b70d0421a438c	d__Bacteria; p__Cyanobacteria; c__Cyanobacteriia; o__Synechococcales; f__Cyanobiaceae; g__Synechococcus_CC9902
AP	415eea08a42a30f9dd2b6e98681ed36b	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Thalassobaculales; f__Thalassobaculaceae; g__Thalassobaculum
AP	47bdf190cb4c41839add1fa0122c61fc	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Caulobacterales; f__Caulobacteraceae; g__Brevundimonas
AP	4b6e8f2a05d35401e1945a4e1d042699	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Halocynthiibacter
AP	55973abe5edb232b265d86b514e01cbe	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Azospirillales; f__Azospirillaceae; g__Nitrospirillum; s__Rhodospirillaceae_bacterium
AP	5cccf187085ee2e9881a3c78d9ba43f5	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Pseudoruegeria

AP	9ba48c9a46a76bad6669e19ce8df3818	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Tateyamaria
AP	b3743a3a74be710357279d9896e0960e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
AP	b532ec4a3aef0cf22d608dc96c608529	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria
AP	bd596c2884ac2fee14364bb59d29bb25	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Methyloligellaceae; g__Methyloceanibacter
AP	dcbea15e4fd8979fa8cc9b92c41410f5	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Corynebacteriaceae; g__Corynebacterium; s__Corynebacterium_lipophiloflavum
AP	e47657e12cd0560645ed2820045583a2	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria
AP	f4f9e1f9e224892f73992c74935b76c0	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
Ap+ CV	3f925bf63ff4ac00a3361834d81d0e21	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
Ap+ Xa+ Xm	2cb9e007f55fa633604e48577e5856b6	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae
Ap+ Xa+ Xm	8bf5a436f3fd1b59424e349679e58c84	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae
Ap+ Xm	0607ef057441744a2715c28f6b142401	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
Ap+ Xm	2e0406e695f2678ab67c3375cb22396b	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.

Ap+ Xm	7544ebc12439b731bf777b2fe1158bea	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
Ap+ Xm	c01174aac8ab604bd95f6e4bf3a6f00d	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
Ap+ Xm	d3e8a3e7e12ecae9569a7ce98a12a729	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
Ap+ Xm	ded2247e13c0bb6cac60dae7058d6710	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Corynebacteriales; f__Nocardiaceae; g__Nocardia; s__Streptomyces_sp.
CV	0324ccb9528df88b2be2a29ac329af42	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	0ad9c37882f33ede0b6ce06d42764471	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	0c8424da0440f9d346e36d6a09fac5c2	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	14b6fa739403d42aa264d5a0599db2ad	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	159dde80822d99635cc1f73e8432bcd6	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	1d000c8f6da99ca20f7df0c284d47d59	d__Bacteria; p__Verrucomicrobiota; c__Verrucomicrobiae; o__Verrucomicrobiales
CV	1e18c3ffb43dd212244ce6a1726cff2f	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	1ec7780bd976287f6049b80f5e43c281	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae

CV	20d1ad4a10a1750ee67c9b79cd8f0afa	d__Bacteria; p__Verrucomicrobiota; c__Verrucomicrobiae; o__Verrucomicrobiales
CV	23d0de9f29bc35e961fed6b9fa55cab9	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	2553b20bd54aba093752d94a1c2b9489	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	260c37c145250b2047a8af4b02ff9fe9	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	26b684bd49001d351551e759fbbfd175	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	292df15e48c7aecf64854f157b4e85dd	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
CV	2c4f50192c5b47e97a9af8db29c37d10	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	2c653d8b715b1c54ee3176363b93d0d1	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum
CV	30af4e4321a1c9cc093cdad086ba38f7	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	3f7422478d81ff497185865e7752534e	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium
CV	44fb8f7e392db3de3c00005a0502f467	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae
CV	5040353f3d078f0dc80f892a664f112c	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae

CV	59292d104035c03865b5253bb1adf2c0	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	608a61b7707cd03f1487dc4db4564319	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Oscillospiraceae
CV	619564fbc904ea00c9bbe988ae5b509e	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum; s__Gadus_morhua
CV	665fc3d5f7488c96b5c49b0bf662917d	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	700f88ea6584bb7a8574b99ed465c8ec	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	79d6411092795e5a66206e959f0ee99d	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium
CV	79e8276069f1702d40d6c9ccabadc10d	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	7df173dedceb287f68750647c4dda1cb	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	83d961ed5675a57834654c808cf8bfd0	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Pygmaiobacter
CV	87544ebc3ec6b1770b0b752a23fc29db	d__Bacteria; p__Verrucomicrobiota; c__Verrucomicrobiae; o__Verrucomicrobiales
CV	8b3ebc481ac2ea7e37fe1dc8f3fa8002	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	9852180a093fdb9150d595f4a029527e	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium

CV	98d6b48de4d45f9f5bfe09652706372c	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
CV	9ba81eb4417081ef337aa3fd396298b7	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	9f4585397b400d34ad0c4161acb84a06	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
CV	a2ac603ac9046139afcd0e63183ec13b	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
CV	aca6d60d651936e532ed644ddbdbd088	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	adaf2c0732976fc458b916fd5cfbe5fa	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	ade0dd5cbdbf9f60917e30c8669bdda0	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	bb80629a64dc4f8a9f85c4873a4e1c8a	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	bbabbcec846b7aefdccc7b7a44ebc109	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium
CV	bee30ddaa8f78e7e793a229892cf80b9	d__Bacteria; p__Verrucomicrobiota; c__Verrucomicrobiae; o__Verrucomicrobiales; f__Rubritaleaceae; g__Persicirhabdus; s__Persicirhabdus_sediminis
CV	beff3a04df9e68bb5ecd8567c953608a	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	c4febb4fea7fbc166a4078b8da06dd32	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus

CV	c5b5ebfe4b8cb7d8aa2c806e3a45eb0a	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum
CV	d0ae1b906d006f7bea30e964c676aebf	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	e0e1917c17cf19bdec22ec15009b613b	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	ece1ce6748f9e8ebba142064ab1a35c8	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Anaerofilum
CV	f2537c3aa97f391acd7e87f07a9122af	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus
CV	f55a631d4316bc637dc3933f2eda9c03	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Faecalibacterium
CV	f81c4dbf1bc50bd6ede2260cfd72e075	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
CV	f8492c3883f685775c192ae7d071b11a	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum
CV +Xa	e9706a6fe3e9a0db10c2d5ec01247f3d	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
XA	43ff89bb05c83dda32be115a0e696c94	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae
XA	c5ae562729c01d8bbc548feb0fc6cfe0	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae
XA	f085234a08bc5515bcd7aa7d5ed3e7cc	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium; s__[Pseudomonas]_geniculata

Xa+ Xm	2c0508a9b5c72b0e32418cd2e87981fa	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
Xa+ Xm	3816cea2cc3f5d4fab29e1f236687d59	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum
Xa+ Xm	70ab9452844f6d800a8eb8b338eac32e	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Beijerinckiaceae; g__Methylobacterium-Methylorubrum

Table 4.3 Total short-chain fatty acid concentrations (mM) in the hindgut of *X. mucosus*, *X. atropurpureus*, *P. chirus*, and *A. purpurescens* from Washington State

Gut region	Wild fish	Lab - Omnivore	Lab-Carnivore	
X. mucosus	3.64 ± 0.79^{b}	2.16 ± 0.41^{a}	1.74	
X. atropurpureus	0.80 ± 1.04^{a}	--	1.48	
P. chirus	0.88 ± 0.93^{a}	--	0.74	
A. purpurescens	1.94 ± 0.35^{ab}	1.52 ± 0.98^{a}	1.25 ± 0.36	*A. purpurescens across diets*
	$F_{3,10}=7.87$	$F_{1,3}=0.491$		$F_{2,7}=1.14$
	P=0.00547	P=0.534		P=0.373

Values are mean (±SD). Comparisons among wild species were made with ANOVA, with differences considered significant at P=0.05. This was followed by a Tukey's HSD multiple comparison test with a family error rate of P=0.05. Values sharing a superscript letter across species are not significantly different. Sample sizes were as follows: *X.* mucosus (wild fish: n=4); *X. atropurpureus*, (wild fish: n=3); *P. chirus*, (wild fish: n=4); *A. purpurescens* (wild fish: n=3). Acetate:propionate:butyrate ratios for total SCFAs were as follows for wild fish: *X. mucosus*=36:0:36; *X. atropurpureus* =70:13:12; *P. chirus* = 54:7:33; *A. purpurescens* = 37:15:20. Note that because the hindgut of the lab-carnivore fish did not have much fluid to extract, we only have an n=1 for *X.* mucosus, *X. atropurpureus,* and *P. chirus*

Table 4.4 Digestive enzyme activities in hindgut of wild *X. mucosus*, *X. atropurpureus*, *P. chirus*, and *A. purpurescens* and laboratory fed *A. purpurescens*

Species	**Alkaline Phosphatase**	**NAGase**	**β-glucosidase**
Wild *X. mucosus*	81.28 ± 86.58	45.96 ± 36.38[a]	2.21 ± 2.82
Wild *X. atropurpureus*	145.41 ± 88.46	93.79 ± 36.38[ab]	0.73 ± 0.92
Wild *P. chirus*	107.75 ± 37.84	121.40 ± 48.25[b]	4.11 ± 4.63
Wild *A. purpurescens*	51.14 ± 75.45	56.82 ± 61.64[ab]	0.77 ± 0.43
Wild comparison stats	$F_{3,27}$=1.441, P=0.253	$F_{3,25}$=3.244, P=0.0388	$F_{3,27}$=1.441, P=0.253
***A. purpurescens* lab omnivore**	36.18 ± 19.60	88.29 ± 30.33	2.33 ± 0.39
***A. purpurescens* lab carnivore**	36.84 ± 29.31	67.27 ± 49.30	3.33 ± 4.04
***A. purpurescens* wild-lab omnivore-lab carnivore comparison stats**	$F_{2,11}$=0.112, P=0.895	$F_{2,12}$=0.461, P=0.642	$F_{2,7}$=2.19, P=0.182

Values are mean (±SD). Comparisons among wild species were made with ANOVA, with differences considered significant at P=0.05. This was followed by a Tukey's HSD multiple comparison test with a family error rate of P=0.05. For β-glucosidase comparing *A. purpurescens* diet comparisons, a boxcox transformation (value=0.3) with an ANOVA and a Kruskal Wallis test was conducted, all coming up with nonsignificant differences. Values sharing a superscript letter across species are not significantly different. Note that there is an n=1 for *X. mucosus*, *X. atropurpureus*, and *P. chirus* consuming a carnivore diet because there was not much fluid to extract for lab-carnivore diet fish.

DISCUSSION

The ultimate goal of this dissertation was to advance our knowledge of dietary specialization in prickleback fishes by integrating organ-level to whole animal level physiology. My second chapter "Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes" fills a critical gap in our understanding of dietary specialization among vertebrates and is novel in integrating molecular and whole organism techniques to reveal how dietary impacts affect the physiology of fishes, providing useful information for fishing, aquaculture, and management agencies to make informed decisions about fish nutrition. The finds from Chapter 2 revealed that prickleback fishes can respond to dietary perturbations in different ways. Consistent with previous studies that found tissue-specific responses when comparing the liver with the intestine, there were few changes in the gene expression of the liver of prickleback fishes fed different diets, and the most responsive pathway is lipid metabolism (De Santis et al. 2015a). Merkin et al. (2012) showed the liver is specialized based on species identity, when comparing liver gene expression profiles with other tissues in vertebrate animals (Merkin et al. 2012). Moreover, different populations of Atlantic salmon fed high- or low-starch diets revealed population-level, not dietary, effects on liver metabolic pathway regulation (Betancor et al. 2018). Similar to my study, the Atlantic salmon liver showed few DEGs in response to dietary variation, unlike their pyloric ceca, stomach, or distal intestine, which showed increased expression of genes involved in lipid metabolism (Jin et al. 2018). Overall, we found that the liver exhibits a more tissue-specific response when comparing different diets and tissues, and it is more tuned to natural diet (Herrera et al. 2022). In summary Chapter 2 provides an important dataset to understand dietary specialization in vertebrates. We showed notable similarities and differences among closely related prickleback fishes in their physiological and

transcriptional responses. Our results highlight the flexibility of the fish digestive and metabolic systems, and how prickleback fishes adjust their physiology to changing dietary conditions in their environment.

Chapter 3 “Diet shifts affect gut and liver function and the distal intestine microbiome of an herbivorous fish” highlighted the interactions between overall fish physiology, gene expression, and the hindgut microbiome, and provided insight into how fish acclimate to dietary perturbations. As discussed in Chapter 2 of this dissertation, the liver transcriptome of other fish species seems to be less susceptible to dietary changes, but the most responsive pathway is lipid metabolism, as we found in *C. violaceus* (De Santis et al. 2015a) (Bernal et al. 2019; Merkin et al. 2012; Betancor et al. 2018). It appears that species-specific patterns of liver gene expression are the norm, and in this study, we found that *C. violaceus* had the ability to modify metabolic pathways in the liver in response to dietary changes (Herrera et al. 2022). These findings suggest far more flexibility in nutrient metabolism than strict dietary specialization in the herbivorous *C. violaceus*. In face of changing environments, it is important to have the ability to shift metabolic pathways in response to different food components and this also challenges how animals are specialized to consume certain diets. The findings from this chapter contributes to a deeper understanding of how marine herbivorous fish acclimate to dietary changes and pave the way for future investigations into the broader implications of these interactions in the context of ecological and evolutionary dynamics.

In chapter 4 “Digestive physiology and individual variation impact the hindgut microbiome of prickleback fishes (Stichaeidae) with different diets” of my dissertation, I focused on the factors that affect the gut microbiome, provided an insight into how diet or host genetics influences the gut microbiome, which is useful information for determining the factors that

influence the vertebrate gut microbiome. I aimed to unravel the determinants of gut microbial community structure in the context of prickleback fish host ecology and evolution. Based on predictions of phylosymbiosis, I expected that there would be a strong relationship between host genetics and microbial communities (Stevens and Hume 1995; Stevens and Hume 1998; Kohl et al. 2018b; Brooks et al. 2016; Ley et al. 2008a). Rather than diet or geographical location being the main determining factors, we found that the gut microbiome changed based on individual variation and species identity within closely-related, sympatric fishes. Individual variation and species identification are known to shape the gut microbiome and our results support the fact that there are species-specific microbial community patterns (Benson et al. 2010; Kohl et al. 2018b).

My dissertation provides an integrative study that utilizes molecular to whole organism techniques to reveal how diet and other factors influences the physiology of fishes. The data from my dissertation advances our understanding of fish physiology, and the factors the influence the vertebrate gut transcriptome and microbiome. This is useful information not only for fishing, aquaculture, and management agencies to make informed decisions about fish nutrition, but also for biomedical research to understand the different factors that can influence the host and its gut microbiome.

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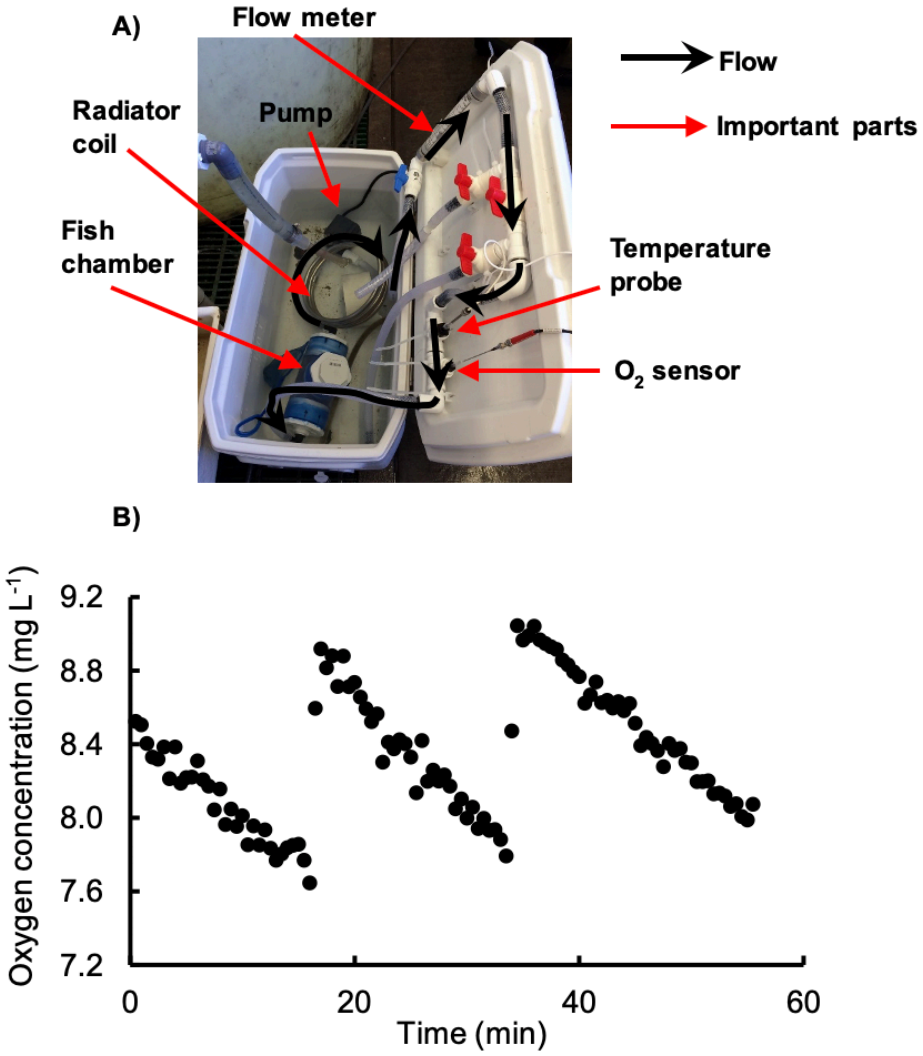
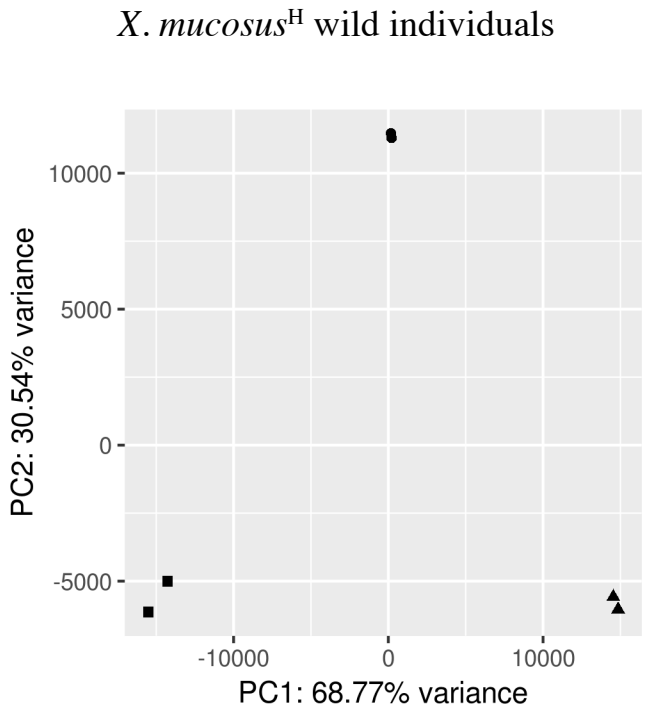
Supplementary Information for Chapter 2: Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes



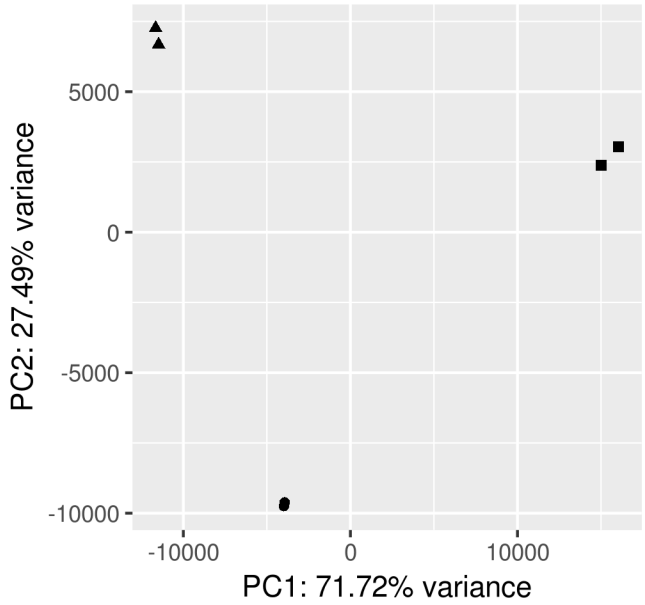
Supplemental Figure S1. A. Respirometer setup showing the chamber submerged in ambient seawater in the closed, recirculating configuration with thermistor and oxygen probe in series. The oxygen consumption of each fish was measured individually in 15-minute intervals after a 30 min acclimation period to the respirometry chamber. Following each 15-min interval, the valves of the system were opened manually to the open configuration to exchange with the flow-through, ambient seawater before being closed again for the next measurement period. B. Representative plot showing the oxygen concentrations in the respirometer over time during measurements of fish metabolic rate. The portions with the negative slopes are the measurement periods when the system was closed (15 min intervals). The system was then opened again for several minutes to be flushed by new seawater from the flow-through system, and then closed

again to take another measurement on the same fish. This process was repeated three times. The above traces are from an individual of *Anoplarchus purpurescens*^{C} that weighed 6.65g.

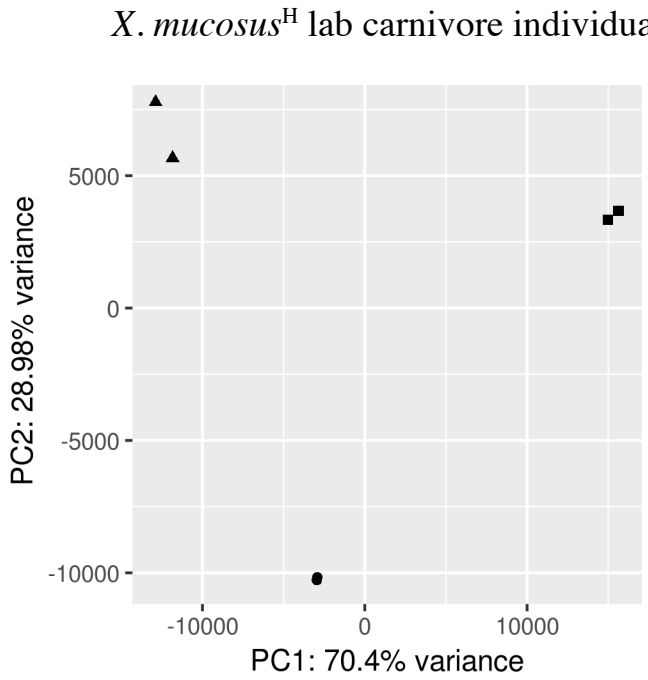


Supplemental Figure S2: *X. mucosus*^{H} wild fish replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

X. mucosus^{H} lab omnivore individuals

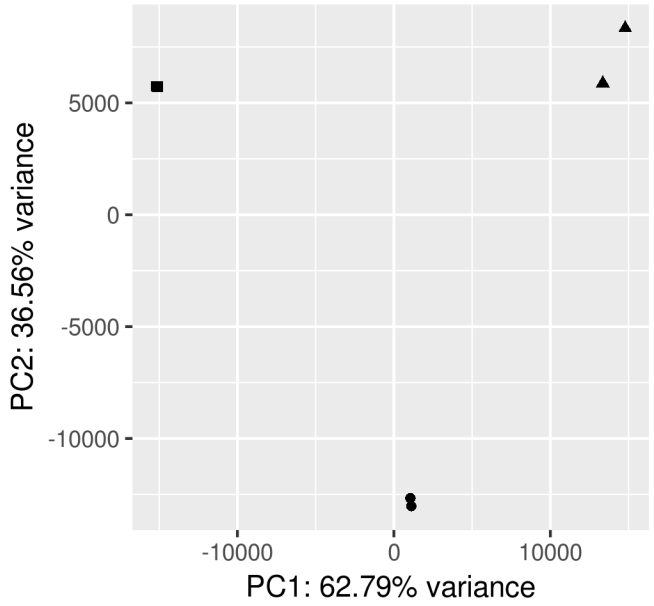


Supplemental Figure S3: *X. mucosus*^{H} laboratory omnivore diet fish replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.



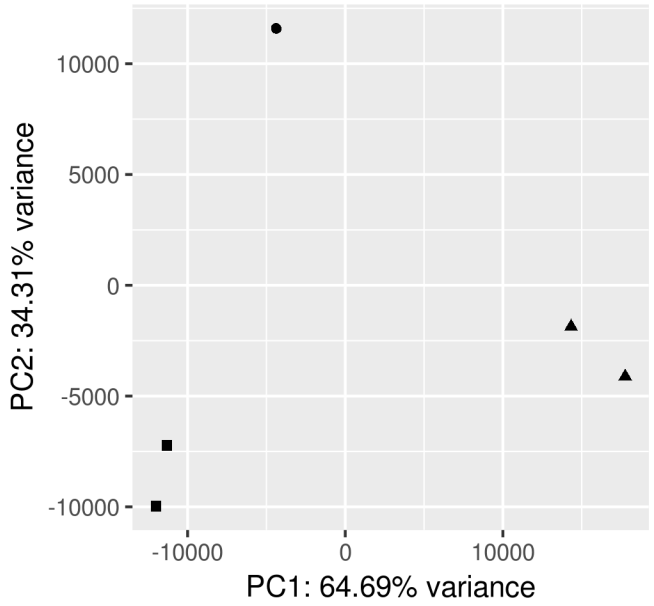
Supplemental Figure S4: *X. mucosus*^{H} laboratory carnivore diet replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

X. atropurpureus^{O} wild individuals



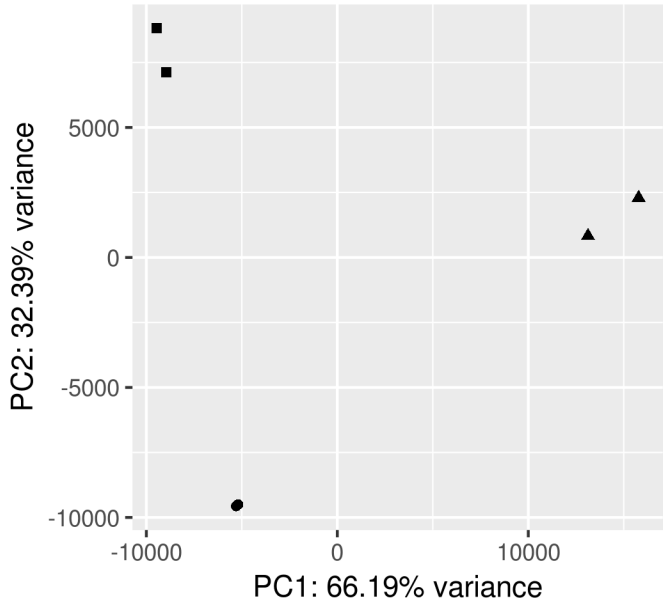
Supplemental Figure S5: *X. atropurpureus*^{O} wild fish replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

X. atropurpureus^{O} lab carnivore individuals



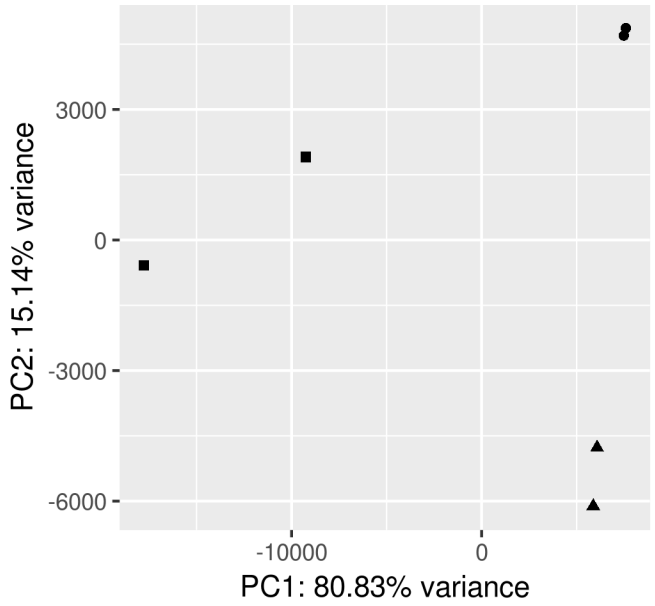
Supplemental Figure S6: *X. atropurpureus*^{O} laboratory carnivore diet replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

P. chirus^{O} wild individuals

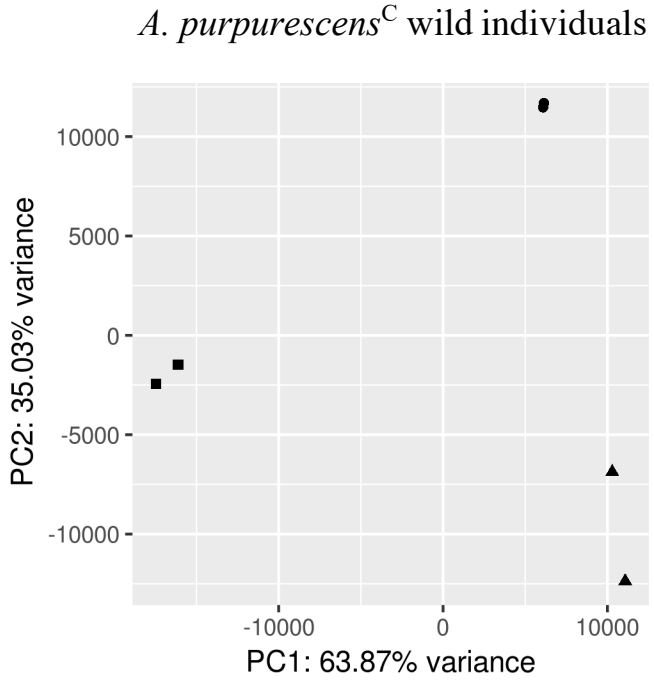


Supplemental Figure S7: *P. chirus*^{O} wild fish replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

P. chirus^{O} lab carnivore individuals

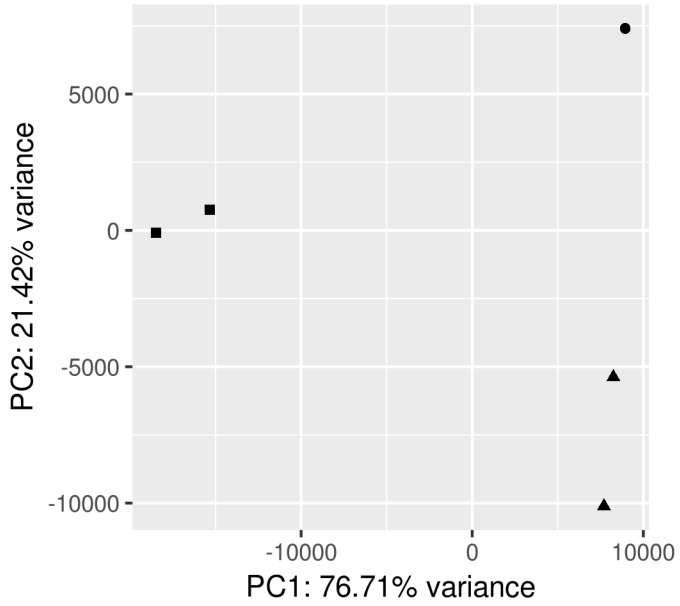


Supplemental Figure S8: *P. chirus*^{O} laboratory carnivore diet replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.



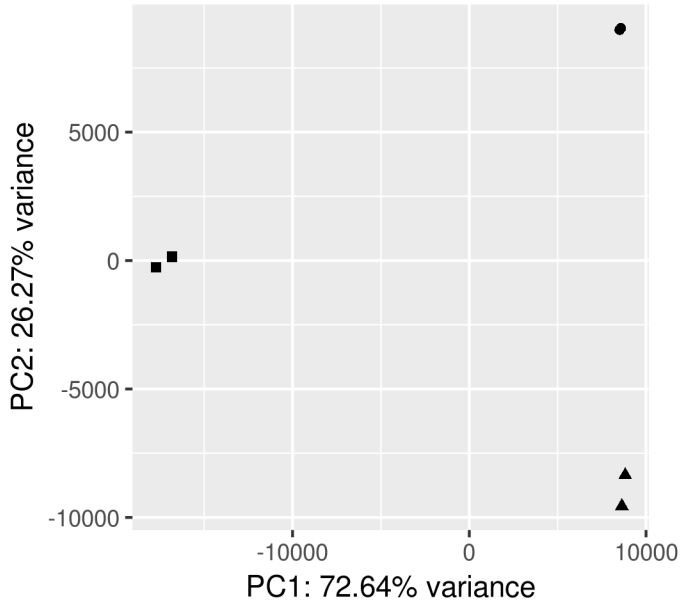
Supplemental Figure S9: *A. purpurescens*^{C} wild fish replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

A. purpurescens^{C} lab omnivore individuals

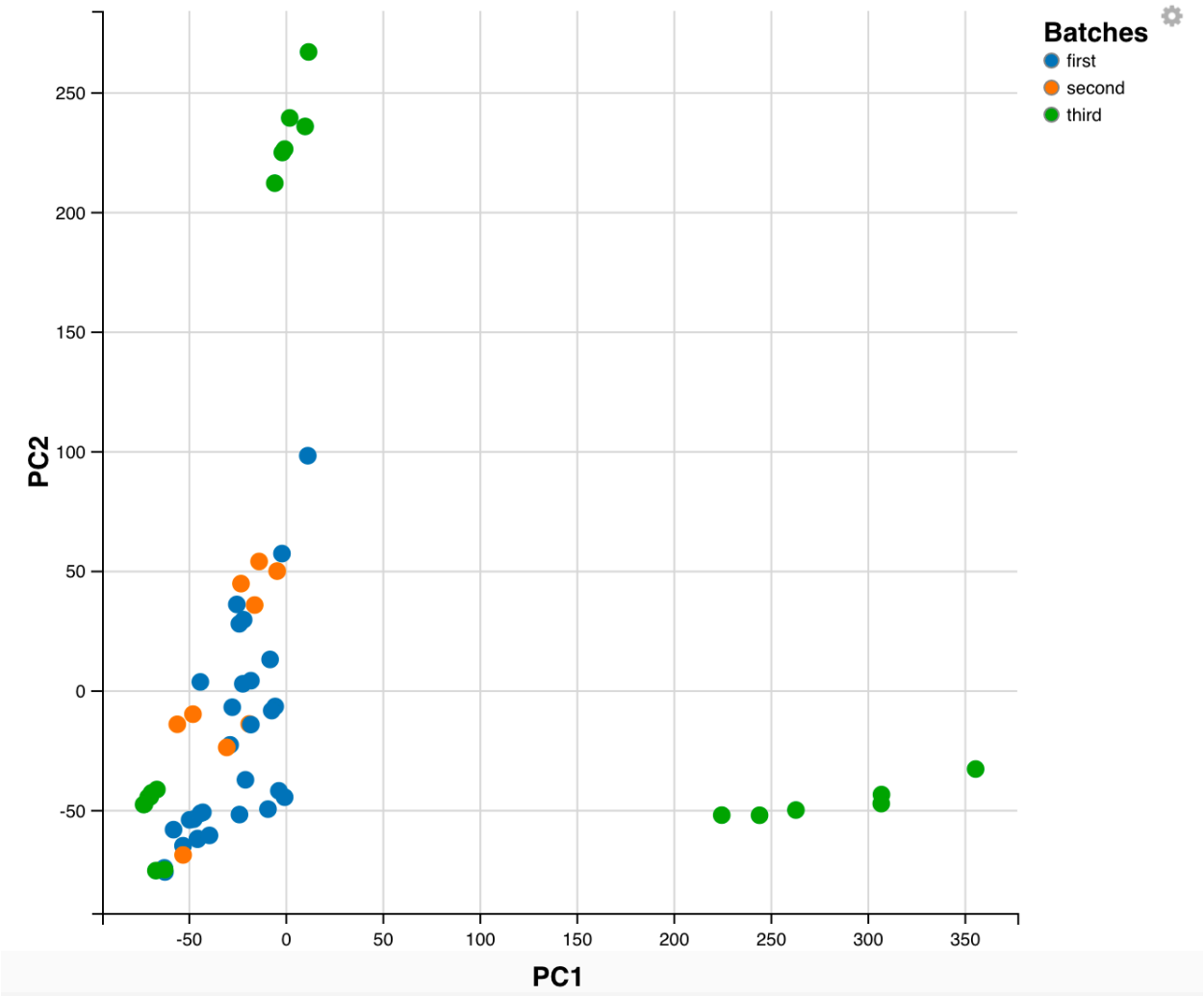


Supplemental Figure S10: *A. purpurescens*^{C} laboratory omnivore diet replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced for transcriptomic data. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.

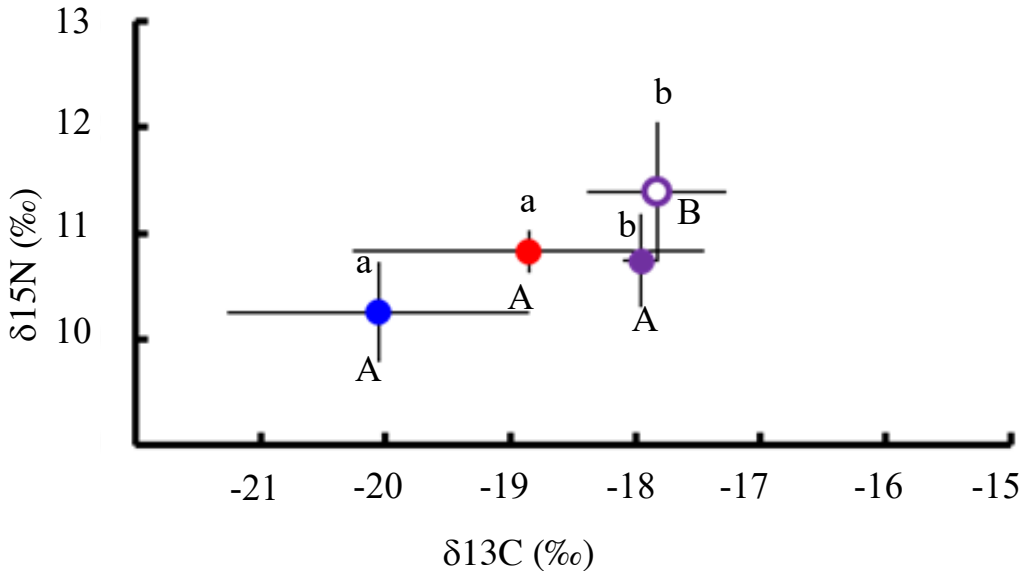
A. purpurescens^{C} lab carnivore individuals



Supplemental Figure S11: *A. purpurescens*^{C} laboratory carnivore diet replicates. PCA plot to depict the quality check analysis of individual replicates within the same species and diet group and across the three tissues we sequenced. Spheres depict Liver replicates, Squares depict pyloric ceca replicates, and triangles depict mid-intestine replicates.



Supplemental Figure S12: PCA plot generated with Batch Quality Check Results. Color indicated batch (blue is first run, orange is second run, green is third run. Samples do not cluster by batch. The standardized Pearson correlation coefficient is 0.87 and the Cramer's V is 0.7, indicating batch does not fully interfere with the signal, with batch 3 showing the most uniqueness because this batch contained the liver samples. All species are represented in each cluster of liver samples (batch 3) on the plot, showing that they are dispersed throughout and not grouping by sample.



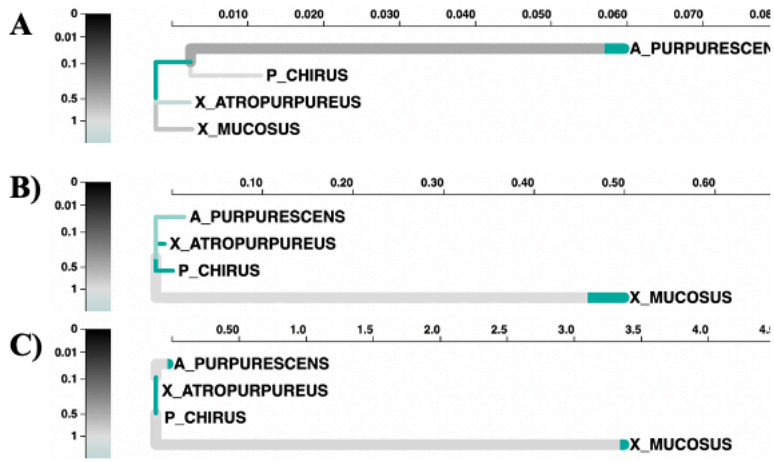
Supplemental Figure S13. Carbon and nitrogen (‰) dual isotope plot of wild-caught prickleback fishes. *X. mucosus*^{H} (blue sphere), *X. atropurpureus*^{O} (purple ring), *P. chirus*^{O} (purple sphere), and *A. purpurescens*^{C} (red sphere). Values are mean ± standard deviation. Interspecific comparisons were made with ANOVA. Significant differences ($P<0.05$) for δ15N indicated with capital letters, whereas lower case letters indicate significant differences in δ13C values. Symbols sharing a capital or lower case letter are not significantly different.

Supplemental Table S1 Stable Isotope ANOVA and Tukey results within each species across diet groups. And wild fish, lab-omnivore fish, and lab-carnivore fish ANOVA and Tukey results				
Species		ANOVA	Tukeys HSD	
X. mucosus[H]	Carbon	P=0.073	Wild-LC	p=0.0808
		$F_{2,7}$=3.893	Wild-LO	p=0.1730
			LO-LC	p=0.8643
	Nitrogen	P=0.00522*	Wild-LC	p=0.0051*
		$F_{2,7}$=12.21	Wild-LO	p=0.0326*
			LO-LC	p=0.3934
X. atropurpureus[O]	Carbon	p=0.263		
		$F_{1,5}$=1.587		
	Nitrogen	p=0.141		
		$F_{1,5}$=3.054		
P. chirus[O]	Carbon	p=7.06e-05 ***		
		$F_{1,5}$=144.2		
	Nitrogen	p=0.00285 **		
		$F_{1,5}$=29.58		
A. purpurescens[C]	Carbon	p=0.787	Wild-LC	p=0.7758
		$F_{2,7}$=0.248	Wild-LO	p=0.9827
			LO-LC	p=0.8826
	Nitrogen	p= 0.00155 **	Wild-LC	p=0.0038*
		$F_{2,7}$=18.73	Wild-LO	p=0.0027*
			LO-LC	p=0.9566
Wild fishes	Carbon	p=0.0258*		
		$F_{3,12}$=4.428		
	Nitrogen	p=0.0355*		
		$F_{3,12}$=3.963		
Lab-Omnivore Diet	Carbon	p=0.666		
		$F_{1,4}$=0.216		
	Nitrogen	p=0.0328*		
		$F_{1,4}$=10.25		
Lab-Carnivore Diet	Carbon	p=0.00319*		
		$F_{3,8}$=11.10		
	Nitrogen	p=0.0526*		
		$F_{3,8}$=3.976		

Supplemental Table S2 Candidate Genes under positive selection in Liver (note there might be more than one GO for each gene)				
Uniprot ID	Full Name	Gene Ontology	Omega (dn/ds) Model M0	% coverage
G6PD_TAKRU	Glucose-6-phosphate 1-dehydrogenase	GO:0051156	0.31363	22.26
ACSA_HUMAN	Acetyl-coenzyme A synthetase , cytoplasmic	GO:0019427	0.31965	17.69
FA10A_DANRE	Fatty acid-binding protein 10-A , liver basic	Not found	0.3819	100
G6PT1_HUMAN	Glucose-6-phosphate exchanger SLC37A4	Not found	0.42308	18.18
TIM21_XENLA	Mitochondrial import inner membrane translocase subunit Tim21	GO:0030150	0.66572	48.71
LIPE_HUMAN	Endothelial lipase	GO:0008283	1.00152	34.20

Supplemental Table S3 Candidate Positively Selected Genes in Pyloric ceca				
Uniprot ID	Full Name	GO	Omega (dn/ds) Model M0	% coverage
TPISB_DANRE	Triosephosphate isomerase B	GO:0006094	0.11128	98.39
RNPL1_MOUSE	Aminopeptidase RNPEPL1	GO:0043171	0.12326	52.64
AQP1_PONAB	Aquaporin-1	GO:0015696	0.38443	76.58
DDHD2_MOUSE	Phospholipase DDHD2	GO:0006888	0.45125	59.94
SER1_DROME	Serine proteases 1/2	GO:0006508	0.48138	89.43
TMC7_CHICK	Transmembrane channel-like protein 7	GO:0006811	0.76999	90.07
CC50B_MOUSE	Cell cycle control protein 50B	GO:0006869	0.82402	94.05
NDUC1_BOVIN	NADH dehydrogenase [ubiquinone] 1 subunit C1 , mitochondrial	Not found	0.91442	89.47
PRS27_MOUSE	Serine protease 27	Not found	0.92613	81.1
ELA1_SALSA	Elastase-1	Not found	1.32135	98.73
PA21B_CANLF	Phospholipase A2	GO:0019731	1.33595	86.3
TPA_HUMAN	Tissue-type plasminogen activator	GO:0007596	1.39125	49.47
TBA_XENLA	Tubulin alpha chain	GO:0007017	12.61093	28.51

Supplemental Table S4 Candidate Positively Selected Genes in mid-intestine				
Uniprot ID	Full Name	GO	Omega (dn/ds) Model M0	
TPISB_DANRE	Triosephosphate isomerase B	GO:0006094	0.11128	98.39
SDHF2_DANRE	Succinate dehydrogenase assembly factor 2 , mitochondrial	GO:0006121	0.11453	100
MA2C1_MOUSE	Alpha-mannosidase 2C1	GO:0006013	0.22929	98.85
ACD11_CHICK	Acyl-CoA dehydrogenase family member 11	Not found	0.28243	80.57
G6PT3_DANRE	Glucose-6-phosphate exchanger SLC37A2	GO:0008643	0.29134	100
SDHB_DANRE	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit , mitochondrial	GO:0009060	0.31831	86.79
PLA2R_MOUSE	Secretory phospholipase A2 receptor	GO:0001816	1.26054	7.13
TRY1_SALSA	Trypsin-1	GO:0007586	1.34063	47.93
NDUA3_MOUSE	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	Not found	1.43125	95.24
TPA_HUMAN	Tissue-type plasminogen activator	GO:0007596	5.42397	47.15



Supplemental Figure S14: An adaptive branch-site random effects likelihood (aBSREL) test for episodic diversification phylogenetic tree constructed for various genes in the pyloric ceca from four prickleback fish species: a) serine protease 27 (PR27), b) tubulin alpha chain (TBA), c) elastase (ELA1). ω is the ratio of nonsynonymous to synonymous substitutions. The color gradient represents the magnitude of the corresponding ω. Branches thicker than the other branches have a $p < 0.05$ (corrected for multiple comparisons) to reject the null hypothesis of all ω on that branch (neutral or negative selection only). A thick branch is considered to have experienced diversifying positive selection.

Supplemental Table S5 Standard length and body mass of wild fishes and fishes fed a carnivore or omnivore diet at the end of the feeding trial					
Diet		*X. mucosus*[H]	*X. atropurpureus*[O]	*P. chirus*[O]	*A. purpurescens*[C]
Wild fish	SL (mm)	163.92±32.71 n=12	131.72±16.65 n=11	100.69±27.68 n=16	108.64±17.85 n=11
	BM (g)	20.43±10.33 n=12	9.05±3.95 n=11	5.16±4.42 n=16	9.61±5.04 n=11
Fish fed Omnivore diet	SL after	159.00±25.17 n=8	-	-	99.5±8.78 n=6
	BM before	15.59±7.19 n=8	-	-	5.99±2.22 n=6
	BM after	16.65±8.46 n=8	-	-	7.04±2.05 n=6
Fish fed Carnivore diet	SL after	156.80±20.91 n=5	131.25±15.19 n=12	97±9.23 n=10	97.29±11.67 n=7
	BM before	13.14±5.06 n=5	7.54±2.85 n=12	3.18±0.72 n=10	5.15±2.14 n=7
	BM after	15.39±6.13 n=5	8.37±3.07 n=12	3.89±1.04 n=10	6.36±2.84 n=7
Wild fishes are separate from fishes fed either an omnivore or carnivore diet in the lab. BM stands for Body Mass and SL stands for Standard Length					

Supplemental Table S6. Growth rate across a four-week feeding trial, and routine metabolic rate of stichaeid fishes fed different diets in the laboratory.

Fish Species	Metabolic Rate (mg O_2 min^{-1} g^{-1})			Growth Rate (% weight gain)		
	Omnivore	Carnivore		Omnivore	Carnivore	
X. mucosus[H]	0.0508 ± 0.0163	0.0369 ± 0.00617	t=0.741 P=0.478	5.40 ± 5.16 %	16.81 ± 2.57 %	t=4.552 P=0.001
X. atropurpureus[O]	--	--		--	11.45 ± 2.57 %	
P. chirus[O]	--	--		--	21.68 ± 8.37 %	
A. purpurescens[C]	0.0340 ± 0.00371	0.0413 ± 0.00672	t=0.936 P=0.373	20.52 ± 12.85 %	22.65 ± 12.02 %	t=0.309 P=0.763

Note: df=11 for growth comparisons. df=9 for metabolic rate comparisons. Values are mean ± standard error.

Supplemental Results: Relative Gene Expression

We used RNA-seq data of the liver, pyloric ceca and mid-intestine to observe the suites of genes that changed with different diets and how species respond to dietary variation. Note that we are only reporting on pathways relevant to digestion and metabolism of specific nutrient classes (Fig. 4, Table 4). If a cluster is not mentioned, yet depicted in the heatmap, then the genes within that cluster were not directly relevant to digestion and nutrient metabolism.

Liver

There were 11 DEGs when comparing wild fish (WF) and laboratory carnivore diet fish (LC) of *X. atropurpureus*^{O}, out of which 36% were annotated (Supplemental Figure S14). Cluster 1 (elevated in wild fish) contained genes important for glucose and fatty acid metabolism (Supplemental Table S7). These proteins are important for energy storage, insulin signaling pathway and glucagon signaling pathway. Cluster 2 (elevated in LC fish) contained one unannotated gene.

P. chirus^{O} stands out with 302 DEGs when comparing liver gene expression among WF and LC *P. chirus*^{O}, out of which 14% of genes were annotated (Supplemental Figure S15). Cluster 1 (elevated in wild fish) contains genes involved in cholesterol metabolism (Supplemental Table S4). Cluster 2 (elevated in LC fish) contains genes for lipid metabolism, fatty acid synthesis, and bile acid biosynthesis.

There are 19 DEGs when comparing WF, laboratory omnivore diet fish (LO) and LC *A. purpurescens*^{C}, out of which 32% of genes were annotated (Supplemental Figure S16). Cluster 1 (elevated in wild fish) consists of genes for cholesterol homeostasis and genes that play a role in controlling the metabolism of fatty acids, specifically glycerophospholipid metabolism and glycerolipid metabolism (Supplemental Table S4).

Pyloric ceca

There were 1226 DEGs when comparing WF to LC *X. atropurpureus*^{O}, out of which 68.1% were annotated (Supplemental Figure S17). Wild *X. atropurpureus* upregulated genes in Cluster 1 (elevated in wild fish), that were involved in digestive processes for chitin degradation, glycolysis, glycogen catabolic process, glycosaminoglycan biosynthesis, bile acid metabolism, proteolysis, carbohydrate metabolic process, collagen metabolic/catabolic process, pentose phosphate pathway, lipid metabolism, and glutamate biosynthetic process (Supplemental Table

S8). LC *X. atropurpureus*^{O} upregulated genes in Cluster 2 (elevated in LC fish), that were involved in lipid metabolism.

Like in the liver, *P. chirus*^{O} showed differing DEGs in comparison to the other species, with only 19 DEGs in the pyloric ceca among WF and LC *P. chirus*^{O}; 94.7% of the genes were annotated (Supplemental Figure S18). Cluster 1 (elevated in wild fish), featured genes involved in carboxypeptidase activity, proteolysis, and carbohydrate binding (Supplemental Table S8). There were no Cluster 2 (elevated in LC fish) genes in *P. chirus*^{O} pyloric ceca.

There were 259 DEGs when comparing WF, LC, and LO *A. purpurescens*^{C}, out of which 62.5% were annotated (Supplemental Figure S19). Cluster 1 (elevated in wild fish) contained genes involved in endopeptidase/trypsin activity and insulin receptor signaling pathway (Supplemental Table S8). Cluster 2 (wild-omnivore genes) contained genes involved in carbohydrate metabolism, bile acid metabolism, cholesterol catabolism, and fatty acid synthesis. Cluster 3 (elevated in the lab genes) contains a large amount of genes, although not directly involved in digestion or metabolism.

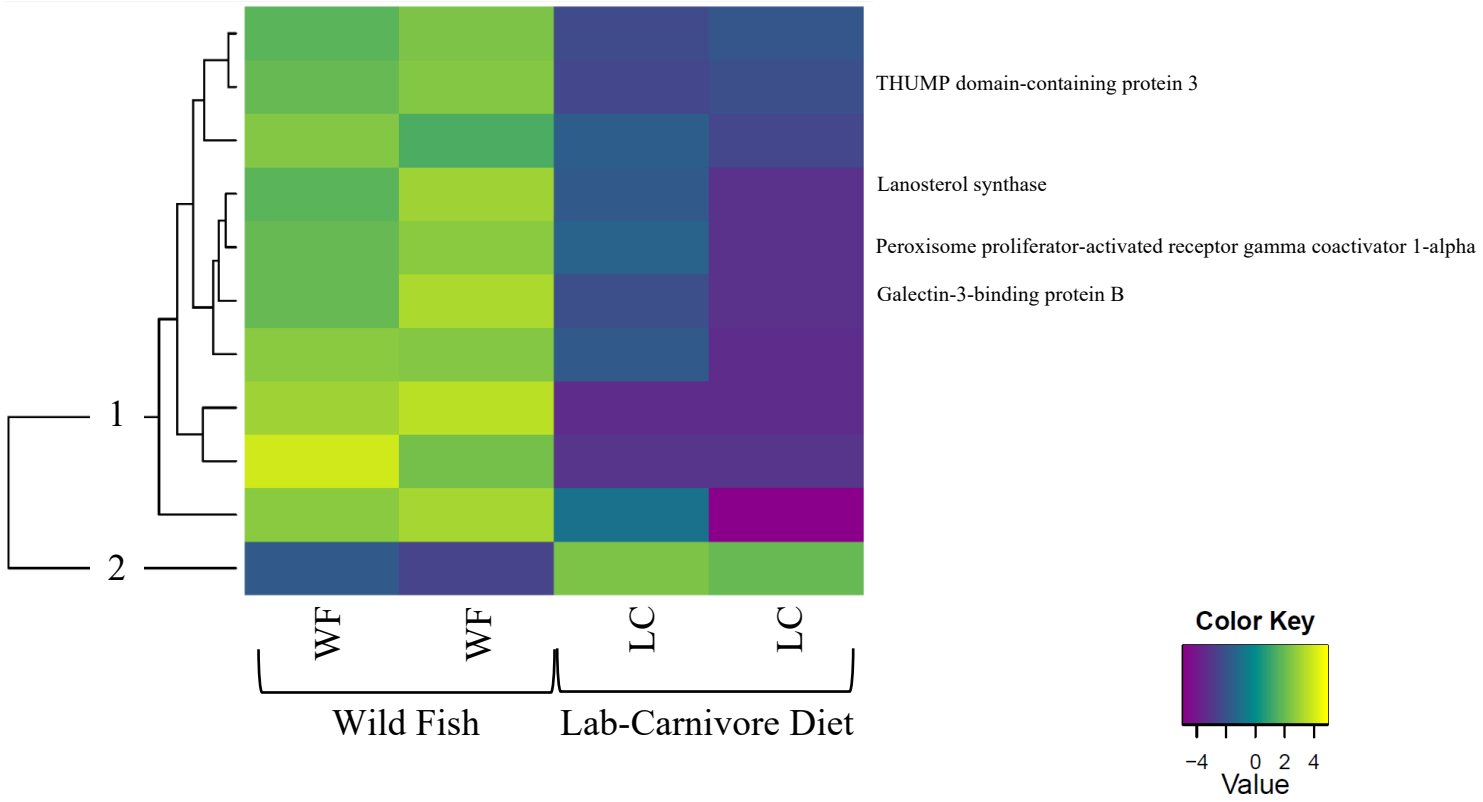
Mid-intestine

There were 343 DEGs for W and LC *X. atropurpureus*^{O}, out of which 83.96% were annotated (Supplemental Figure S20). Cluster 1 (elevated in wild fish) contained genes are involved in glycolysis, cholesterol biosynthesis, lipid metabolism, carbohydrate metabolism, protein metabolism, fatty acid biosynthesis, and pentose phosphate pathway (Supplemental Table S9). Cluster 2 (elevated in LC fish) genes are involved in cholesterol metabolism.

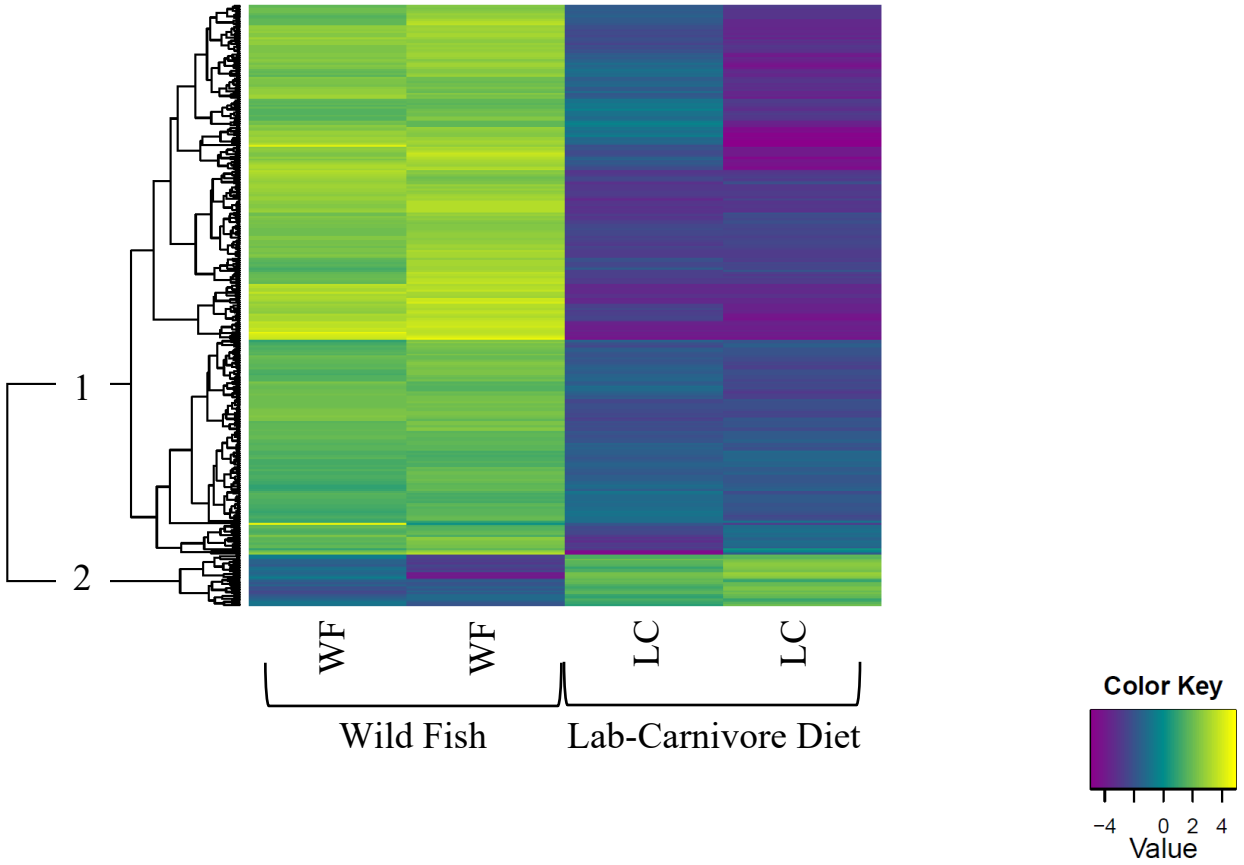
There were 298 DEGs for WF and LC *P. chirus*^{O}, out of which 90.6% were annotated (Supplemental Figure S21). Cluster 1 (elevated in wild fish) contained 293 of the genes, including those involved in gluconeogenesis, protein deubiquination, creatine metabolic process, and calcium ion transport (Supplemental Table S9). Five genes not directly involved in digestion or nutrient metabolism composed Cluster 2 (elevated in LC fish).

There were 872 DEGs for WF, LC, and LO *A. purpurescens*^{C}, out of which 82.2% were annotated (Supplemental Figure S22). Cluster 1 (elevated in wild fish) contained genes involved in mannose metabolism, glycogen catabolism, insulin signaling, gluconeogenesis, glucose homeostasis and lipid catabolism (Supplemental Table S9). Cluster 2 (wild-omnivore genes) contained genes involved in bile acid metabolism. Cluster 4 (wild-carnivore genes) contained genes involved in proteolysis. Cluster 5 (carnivore genes) contained genes involved in collagen

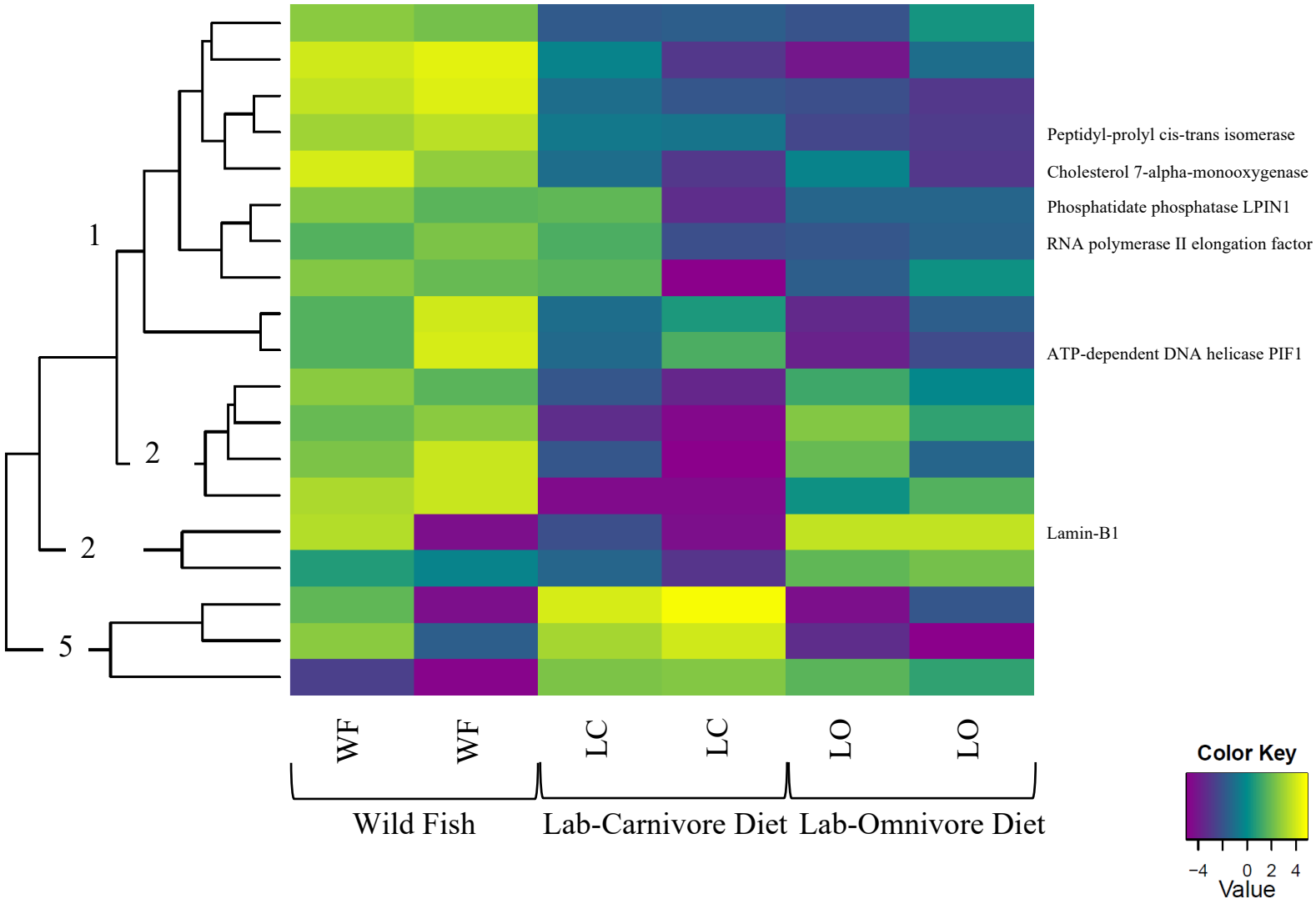
catabolism. Cluster 6 (omnivore genes) contained genes involved in cholesterol biosynthesis and glucose metabolism.



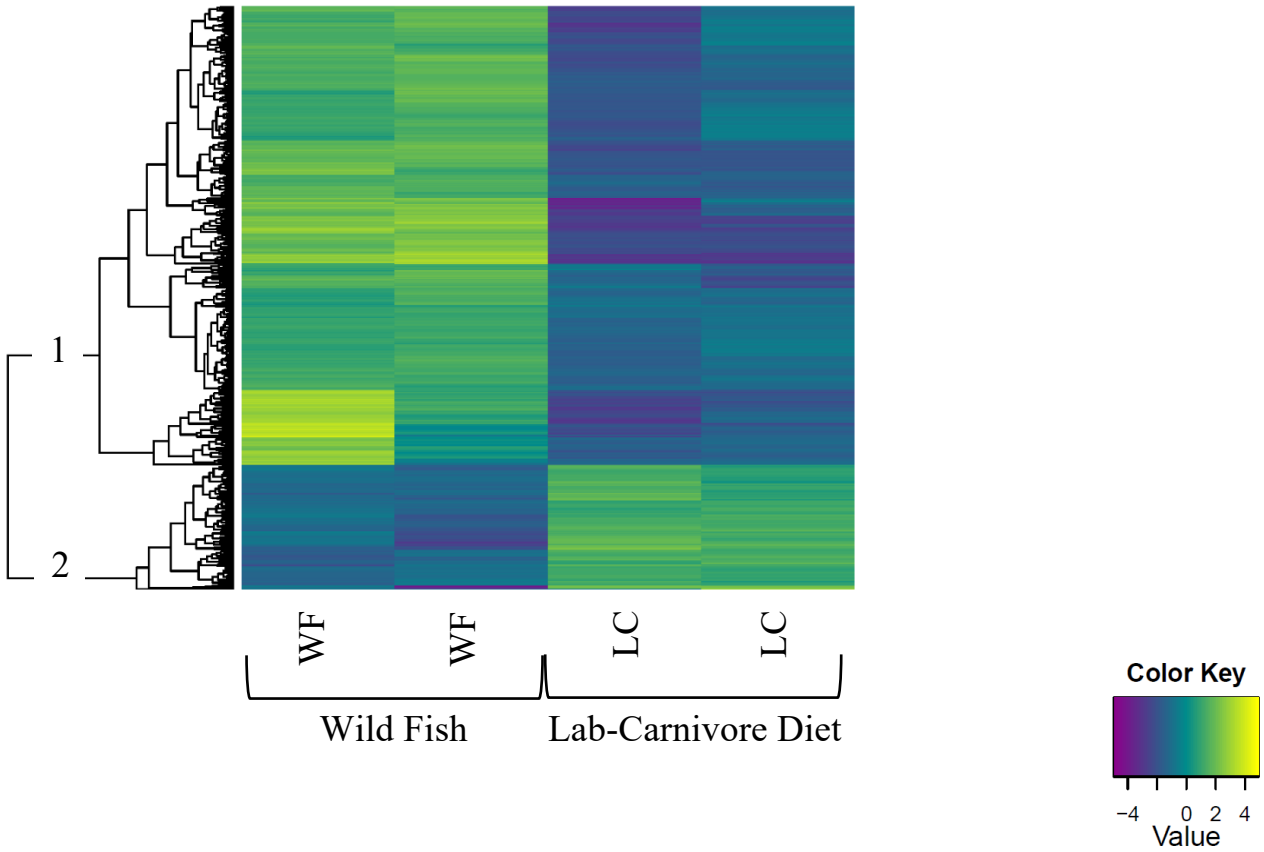
Supplemental Figure S15: Differential gene expression depicted as a heatmap in the liver of *X. atropurpureus* [O]. Yellow indicates elevated relative expression, whereas blue indicates low expression. Each row is a single gene, and genes are clustered in a dendrogram (on left of each heatmap) by similarity of expression patterns. The various clusters of genes are described in Table 4. Each column represents the gene expression in a single tissue from an individual fish, with WF = wild-caught fish, LO = fish fed an omnivore diet in the laboratory (in the case of *X. mucosus* [H] and *A. purpurescens* [C]), and LC = fish fed a carnivore diet in the laboratory.



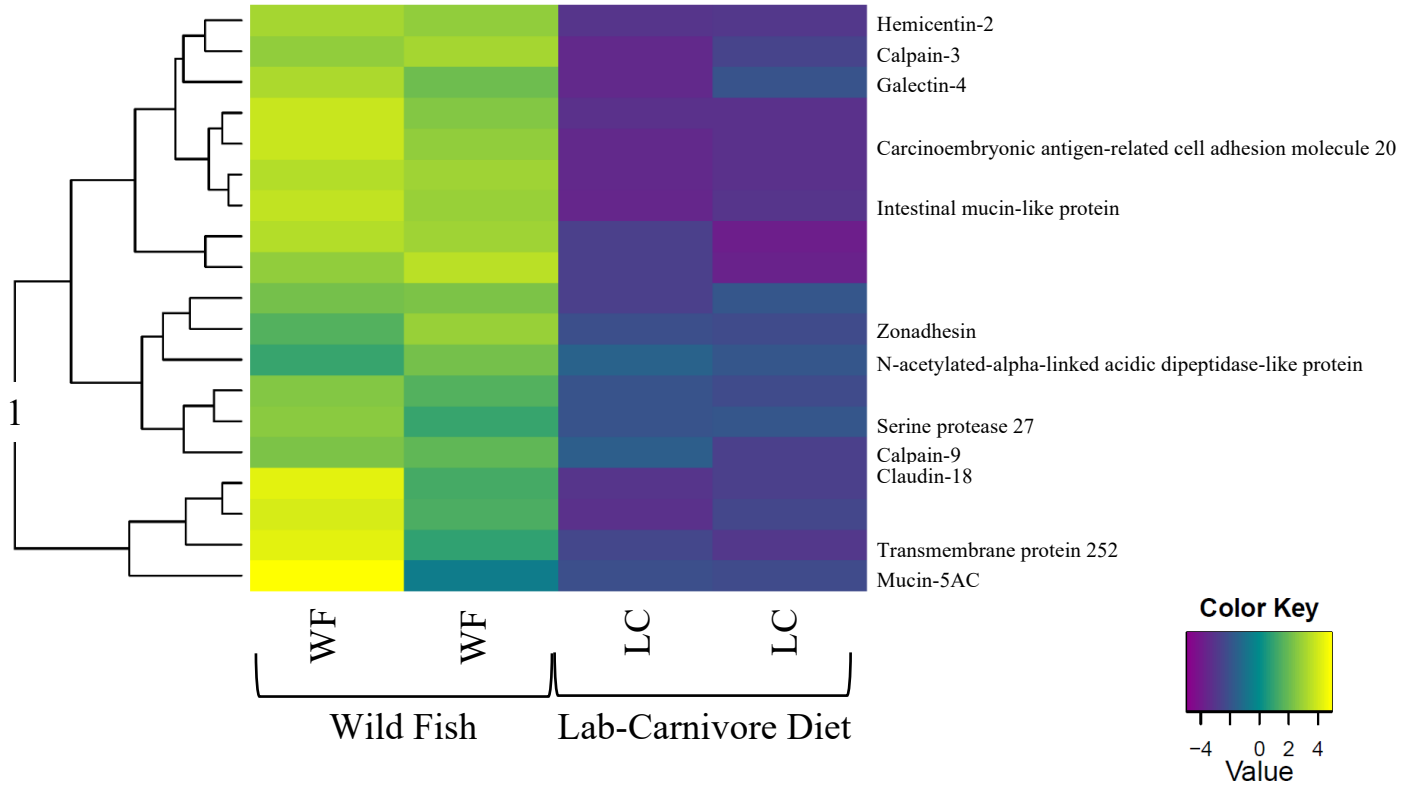
Supplemental Figure S16: As described for Figure S14, but depicting the liver in *P. chirus* O.



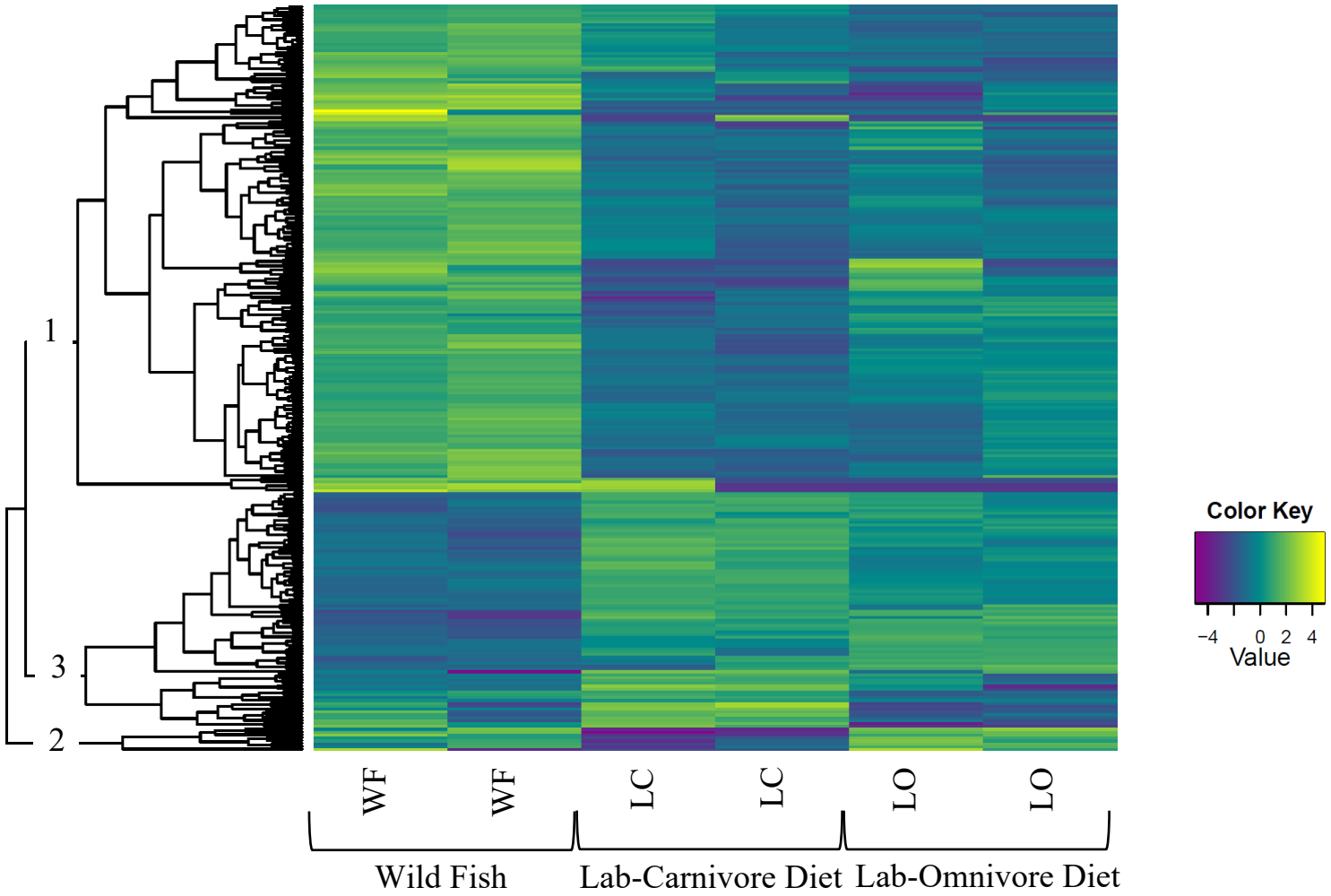
Supplemental Figure S17: As described for Figure S14, but depicting the liver in *A. purpurescens* [C].



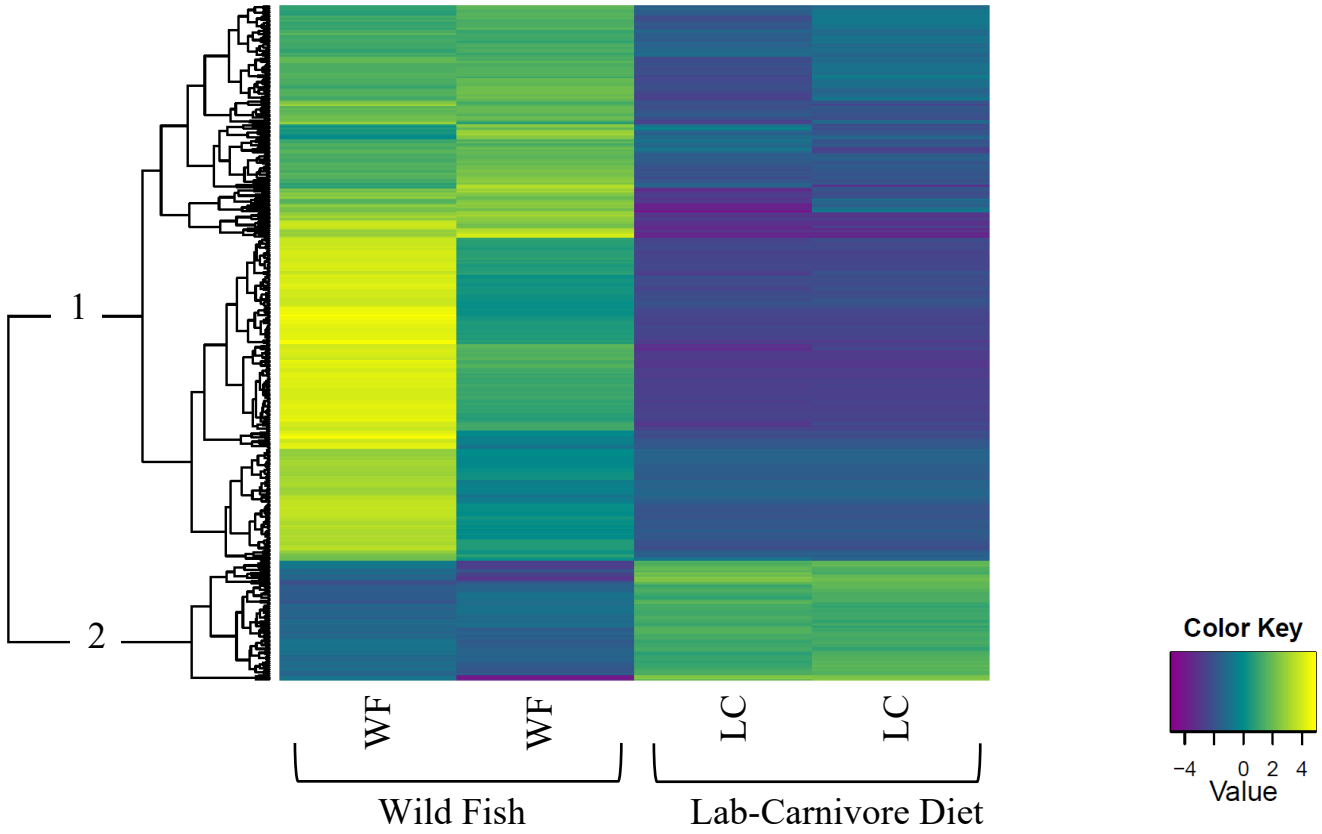
Supplemental Figure S18: As described for Figure S14, but depicting the pyloric ceca in *X. atropurpureus* [O].



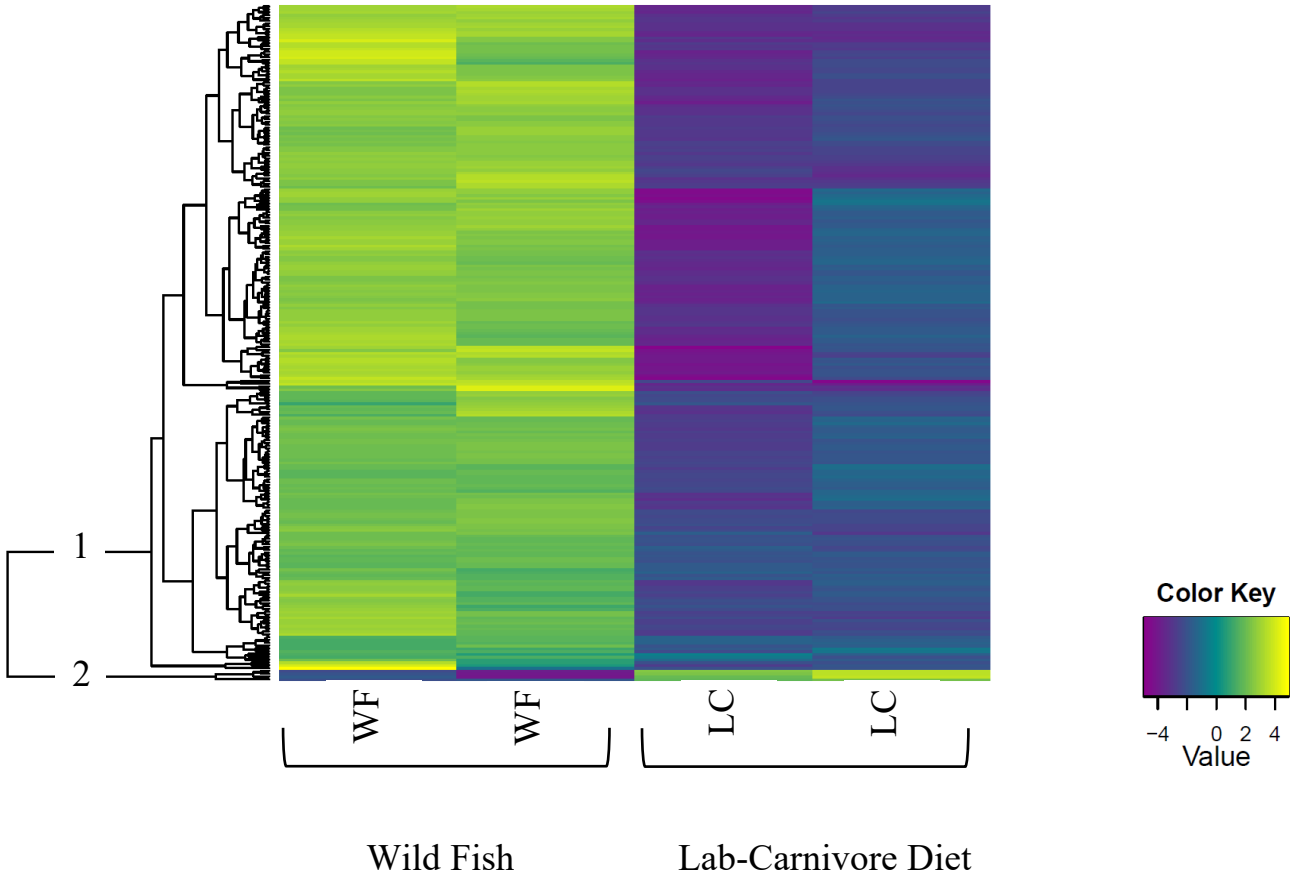
Supplemental Figure S19: As described for Figure S14, but depicting the pyloric ceca in *P. chirus* O.



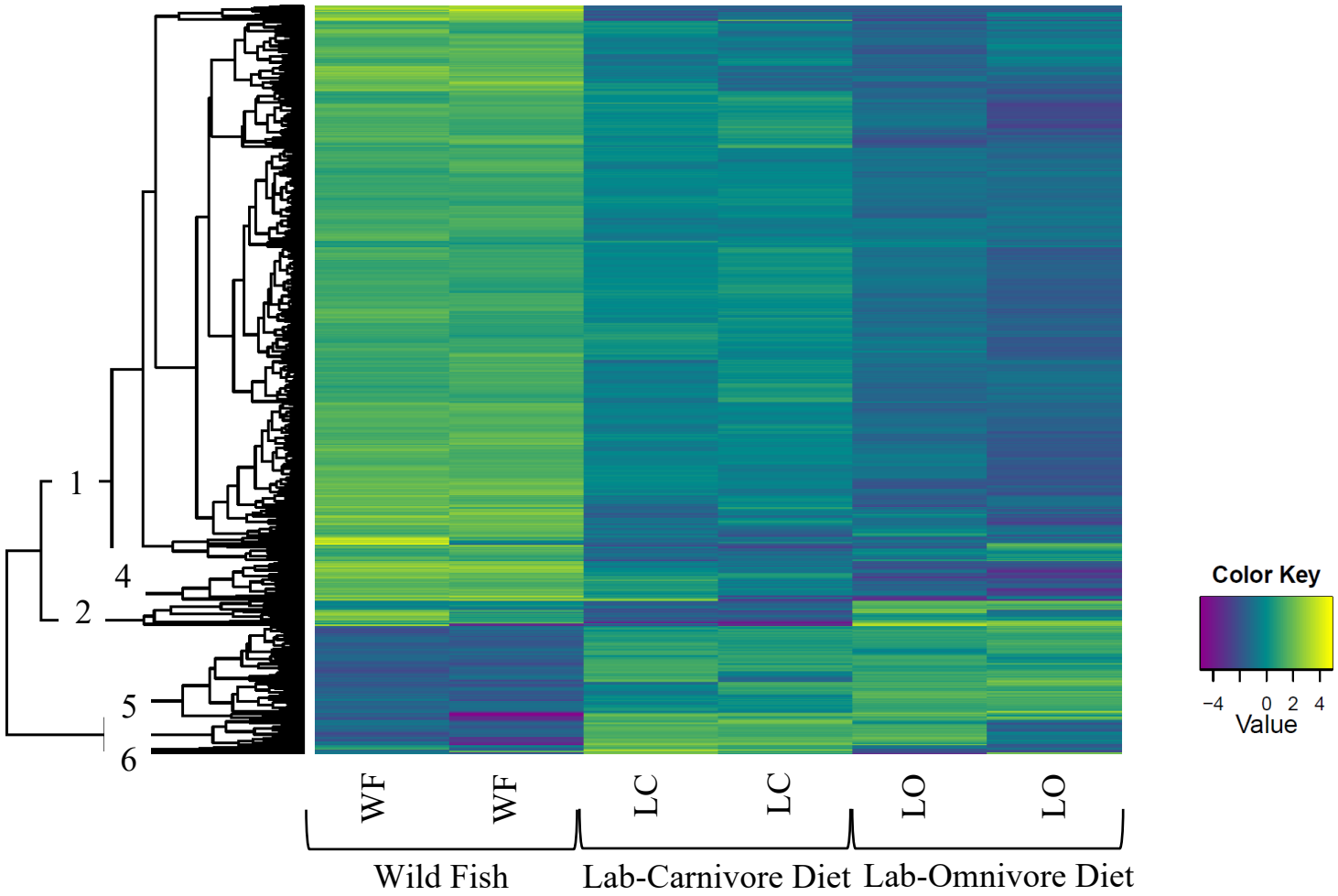
Supplemental Figure S20: As described for Figure S14, but depicting the pyloric ceca in *A. purpurescens* [C].



Supplemental Figure S21: As described for Figure S14, but depicting the mid-intestine in *X. atropurpureus* [O].



Supplemental Figure S22: As described for Figure S14, but depicting the mid-intestine in *P. chirus* [O].



Supplemental Figure S23: As described for Figure S14, but depicting the mid-intestine in *A. purpurescens* [C].

Supplemental Table S7 Differentially Expressed Genes relevant to metabolism in Liver							
Species	Cluster	Gene	Function	WF fish	LC fish	LO fish	PSG in WF fish
Xiphister atropurpureus [O]	1	Lanosterol synthase	cholesterol biosynthesis	High (++++)	Low (+)	N/A	
	1	peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Coordinates genes involved in glucose and fatty acid metabolism	High (++++)	Low (+)	N/A	
Phytichthys chirus [O]	1	Cholesterol side-chain cleavage enzyme	cholesterol metabolism	High (++++)	Low (+)	N/A	
	2	Apolipoprotein	lipid metabolism	Low (+)	High (+++++)	N/A	
	2	Glucose-6-phosphate 1-dehydrogenase	Pentose phosphate pathway: produces NADPH for fatty acid and nucleic acid synthesis	Low (+)	High (+++++)	N/A	*
	2	Sterol 26-hydroxylase	bile acid biosynthesis	Low (+)	High (+++++)	N/A	
Anoplarchus purpurescens [C]	1	Cholesterol 7-alpha monooxygenase	cholesterol homeostasis	High (+++++)	Low (+)	Low (+)	
	1	phosphatidate phosphatase	controls the metabolism of fatty acids	High (+++++)	Low (++)	Low (++)	
PSG: Positively Selected Gene when comparing sequences (PAML and Datamonkey) among wild-caught fishes of the four prickleback species. Gradient of expression is depicted by plus signs, in that one (+) is low expression to a roughly 5 fold increase (+++++).							

Supplemental Table S8 Differentially Expressed Genes relevant to digestion in Pyloric ceca							
Species	Cluster	Gene	Function	WF fish	LC fish	LO fish	PSG in WF fish
X. atropurpureus [O]	1	acidic endochitinase SP2	chitin degradation	High (++++)	Low (+)	N/A	
	1	chitinase A	chitin degradation	High (++++)	Low (+)	N/A	
	1	phosphoglycerate kinase	Glycolysis	High (++++)	Low (+)	N/A	
	1	ADP-dependent glucokinase	Glycolysis	High (++++)	Low (+)	N/A	
	1	glucose-6-phosphate isomerase	Glycolysis	High (++++)	Low (+)	N/A	
	1	fructose-bisphosphate aldolase 2	Glycolysis	High (++++)	Low (+)	N/A	
	1	triosephosphate isomerase	Glycolysis	High (++++)	Low (+)	N/A	
	1	glycogen phosphorylase 1	glycogen catabolic process	High (++++)	Low (+)	N/A	
	1	2-phosphoxylose phosphatase	glycosaminoglycan biosynthesis	High (++++)	Low (+)	N/A	
	1	glypican-5	glycosaminoglycan biosynthesis	High (++++)	Low (+)	N/A	
	1	glycerol-3-phosphate dehydrogenase	lipid metabolism	High (++++)	Low (+)	N/A	
	1	gastrotropin	bile acid metabolism	High (++++)	Low (+)	N/A	
	1	Transmembrane protease serine 7	proteolysis	High (++++)	Low (+)	N/A	
	1	Carboxypeptidase M	Proteolysis	High (++++)	Low (+)	N/A	
	1	Puromycin-sensitive aminopeptidase	Proteolysis	High	Low (+)	N/A	

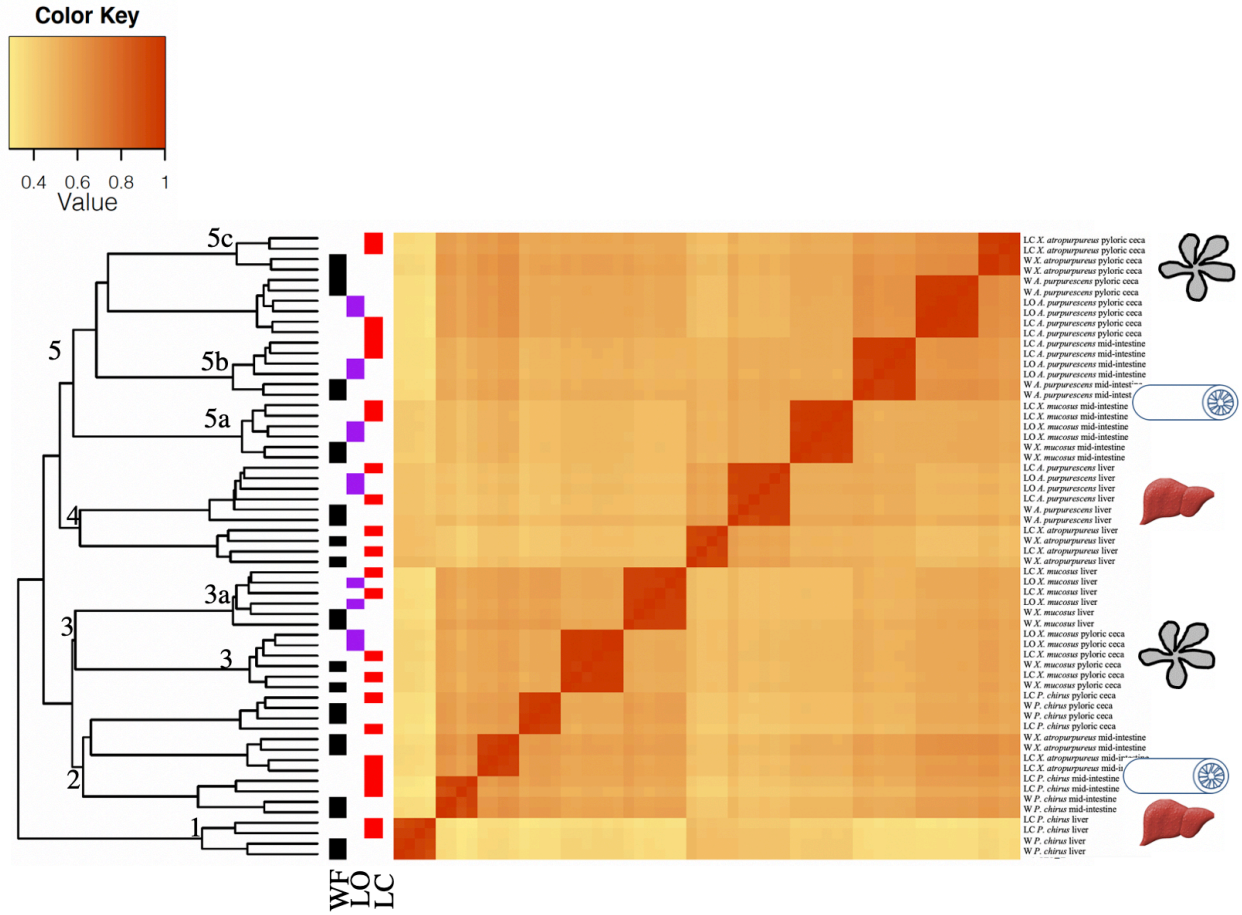
				(++++)			
	1	Pepsin A	Proteolysis	High (++++)	Low (+)	N/A	
	1	L-lactate dehydrogenase	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	Lactoylglutathione lyase	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	Beta-1,4 N-acetylgalactosaminyltransferase 1	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	72 kDa type IV collagenase	collagen catabolic process	High (++++)	Low (+)	N/A	
	1	Prolyl 3-hydroxylase 3	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	Malate dehydrogenase	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	Transketolase	pentose phosphate pathway	High (++++)	Low (+)	N/A	
	1	Collagenase 3	carbohydrate metabolic process	High (++++)	Low (+)	N/A	
	1	Malonyl-CoA-acyl carrier protein transacylas	lipid metabolism	High (++++)	Low (+)	N/A	
	1	Glutamate dehydrogenase 1	glutamate biosynthetic process	High (++++)	Low (+)	N/A	
	2	glycerol-3-phosphate dehydrogenase	cellular lipid metabolic process	Low (++)	High (+++)	N/A	
P. chirus [O]	1	N-acetylated-alpha-linked acidic dipeptidase-like protein	carboxypeptidase activity	High (++++)	Low (+)	N/A	
	1	Calpain 9	Proteolysis	High (++++)	Low (+)	N/A	
	1	Calpain 3	Proteolysis	High (++++)	Low (+)	N/A	
	1	Serine protease 27	Proteolysis	High (++++)	Low (+)	N/A	*

	1	Galectin 4	Carbohydrate binding	High (++++)	Low (+)	N/A	
A. purpurescens[C]	1	Serine protease 27	Endopeptidase/trypsin activity	High (++++)	Low (+)	Low (+)	*
	1	Insulin receptor substrate 2A and 2B	Insulin receptor signaling pathway	High (++++)	Low (+)	Low (+)	
	2	Lactase-phlorizin hydrolase	Carbohydrate metabolism	High (++++)	Low (+)	High (+++++)	
	2	Gastrotropin	Bile acid metabolism	High (++++)	Low (+)	High (+++++)	
	2	Cholesterol 7-alpha-monooxygenase	Cholesterol catabolism	High (++++)	Low (+)	High (+++++)	
	2	Fatty acid synthase	Fatty acid synthesis	High (++++)	Low (+)	High (+++++)	
PSG: As described in Table S4. Gradient of expression is depicted by plus signs, in that one (+) is low expression to a roughly 5 fold increase (+++++).							

Supplemental Table S9 Differentially Expressed Genes relevant to digestion in Mid-intestine							
Species	Cluster	Gene	Function	WF fish	LC fish	LO fish	PSG in WF fish
X. atropurpureus[O]	1	Fructose-bisphosphate adolase 2	Glycolysis	High (+++++)	Low (+)	N/A	
	1	Glycogen phosphorylase 1	Glycogen catabolic process	High (+++++)	Low (+)	N/A	
	1	Glucose-6-phosphate isomerase	Glycolysis	High (+++++)	Low (+)	N/A	
	1	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	High (+++++)	Low (+)	N/A	
	1	Phosphoglycerate kinase	Glycolysis	High (+++++)	Low (+)	N/A	
	1	Pyruvate kinase	Glycolysis	High (+++++)	Low (+)	N/A	
	1	Lanosterol synthase	Cholesterol biosynthesis	High (+++++)	Low (+)	N/A	
	1	Endothelial lipase	Lipid metabolism	High (+++++)	Low (+)	N/A	
	1	Malate dehydrogenase	Carbohydrate metabolism	High (+++++)	Low (+)	N/A	
	1	L-lactase dehydrogenase	Carbohydrate metabolism	High (+++++)	Low (+)	N/A	
	1	Aminopeptidase	Fatty acid biosynthesis	High (+++++)	Low (+)	N/A	
	1	Prolyl endopeptidase	Fatty acid biosynthesis	High (+++++)	Low (+)	N/A	
	1	Fatty acid synthase	Fatty acid biosynthesis	High (+++++)	Low (+)	N/A	
	1	Transketolase	Pentose phosphate pathway	High (+++++)	Low (+)	N/A	

	2	Apolipoprotein B-100	Cholesterol metabolism	Low (+)	High (++++)	N/A	
Phytichthys chirus[O]	1	Pyruvate carboxylase	Gluconeogenesis	High (+++++)	Low (+)	N/A	
	1	Ubiquitin carboxyl-terminal hydrolase 24	Protein deubiquination	High (+++++)	Low (+)	N/A	
	1	Ubiquitin carboxyl-terminal hydrolase 34	Protein deubiquination	High (+++++)	Low (+)	N/A	
	1	Sodium- and chloride-dependent creatine transporter 1	Ion transport	High (+++++)	Low (+)	N/A	
	1	Sarcoplasmic/endoplasmic reticulum calcium atpase 1	Calcium ion transport	High (+++++)	Low (+)	N/A	
	1	Sarcoplasmic/endoplasmic reticulum calcium atpase 2	Calcium ion transport	High (+++++)	Low (+)	N/A	
A. purpurescens[C]	1	alpha-mannosidase	mannose metabolism	High (++++)	Low (+++)	Low (+++)	
	1	L-fucose kinase	glycogen catabolism	High (++++)	Low (+++)	Low (+++)	
	1	insulin receptor substrate	insulin signaling	High (++++)	Low (+++)	Low (+++)	
	1	insulin receptor substrate 2-B	insulin signaling	High (++++)	Low (+++)	Low (+++)	
	1	pyruvate carboxykinase	gluconeogenesis	High (++++)	Low (+++)	Low (+++)	
	1	phosphoenolpyruvate carboxykinase	glucose homeostasis	High (++++)	Low (+++)	Low (+++)	
	1	lipase	lipid catabolism	High (++++)	Low (+++)	Low (+++)	
	2	Gastropin	bile acid metabolism	High (++++)	Low (+)	High (++++)	
	4	aminopeptidase	proteolysis	High (+++++)	High (++++)	Low (+)	
	5	Stromelysin-3	collagen catabolism	Low (+)	High (++++)	Low (+)	
	6	diphosphomevalonate decarboxylase	cholesterol biosynthesis	Low (+)	Low (+)	High (+++)	
	6	lanosterol synthase	cholesterol biosynthesis	Low (+)	Low (+)	High (+++)	

	6	GDP-D-glucose phosphorylase 1	glucose metabolism	Low (+)	Low (+)	High (+++)	
PSG: As described in Table S4. Gradient of expression is depicted by plus signs, in that one (+) is low expression to a roughly 5 fold increase (+++++).							



Supplementary Figure S24. Correlation matrix of expressed genes in the liver, pyloric ceca, and mid intestine of four prickleback species caught from the wild (WF), or fed omnivore (LO) or carnivore (LC) diets in the laboratory. Clustering of WF are depicted by black bars in between the dendrogram and the correlation matrix, whereas LO fishes are depicted by purple bars, and LC fishes by red bars. All sample names are depicted on the right side (60 individual tissues in total), with symbols for each tissue type used to emphasize clustering. Correlation matrix created with the Trinity toolkit "PtR" and Pearson correlation as sample distances. We emphasize five distinct clusters: 1: *Phytichthys chirus*^{O} liver. 2: Mid intestine of *P. chirus*^{O} and *X. atropurpureus*^{O}. 3: broken into two clusters, 3a: *X. mucosus*^{H} liver, 3b: *X. mucosus*^{H} pyloric ceca. 4: *Anoplarchus purpurescens*^{C} and *X. atropurpureus*^{O} liver. And, 5: broken into three clusters, 5a: *Anoplarchus purpurescens*^{C} and *X. atropurpureus*^{O} pyloric ceca, 5b: *A. purpurescens*^{C} mid intestine, 5c: *X. mucosus*^{H} mid intestine.

Supplemental Discussion

Metabolic Rate

Contrary to our expectation that fishes consuming the carnivore diet in the laboratory would have higher metabolic rates than fishes consuming the omnivore diet, routine metabolic rate did not vary among the species or intra-specifically on the different diets, suggesting that body mass is still one of the main determinants of metabolic rate in fishes, and these fishes are all similar in size in comparison to the range of sizes fishes can attain (Gillooly et al. 2001; Clarke and Johnston 1999; Ikeda 2016). More detailed measures of metabolic rate across longer time scales undoubtedly would show differences in Specific Dynamic Action for fishes consuming the different diets in the laboratory (Secor 2009), but we only measured routine metabolic rate. Given the short period of time over which we measured metabolic rate, our results were possibly influenced by stress and thus, more detailed analyses of metabolic rate in prickleback fishes are needed (Killen et al. 2021).

Clarke A, Johnston NM (1999) Scaling of metabolic rate with body mass and temperature in teleost fish. J Animal Ecol 68 (5):893-905. doi:10.1046/j.1365-2656.1999.00337.

Ikeda T (2016) Routine metabolic rates of pelagic marine fishes and cephalopods as a function of body mass, habitat temperature and habitat depth. J Exp Mar Biol Ecol 480:74-86. doi:https://doi.org/10.1016/j.jembe.2016.03.012

Killen SS, Christensen EAF, Cortese D, Závorka L, Norin T, Cotgrove L, Crespel A, Munson A, Nati JJH, Papatheodoulou M, McKenzie DJ (2021) Guidelines for reporting methods to estimate metabolic rates by aquatic intermittent-flow respirometry. J Exp Biol 224 (18):jeb242522. doi:10.1242/jeb.242522

Gillooly JF, Brown JH, West GB, Savage VM, Charnov EL (2001) Effects of Size and Temperature on Metabolic Rate. Science 293 (5538):2248. doi:10.1126/science.1061967

Secor SM (2009) Specific dynamic action: a review of the postprandial metabolic response. J of Comp Physiol B 179 (1):1-56. doi:10.1007/s00360-008-0283-7

Supplementary Table S10 Annotated Gene IDs of vectors in PCA plot (Figure 6)
Liver

Genes associated with *X. mucosus*[H] (towards the left of the PCA plot)	Apolipoprotein B-100
	Probable bifunctional E2/E3 enzyme R795
	CASP8 and FADD-like apoptosis regulator
	Complement factor I
	3-hydroxyanthranilate 3,4-dioxygenase
Genes associated with *X. atropurpureus*[O] (upwards of the PCA plot)	Apolipoprotein B-100
	Very long-chain acyl-CoA synthetase
	Complement factor H-related protein 2
	RNA 3'-terminal phosphate cyclase
	Pyrethroid hydrolase Ces2e
Pyloric Ceca	
Genes associated with *X. mucosus*[H] (bottom left of the PCA plot)	Plectin
	Uncharacterized protein 075L
	Sodium channel protein type 4 subunit alpha B
Genes associated with *A. purpurescens*[C] (bottom right of the PCA plot)	ATP-citrate synthase
Mid-intestine	
Genes associated with *X. mucosus*[H] (bottom left of the PCA plot)	Agmatinase, mitochondrial
	Serine/threonine-protein kinase 16
	Deoxyribonuclease gamma
	Antizyme inhibitor 1
	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform
Genes associated with *A. purpurescens*[C] (upwards of the PCA plot)	Peptidyl-prolyl cis-trans isomerase FKBP3

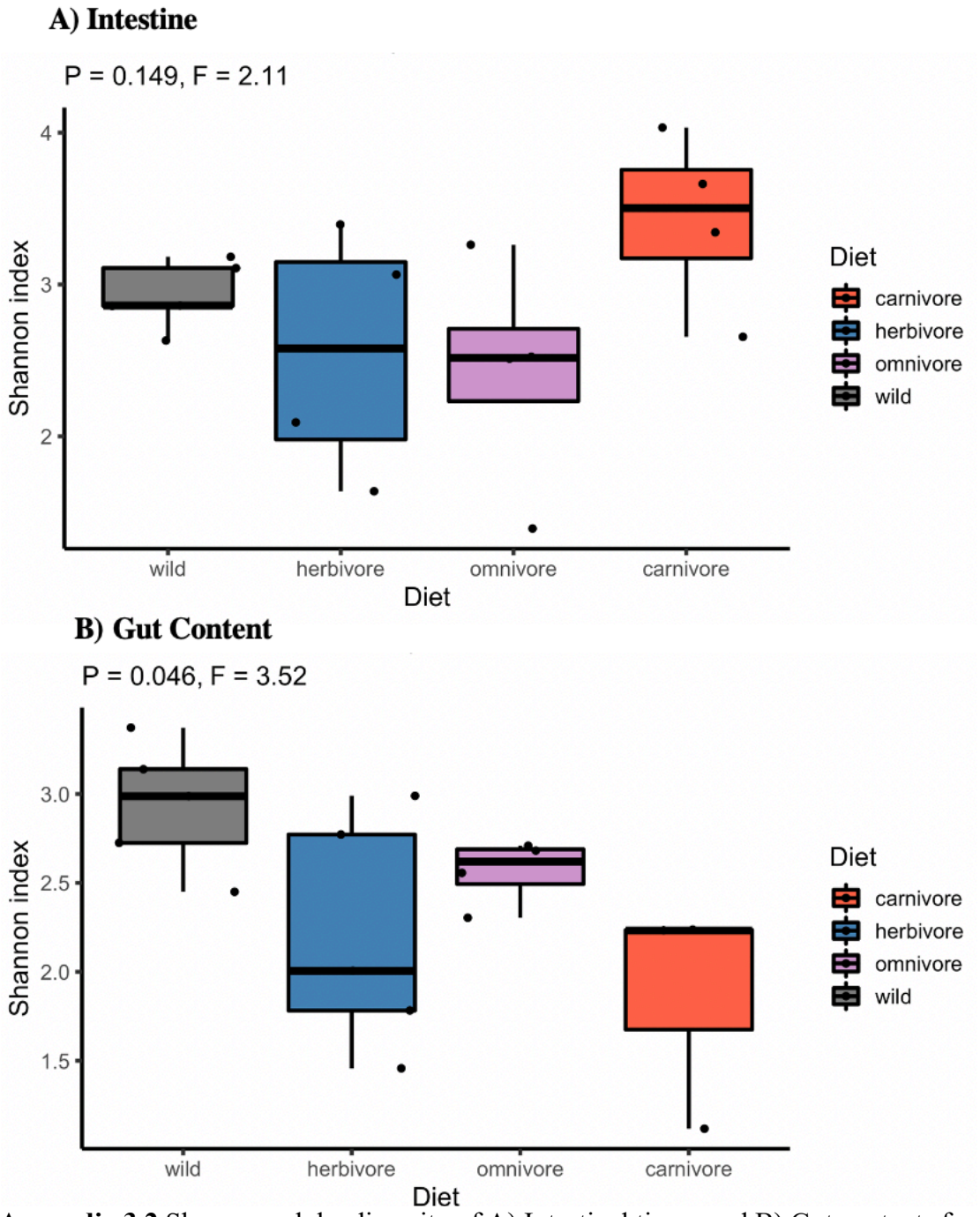
Appendix and Supplementary Information for Chapter 3

Diet shifts affect gut and liver function and the distal intestine microbiome of an herbivorous fish

Appendix 3.1. Digestive enzyme activities (μmol product · min^{-1} · g^{-1}) in the distal intestine tissue of *C. violaceus* fed different diets in the laboratory for six months.

Enzyme	Carnivore diet	Omnivore diet	Herbivore diet	Wild†	
Aminopeptidase	2.19 ± 0.94	3.26 ± 1.29	2.51 ± 0.87	3.61 ± 0.77	$F_{3,23}$=2.865, P=0.056
Maltase	0.45 ± 0.31	0.68 ± 0.21	0.59 ± 0.30	0.41 ± 0.16	$F_{3,23}$=1.642, P=0.207
Lipase	3.08 ± 2.71	1.74 ± 1.57	1.09 ± 0.92	2.58 ± 1.58	$F_{3,23}$=2.192, P=0.116
β-glucosidase	0.39 ± 0.24[a]	0.39 ± 0.25[a]	0.75 ± 0.47[ab]	1.13 ± 0.60[b]	**$F_{3,23}$=5.033, P=0.008**
NAGase*	0.17 ± 0.08[b]	0.26 ± 0.11[b]	0.16 ± 0.06[b]	0.06 ± 0.01[a]	**$F_{3,23}$=12.88, P<0.001**

Values are mean ± standard deviation. Values for a particular enzyme compared among the fish on the different diets with ANOVA. Those values sharing a superscript letter for a given enzyme are not statistically significantly different. * NAGase = N-acetyl-β-D-glucosaminidase. † Data on wild-caught fish from German et al. (2015).

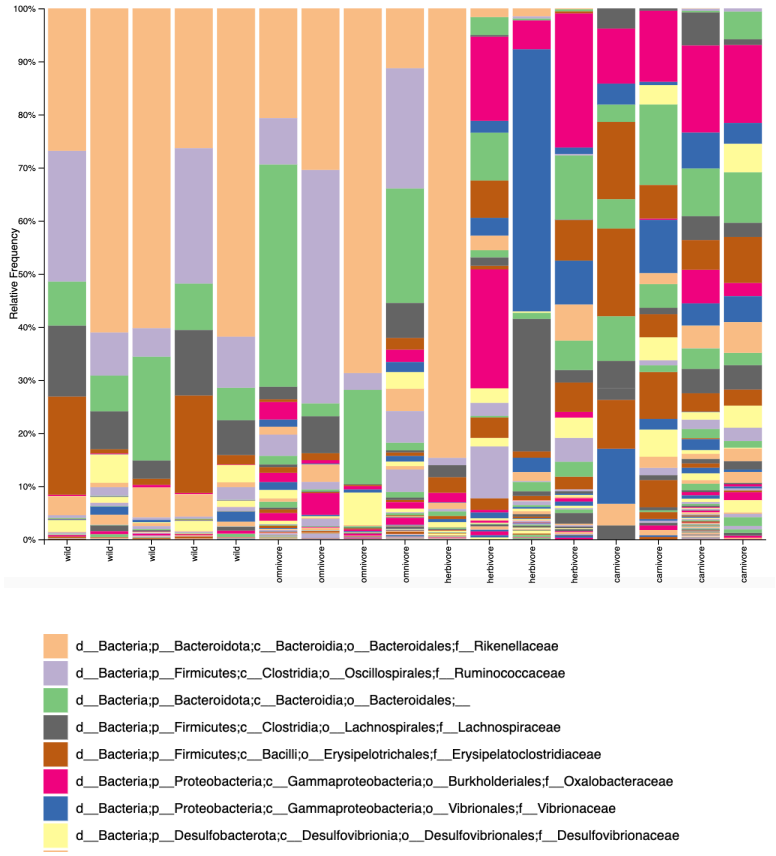


Appendix 3.2 Shannons alpha diversity of A) Intestinal tissue and B) Gut contents for wild fish and fish fed different diets in the laboratory.

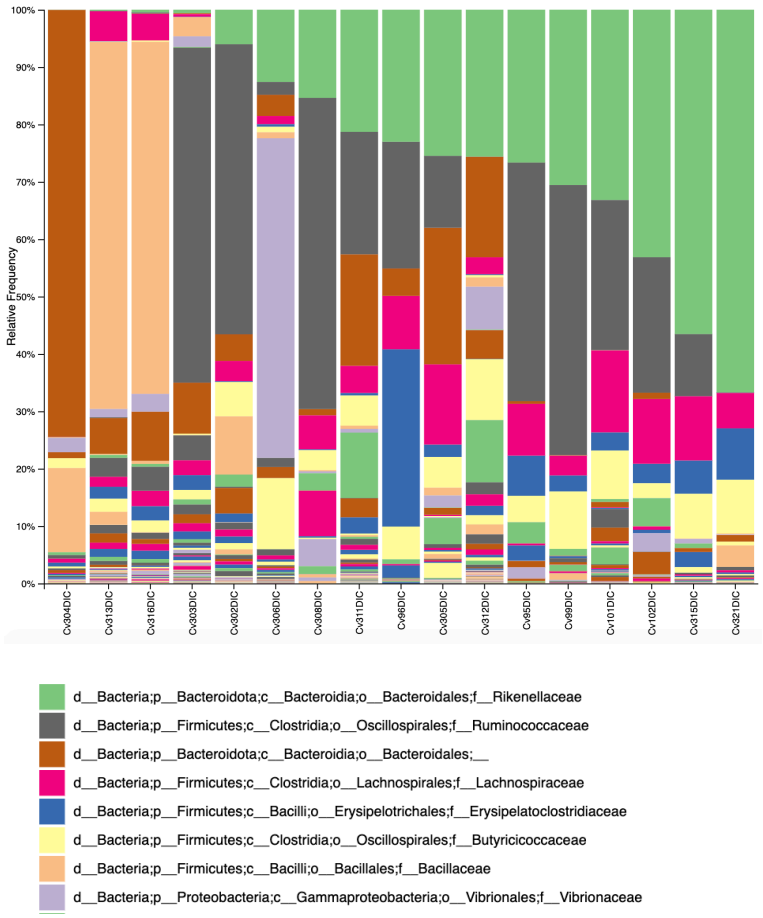
Appendix 3.3 Table 1 Vector for NMDS plot of intestine tissue only	
Direction of vector	**Taxa**
LH and LC	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter
LH and LC	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Massilia
LH and LC	d__Bacteria; p__Bacillota; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Anoxybacillus
LH and LC	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Chromobacteriaceae; g__Vogesella
WF and LO	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Vibrionales; f__Vibrionaceae; g__Vibrio; s__Vibrio_renipiscarius

Appendix 3.3 Table 2 Vector for NMDS plot of gut contents only	
Direction	Taxon for vectors
left	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Anaerostignum; s__Gadus_morhua
left	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae
left	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae
left	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Butyricicoccaceae; g__Butyricicoccus; s__Butyricicoccus_pullicaecorum
left	d__Bacteria; p__Bacillota; c__Bacilli; o__Erysipelotrichales; f__Erysipelatoclostridiaceae; g__Erysipelatoclostridium; s__Erysipelotrichaceae_bacterium
left	d__Bacteria; p__Bacillota; c__Clostridia; o__Oscillospirales; f__Ruminococcaceae; g__Angelakisella; s__uncultured_bacterium
right	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Paracoccus

right	d__Bacteria; p__Bacillota; c__Clostridia; o__Lachnospirales; f__Lachnospiraceae; g__Epulopiscium
top	d__Bacteria; p__Pseudomonadota; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae
top	d__Bacteria; p__Pseudomonadota; c__Gammaproteobacteria; o__Burkholderiales; f__Chromobacteriaceae; g__Vogesella
top	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Microbacteriaceae
top	d__Bacteria; p__Bacillota; c__Bacilli; o__Exiguobacterales; f__Exiguobacteraceae; g__Exiguobacterium



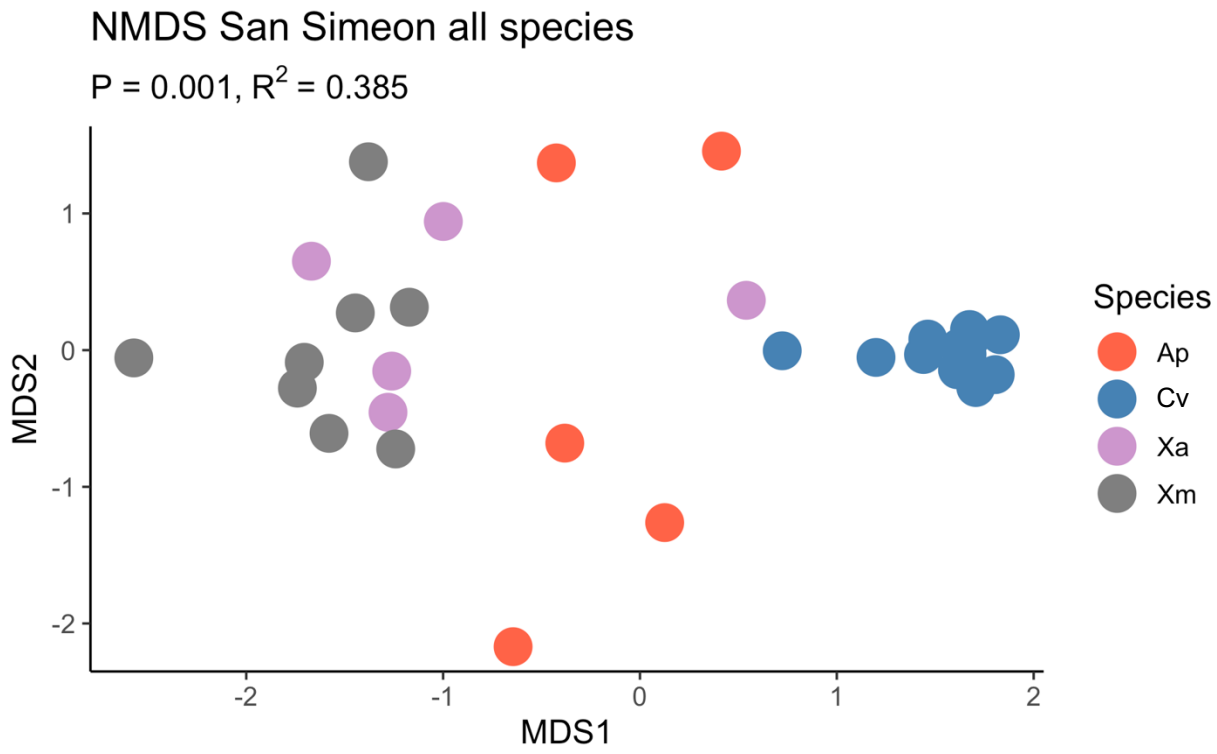
Appendix 3.4 Figure 1 Stacked bar plot for intestinal tissue of relative frequency of microbial communities at the Family level. The key depicts the top ten abundant microbial Families.



Appendix 3.4 Figure 2 Stacked bar plot for gut contents of relative frequency of microbial communities at the Family level. The key depicts the top ten abundant microbial Families.

Appendix and Supplementary Information for Chapter 4

Digestive physiology and individual variation impact the hindgut microbiome of prickleback fishes (Stichaeidae) with different diets



Appendix 4.1 NMDS plot of all host species from San Simeon, CA. Ap: *A. purpurescens*; Cv: *Cebidichthys violaceus*; Xa: *X. atropurpureus*; and Xm: *Xiphister mucosus*