Comparative Biochemistry and Physiology, Part A 285 (2023) 111508

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

When digestive physiology doesn't match "diet": *Lumpenus sagitta* (Stichaeidae) is an "omnivore" with a carnivorous gut

Daniel R. Rankins *, Michelle J. Herrera, Michelle P. Christensen, Alisa Chen, Newton Z. Hood, Joseph Heras [1], Donovan P. German

Department of Ecology and Evolutionary Biology, University of California, Irvine, 321 Steinhaus Hall, Irvine, CA 92697-2525, USA

ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Digestion
Microbiome
Transcriptomics
Gut
Intestine
Prickleback fish
Physiology

ABSTRACT

What an animal ingests and what it digests can be different. Thus, we examined the nutritional physiology of *Lumpenus sagitta*, a member of the family Stichaeidae, to better understand whether it could digest algal components like its better studied algivorous relatives. Although *L. sagitta* ingests considerable algal content, we found little evidence of algal digestion. This fish species has a short gut that doesn't show positive allometry with body size, low amylolytic activity that actually decreases as the fish grow, no ontogenetic changes in digestive enzyme gene expression, elevated *N*-acetyl-glucosaminidase activity (indicative of chitin breakdown), and an enteric microbial community that is consistent with carnivory and differs from members of its family that consume and digest algae. Hence, we are left concluding that *L. sagitta* is not capable of digesting the algae it consumes, and instead, are likely targeting epibionts on the algae itself, and other invertebrates consumed with the algae. Our study expands the coverage of dietary and digestive information for the family Stichaeidae, which is becoming a model for fish digestive physiology and genomics, and shows the power of moving beyond gut content analyses to better understand what an animal can actually digest and use metabolically.

1. Introduction

The vertebrate digestive system is dynamic, responding to a variety of pH, osmotic, and nutrient content challenges on a daily basis (Karasov and Douglas, 2013; Karasov and Martínez del Rio, 2007). With the term "gut" defined as the entire gastrointestinal tract (mouth to anus), diet is the factor that has the most influence on the gut, affecting gene expression, gut size, digestive enzyme activities, nutrient transport rates, mucosal surface area, and enteric microbial diversity (Baldo et al., 2017; Baldo et al., 2023; Davis et al., 2013; Egerton et al., 2018; German and Horn, 2006; German et al., 2004; German et al., 2015; Herrera et al., 2022; Horn, 1989; Karasov and Hume, 1997; Kramer and Bryant, 1995b; Leigh et al., 2022; Leigh et al., 2018a; Leigh et al., 2018b; Skea et al., 2005, 2007; Stevenson et al., 2022; Wagner et al., 2009; Sparagon et al., 2022). Although there are a growing number of studies on fish digestive systems (e.g., Castro-Ruiz et al., 2021; Clements et al., 2017; Crossman et al., 2005; German, 2011; Sabapathy and Teo, 1993; Tengjaroenkul et al., 2000), there are more studies of gut structure and function in ecological and evolutionary contexts for terrestrial vertebrate animals (Choat and Clements, 1998; Clements et al., 2017; Karasov and Martínez del Rio, 2007; Nie et al., 2019; Stevens and Hume, 1995). Thus, there is a crucial need to better understand fish nutritional ecology in a changing world, particularly in ecological and evolutionary contexts (e.g., Clements et al., 2017; Leigh et al., 2018a; Leigh et al., 2018b; Clark et al., 2023), which may then aid efforts in fisheries management and aquaculture.

One of the largest disconnects in the fish nutritional ecology literature is between what a fish ingests and what it actually digests and assimilates (Clements et al., 2017; Karasov and Douglas, 2013; Raubenheimer et al., 2005). With aquaculture (albeit on a limited number of mostly carnivorous species) as the exception, there are numerous studies of fishes focusing on bite rates on specific resources (e.g., Duran et al., 2019), or on gut content analyses (e.g., Choat et al., 2002), with comparatively fewer studies examining what the fish's digestive system can actually digest, and therefore, what fuels may be available to the fish metabolically (Clements et al., 2017; Crossman et al., 2005; Nie et al., 2019). This has led to assumptions about the trophic roles of fishes that may be incorrect. For instance, parrotfishes

* Corresponding author.
E-mail address: rankinsd@uci.edu (D.R. Rankins).
[1] Current address: Department of Biology, California State University, San Bernardino, CA 92407 USA.

https://doi.org/10.1016/j.cbpa.2023.111508
Received 31 March 2023; Received in revised form 21 August 2023; Accepted 21 August 2023
Available online 23 August 2023

(Labridae: Scarinae) were thought to ingest and digest macroalgae from the reef surface based on feeding observations, whereas their nutritional physiology (Crossman et al., 2005; Clements et al., 2017) based on multiple lines of evidence shows that many species are microphagous, targeting microscopic photoautotrophs to meet their nutritional needs (Clements et al., 2017; Nicholson and Clements, 2020; Nicholson and Clements, 2021). Similarly, wood-eating catfishes (Loricariidae) were assumed to be able to digest wood (Nelson et al., 1999; Schaefer and Stewart, 1993) until more detailed analyses of their gut structure and function showed that they could digest very little from wood itself, and instead, are reliant on the microbial biomass degrading the wood as their food source (German, 2009; German and Bittong, 2009; German and Miles, 2010; German et al., 2010b; Lujan et al., 2011; McCauley et al., 2020). Furthermore, bonnethead sharks, which incidentally consume considerable amounts of seagrass (Bethea et al., 2011; Bethea et al., 2007), were assumed to not digest seagrass because shark guts aren't optimized to digest plant material. Analysis of the bonnethead digestive system, however, showed that they could indeed digest and assimilate seagrass molecules (Leigh et al., 2018b, 2021). Hence, it is crucial to study the nutritional physiology of fishes (including their digestive physiology) to discern what they are capable of digesting from what they ingest.

The family Stichaeidae has become a model system for understanding fish digestion (Fig. 1). With dietary diversity, ontogenetic dietary shifts, sister taxa with different diets, convergent evolution of herbivory, and many sympatric species with different diets, there are ample opportunities to investigate how the fish gut responds to different nutrient loads, and any adaptations that have arisen allowing for dietary specialization within stichaeid fishes (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). The herbivores appear to be specialists, digesting algae with high efficiency (Fris and Horn, 1993; Horn et al., 1986). The herbivores and omnivores have elevated amylolytic activity in their guts, and one of the carnivores (*A. purpurescens*) has elevated aminopeptidase activity in its gut (German et al., 2016; German et al., 2014; German et al., 2004; German et al., 2015). The omnivorous *Phytichthys chirus* has elevated chitinolytic activity in its gut reflecting its intake of crustaceans (German et al., 2015). Some of the herbivores show increased gene copy number for amylase (German et al., 2016; Heras et al., 2020) underpinning elevated amylolytic activity and greater algal starch digestion. One of the herbivores (*Cebidichthys violaceus*) has active hindgut microbial fermentation aiding in algal digestion, whereas the other herbivore (*Xiphister mucosus*) does not (German et al., 2015), although the level of microbial fermentation in *C. violaceus* is still lower than many other herbivorous fishes that are known to be reliant on microbial fermentation to subsist on algal diets (e.g., Clements and Choat, 1995; Clements et al., 2017). Gut size shows the typical relationship with intake: those fishes consuming more plant material have higher intake, and thus, a longer gut to allow for more efficient digestion (Fig. 1; German et al., 2014;

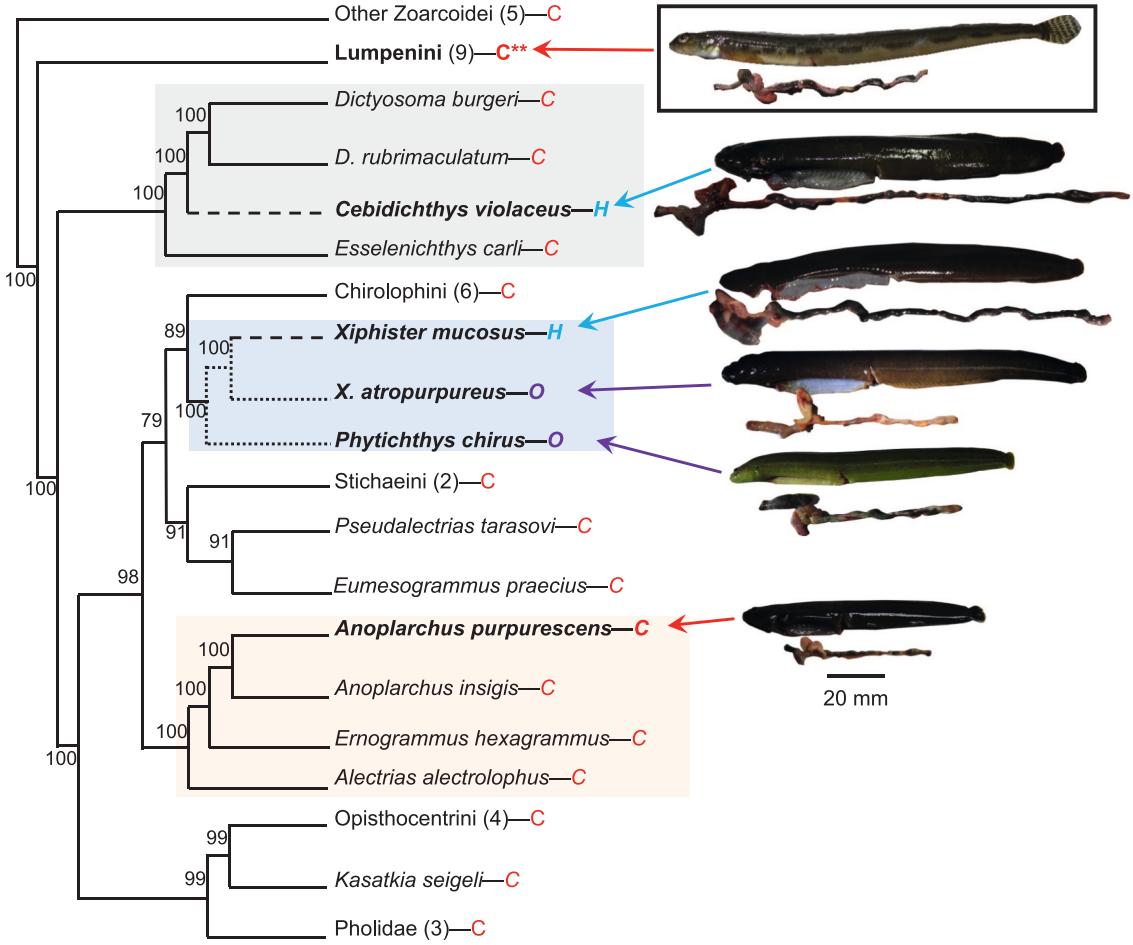

Fig. 1. Phylogenetic relationships of the polyphyletic family Stichaeidae based on 2100 bp of *cytb*, *16S*, and *tomo4c4* genes (Kim et al., 2014). Bayesian posterior probabilities are indicated on nodes. Studied taxa are bolded, and photos are shown with their digestive systems beneath their bodies. Note the differences in gut size. **H** = herbivory, **O** = omnivory, **C** = carnivory. *Lumpenus sagitta* is boxed. Evolution of herbivory (— — — —) and omnivory (.....) are shown. Numbers in parentheses show number of taxa evaluated at that branch. Shading highlights those clades within the family Stichaeidae that showed intertidal invasion where most species are intertidal. Remaining clades (including *L. sagitta*) are subtidal.

German and Horn, 2006; Herrera et al., 2022). Moreover, stichaeid species showing ontogenetic dietary shifts have guts that get disproportionately longer as the fish grow, whereas those species that are carnivorous throughout life (e.g., *Anoplarchus purpurescens*) do not show positive allometry of gut size with body size (German et al., 2014; German and Horn, 2006). Up to this point, all of the studies of stichaeid digestion have been focused on intertidal species since these groups show the most dietary diversity, even though the majority of the family is subtidal and mostly carnivorous (Fig. 1; German et al., 2015; Kim et al., 2014). Thus, the point of this study is to expand the coverage of stichaeid species for studies of digestion to include a subtidal species, *Lumpenus sagitta*. This species may consume a considerable amount of algae in some locations (Tresierra-Aguilar, 1980), although stable isotopic data show that species of *Lumpenus* are more enriched in $\delta^{15}N$, suggesting carnivorous protein sources (Hindell et al., 2012; Tamelander et al., 2006). There is no information on how the diet of *L. sagitta* may change as the fish grows, nor what their gut may be capable of digesting. Therefore, we collected a range of sizes of this species to examine their diet and digestive physiology to compare them with other, well-studied stichaeid species.

In *L. sagitta* we examined stomach contents, gut length, digestive enzyme activities of the stomach and intestine, the gene expression (transcriptomics) of the pyloric caeca, and the microbial diversity of their distal intestines. We outline our hypotheses in Table 1. For diet, gut size, and digestive enzyme activities, we examined how these changed as the fish grew. If *L. sagitta* consumes more algae as they grow, as occurs in other stichaeid fishes that consume algae as adults (i.e., *C. violaceus*, *P. chirus*, *X. mucosus*, *X. atropurpureus*; German et al., 2014; German and Horn, 2006; German et al., 2004), then they should show the concomitant positive allometry of gut size relative to body size (German et al., 2014), and ontogenetic changes in digestive enzyme activities, particularly an increase in amylase activity, and a decrease in aminopeptidase activity (German et al., 2014; German et al., 2004; German et al., 2015). Chitinolytic activities are low in most of the herbivores or omnivores (German et al., 2015). Although we have examined dietary impacts on gut transcriptomics in stichaeid fishes (Herrera et al., 2022), we have not done so in an ontogenetic manner (outside of specific genes; Gawlicka and Horn, 2006; Kim et al., 2014). Hence, our transcriptomic analyses are more exploratory in nature to observe what gene expression may change as *L. sagitta* grows, and whether gene expression patterns may match some of the digestive enzyme activity data we gather (Herrera et al., 2022). Finally, if *L. sagitta* is omnivorous/herbivorous as it gets larger, we would expect the distal intestine microbial diversity of this species to roughly resemble that of the other stichaeid species (perhaps to the family or genus level; Baldo et al., 2017) that consume considerable algal content. Overall, this broad suite of traits should provide insight into whether this subtidal stichaeid species can indeed digest algae like its more well-studied relatives that reside in the intertidal zone, and may expand the dietary, and digestive diversity of fishes in the family. Moreover, our investigation further shows the importance of measuring aspects of digestive physiology to discern what an animal can digest as opposed to only studying gut contents.

2. Materials and methods

2.1. Fish capture and tissue preparation

Thirteen individuals of *Lumpenus sagitta* were collected by beach seine at Jackson Beach, San Juan Island, Washington, USA (48.520 N, 123.011 W) in June 2016. The fish ranged in size from 79 to 213 mm standard length (average ± standard deviation: 134.85 ± 50.14 mm), which ranges from juvenile to adult (Tresierra-Aguilar, 1980). The fish were transported live in seawater to Friday Harbor Laboratories (Friday Harbor, WA) where they were dissected within three hours of capture. 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). 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 esophageal sphincter to the distal-most end of the intestine. The measured guts 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 caeca were excised. The intestine was divided into two sections of equal length and the sections were designated as the proximal or distal intestine (German et al., 2015; Herrera et al., 2022). The contents of the stomach and intestine were emptied into their own vials and the tissues were rinsed with ice cold 25 mM Tris HCl, pH 7.5, to ensure no digesta remained adhered to the gut tissue. From four individuals (standard lengths of 165, 117, 86, and 83 mm), approximately 100 mg of the pyloric caeca 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. The remaining portions of the tissues, stomach, and intestinal contents were frozen on dry ice and transferred to −80 °C freezer for storage for digestive enzyme activity assays, and other uses.

2.2. RNA isolation and library preparation

Approximately four months after capture, total RNA from the pyloric caecal samples (20-50 mg) from the four individual fish of varying lengths were isolated using TRIzol reagent (Thermo Fisher Scientific) following the manufacture's protocol. Samples were quantified (ng/μl) using an RNA Nanodrop and RNA quality was determined by Bioanalyzer (RNA Integrity >7) at the UC Irvine Genomics Research and Technology Hub. 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 run in a single lane, in a single paired-end 100 bp run on a HiSeq 2500 (Illumina, San Diego, CA) by the UCI Genomics Research and Technology Hub. All transcriptomic data generated were deposited into NCBI Archive with accession number PRJNA947617.

Table 1
Predictions for digestive system features in *Lumpenus sagitta* if this species digests algae like other herbivorous or omnivorous stichaeid fishes. Included are predictions for potential ontogenetic changes, and in comparison to other stichaeid species that are known to consume and digest algae.

Analysis	% Dietary algae	Gut length	Transcriptomics	Microbiome	Amylolytic	Chitinolytic	Proteolytic	Lipolytic
Ontogenetic	Increase	Positive allometry	Increased expression of genes involved in carbohydrate digestion/metabolism; Decreased expression of genes involved in protein digestion/metabolism	N/A	Increase	Decrease	Decrease	Increase
Comparative	Elevated intake	Longer	N/A	Similarity with herbivores/ omnivores	Elevated activity	Low activity	Low activity	Elevated activity

2.3. Assembly of sequence reads and relative expression levels

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). A *de-novo* assembly using Trinity v r2015–2.1.1 was conducted, in where we selected the largest individual (165 mm SL) as the reference assembly.

2.4. Annotation of genes

Annotation was conducted with Trinotate v3.0.0 annotation suite for genes under differential expression, the full transcripts of the 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.

2.5. Ontogenetic differential expression analysis

A *de-novo* assembly using Trinity v r2015–2.1.1 was conducted, for which we selected the largest *L. sagitta* individual (165 mm SL) 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). Relative expression levels of all genes expressed in the pyloric caeca 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 different sizes. Then, we generated heatmaps using EdgeR (Bioconductor v3.2) with a false discovery rate (FDR) of 0.2 and a dispersion value of 0.4. We chose a high FDR cutoff (typically <0.001; e.g., Herrera et al., 2022) because there were zero Differentially Expressed Genes (DEGs) until we hit an FDR of 0.2, at which there were three DEGs. There were more DEGs at an FDR of 0.5 (see Supplemental Fig. S2), but this is such a high cutoff for FDR it is meaningless in terms of actual DEGs having biological significance.

2.6. Gut microbiome analysis

Sample DNA was isolated from the distal intestine tissues and contents of the same four *L. sagitta* (165, 117, 86, and 83 mm SL) used for the transcriptomic analyses. These data were compared to the microbiomes isolated from the distal intestine tissues and contents of wild-caught *X. mucosus*, *X. atropurpureus*, *P. chirus*, and *A. purpurescens* collected from Deadman's Bay, San Juan Island, Washington, USA (48.510° N, 123.140° W; Herrera et al., 2022) within two weeks of the *L. sagitta* collection, in June 2016. 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) (Caporaso et al., 2012; Walters et al., 2016) using the Earth Microbiome Project primers (515F [barcoded] and 926R; Caporaso et al., 2012; Walters et al., 2016). Using MiSeq v3 chemistry (PE300 sequencing length), the libraries were sequenced at the UC Irvine Genomics Research and Technology Hub. This resulted in 17.4 M reads passing filter (21% of that is phiX) with an overall >Q30 80.4%. 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). 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., 2012). 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. To determine which microbial taxa were driving differences among the fish host species, we ran indicator species analysis, which shows which microbial taxa are uniquely associated with particular fish host species (De Cáceres et al., 2012). Furthermore, to observe which 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 among 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. All microbiome data generated were deposited into NCBI Archive with accession number PRJNA949661.

2.7. Tissue homogenates, stomach content analyses, and digestive enzyme activity measurements

Gut tissues from each gut region from individual fish were weighed and homogenized following German et al. (2015). Intestinal tissues were homogenized in 25 mM Tris-HCl, pH 7.5, whereas stomach tissue was homogenized in 100 mM citric acid-sodium citrate buffer, pH 5.0. The supernatants of homogenates were collected and stored in small aliquots (100–200 μl) at –80 °C until just before use in spectrophotometric or fluorometric assays of digestive enzyme activities.

Stomach contents were analyzed from each *L. sagitta* specimen following Boyle and Horn (2006). Contents were defrosted, removed from the centrifuge vial in which they were stored (frozen) and placed into a petri dish filled with deionized water. Under a dissecting microscope (AmScope, Irvine, CA) contents were separated into taxonomic groups. Algae and invertebrates were separated by species (where possible), and prey items were damp-dried and weighed to the nearest 0.001 g. Diets were quantified using prey biomass rather than number of individual prey items to allow a direct comparison between the different individuals. Following German et al. (2004), the *L. sagitta* diet was condensed to the average percent algal or animal material in their stomachs for qualitative comparison with other prickleback species (German et al., 2015).

All digestive enzyme 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). 15 °C was chosen because this is towards the upper range of the temperatures experienced in the Salish Sea during the summer months (Crummett, 2020), and was also the temperature used for enzyme assays in our previous investigation (German et al., 2015), which we used as a source for comparative data in the present study on *Lumpenus sagitta*. General elements of each enzyme assay (substrate, pH, citation) are presented in

Table 2
Summary of enzyme assay methods used in this study of digestive enzyme activities in prickleback fishes.

Enzyme	Nutrient target	Substrate	Substrate concentration	Method citation
Gastric enzymes[a]				
Pepsin	Protein	Hemoglobin	2% (mass/volume)	Anson, 1938, German et al., 2004
Chitinase	Carbohydrate	Chitin	5 mg/ml	Jeuniaux, 1966, German and Bittong, 2009
Intestinal and pancreatic enzymes[b]				
Amylase	Carbohydrate	Potato Starch	1% (mass/volume)	German et al., 2004
Maltase	Carbohydrate	Maltose	56 mM	German and Bittong, 2009, Dahlqvist, 1968
NAGase[†]	Carbohydrate	MUB-NAG[*]	0.2 mM	German et al., 2011
Trypsin	Protein	BAPNA[*]	2 mM	Erlanger et al., 1961, German et al., 2004
Aminopeptidase	Protein	APNA[*]	2.04 mM	German and Bittong, 2009
Carboxyl ester lipase	Lipids	4NP-Myristate[*]	0.55 mM	German and Bittong, 2009

[a] Pepsin assays were run at pH 2, Chitinase at pH 4.5.
[b] All intestinal and pancreatic enzyme activities were measured at pH 7.5.
[†] NAGase: *N*-acetyl-β-D-glucosaminidase.
[*] MUB-NAG: 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide; BAPNA: Nα-benzoyl-L-arginine-p-nitroanilide hydrochloride; APNA: L-alanine-p-nitroanilide HCl; 4NP-Myristate: 4-nitrophenyl myristate.

Table 2. All assay protocols generally followed methods detailed in German and Bittong (2009), as described in German et al. (2015). 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. Each enzyme assay included blanks consisting of substrate only and homogenate only (in buffer) to simultaneously account for endogenous substrate and/or product in the tissue homogenates and substrate solutions.

2.7.1. Gastric enzyme assays

Pepsin activity was expressed in U (μmol of L-tyrosine liberated per minute) per gram wet weight of gut tissue based on a L-tyrosine standard curve.

Chitinase activities were measured as the amount of *N*-acetyl-β-D-glucosamine (NAG) released from chitin hydrolysis, and quantified following the method of Reissig et al. (1955). The chitinase activity was determined from a NAG standard curve and expressed in U (1 μmol NAG liberated per min) per gram wet weight of gut tissue.

2.7.2. Pancreatic and intestinal enzyme assays

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.

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.

N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol product released per minute per gram wet weight tissue) was calculated from the methylumbelliferone (MUB) standard curve.

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 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.

Carboxyl ester 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.

2.8. Statistical analyses

Correlation analysis was used to determine how percent algal material in the diet and the activity of each of the seven digestive enzymes varied with SL in *L. sagitta*. Correlations (Spearman's rank correlation coefficient, ρ) were employed to examine the relationship with size. Gut allometry was examined in *L. sagitta*, *X. mucosus*, *X. atropurpureus*, *P. chirus*, *C. violaceus*, and *A. purpurescens* using linear regression contrasting relative gut length with SL. Data from the other species came from German et al. (2014). Slopes of regression lines were compared using ANCOVA followed by Tukey's multiple comparisons test with $P = 0.05$. To contrast digestive enzyme activity levels of *L. sagitta* with other prickleback species, we used ANOVA, followed by a Tukey's HSD, to compare the digestive enzyme activities of the stomach, pyloric caeca, proximal intestine, and distal intestines individually among *L. sagitta*, *X. mucosus*, *X. atropurpureus*, *P. chirus*, *C. violaceus*, and *A. purpurescens* (using data from German et al., 2015 for the intestinal enzyme data for species other than *L. sagitta*). Note that the herbivorous *C. violaceus* wasn't included in the microbiome analyses because this fish species is not found in Washington, like the other species. Thus, for the microbiome analyses, we limited the fish taxa to those that are sympatric with *L. sagitta*. Prior to all significance tests, Levene's and Bartlett's tests were performed to ensure the appropriateness of the data for parametric analyses. Where necessary, data were transformed using a Box-Cox transformation to meet the assumptions of ANOVA. Some enzyme activities (aminopeptidase and trypsin) still didn't meet requirements for parametric tests and thus were analyzed with Kruskal-Wallis tests followed by Pairwise Wilcoxon Rank Sum tests for multiple comparisons with a Bonferoni correction. All statistics were run in R (version 4.1.2).

3. Results

3.1. Pyloric caeca transcriptomics

Astonishingly, there were no ontogenetic patterns in gene expression in the pyloric caeca of *L. sagitta* (Supplemental Fig. S1). We examined the pyloric caecal gene expression in four individuals ranging in size from 83 to 165 mm SL and observed only three DEGs with a FDR cutoff of 0.2 because we could not detect any DEGs below this cutoff. The three alleged DEGs are Compliment Receptor 1, Signal Recognition Particle 54, and an uncharacterized protein (Supplemental Fig. S1). However, the only pattern is driven by relatively low expression of these genes in the *L. sagitta* individual that was 117 mm SL, and thus, even these three genes don't show an ontogenetic pattern. Thus, the pyloric caecal gene expression patterns were nearly identical among the four individuals.

3.2. Gut length

Each of the four prickleback species that are known to consume considerable amounts of algae (*C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *P. chirus*) in their diets showed a positive relationship between relative gut length and body length, showing that as their bodies get larger, their guts get disproportionately longer, whereas *A. purpurescens*

(carnivore) and *L. sagitta* showed little relationship between these variables (Table 3; Fig. 2). The magnitude of these relationships (i.e., slopes) varied according to diet, with the two herbivores, *C. violaceus* and *X. mucosus*, having statistically significantly steeper slopes between gut length and body length than all of the other species, and *C. violaceus* had a steeper slope than *X. mucosus* (Table 3). The omnivorous *X. atropurpureus* and *P. chirus* had intermediate slopes between gut length and standard length, and differed from each other, but were statistically different from the herbivores and carnivores. The carnivorous *A. purpurescens* and *L. sagitta* showed no significant relationship between gut size and body size, and had slopes that differed from one another and all other species except *P. chirus* (Table 3; Fig. 2).

3.3. Diet and digestive enzyme activities

Algae (mostly *Ulva* sp. with some Rhodophytes occasionally) was the dominant item by mass in most of the *L. sagitta* stomachs, composing between 5.66 and 100% of stomach material by mass (Table 4). The remainder was crustaceans, polychaete worms, mollusks, and some detritus. There was no correlation of dietary algal content with standard length ($P = 0.48$; Table 4). Three digestive enzymes showed some correlation between activity level and body size in *Lumpenus sagitta*. Aminopeptidase showed a negative correlation, decreasing in activity in each of the proximal and distal intestine of the fish as they grew (Table 4). Similarly, amylase activities showed a negative correlation with body size, but only for the proximal intestine. Lipase activities showed differing relationships with body size depending on gut region, with the proximal intestine activities having a significantly positive correlation with standard length, whereas the distal intestine correlation was negative among these variables (Table 4). No other enzymes showed any relationship of activity level with body size in *L. sagitta*.

All enzyme activity data, including the statistical analyses, can be found in Supplemental Tables S1 (gastric enzymes) and S2 (intestinal enzymes) in the online version of this manuscript. For the gastric enzymes, pepsin activity was significantly higher in the omnivorous *P. chirus* and the carnivorous *A. purpurescens* and *L. sagitta* than in the other omnivorous and herbivorous species, although *X. atropurpureus* pepsin activity was not statistically different from that of *L. sagitta* ($F_{5,40} = 30.11$ $P = 0.01$; Fig. 3). For chitinase, the omnivorous *P. chirus* and carnivorous *A. purpurescens* had had significantly higher activities than the other species ($F_{5,28} = 16.73$; $P = 0.01$; Fig. 3). The omnivorous *X. atropurpureus* and *L. sagitta* had activities that were not different from each other, but higher than the herbivorous species, although the chitinase activity of *L. sagitta* wasn't statistically distinguishable from the herbivores (Fig. 3).

For this section, we will report the most interesting comparisons for the digestive enzyme activities among the species for the proximal intestine, which is the intestinal region with the highest enzyme activities in many of the species (German et al., 2015). Aminopeptidase activities showed strong dietary affinity with the carnivorous *A. purpurescens* having significantly higher activity than all other species, followed by the omnivorous *P. chirus* and *X. atropurpureus* ($F_{5,36} = 25.42$; $P = 0.01$; Fig. 4). The two herbivorous species (*C. violaceus* and *X. mucosus*) had the lowest aminopeptidase activities, and were not different from *L. sagitta* in activity level for this enzyme (Fig. 4). Amylase activity in

Table 3
Regression statistics of relative gut length (RGL) contrasted with SL for six species of prickleback fishes.

Species	Equation of the line	r^2	*P*	Slope comparison
Cebidichthys violaceus (H)	y = 0.0061× + 0.549	0.917	<0.001	e
Xiphister mucosus (H)	y = 0.0056× + 0.377	0.936	<0.001	d
Xiphister atropurpureus (O)	y = 0.0025× + 0.490	0.804	<0.001	c
Phytichthys chirus (O)	y = 0.0021× + 0.410	0.487	0.019	ab
Anoplarchus purpurscens (C)	y = 0.0004× + 0.526	0.017	0.657	b
Lumpenus sagitta (C)	y = −0.0001× + 0.769	0.224	0.071	a

P values indicate the significance of the relationship between relative gut length and body length. Slopes were compared among species for each contrast with ANCOVA followed by a Tukey's multiple comparisons test. Significance was set at $P = 0.05$. Slopes that share a letter are not significantly different. Sample sizes are as follows, *X. mucosus* ($n = 68$), *X. atropurpureus* ($n = 73$), *P. chirus* ($n = 36$), *C. violaceus* ($n = 72$), *A. purpurescens* ($n = 84$), and *L. sagitta* ($n = 13$). H = herbivore, O = omnivore, C = carnivore. Data from species other than *L. sagitta* from German et al. (2014).

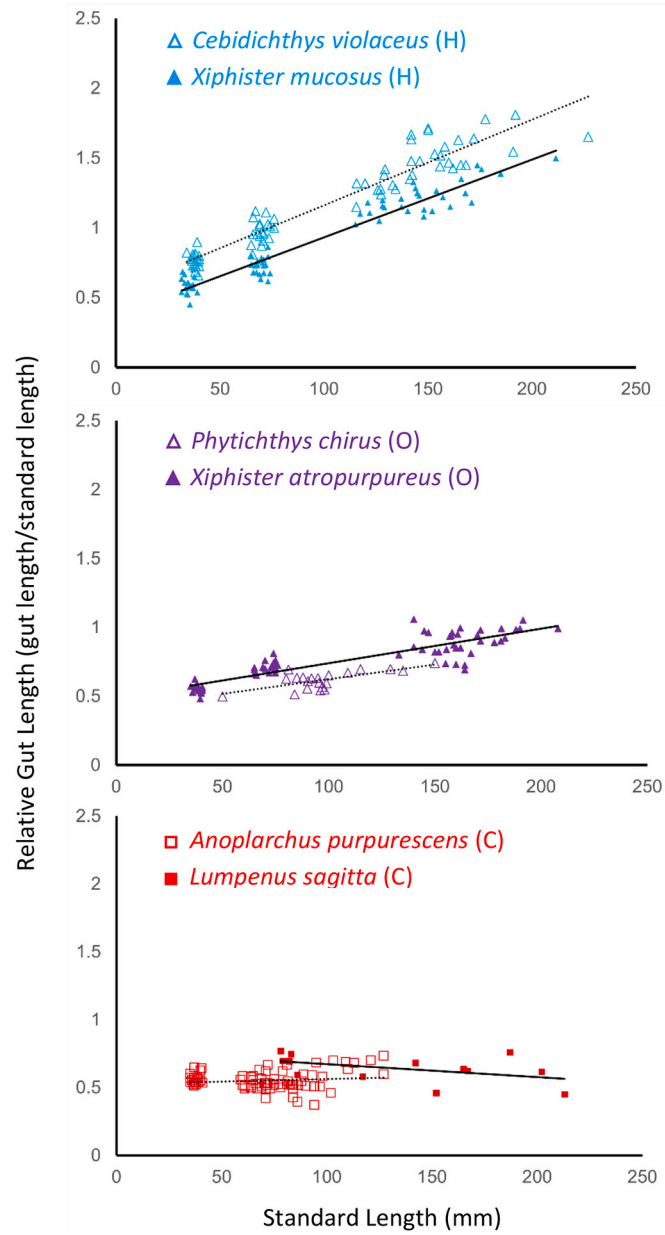

Fig. 2. Regressions of relative gut length {gut length (mm) x standard length $(mm)^{-1}$} as a function of standard length. Plots are broken up by diet, with herbivores (top), omnivores (middle), and carnivores (bottom) in different plots. Phylogenetic relationships shown in Fig. 1. Regression statistics can be found in Table 2. **H** = herbivory, **O** = omnivory, and **C** = carnivory. Data on species other than *Lumpenus sagitta* from German et al. (2014).

Table 4
Percent algae in the diet and digestive enzyme activities (U * g gut tissue $^{-1}$), and the correlation of each with standard length (SL) in *Lumpenus sagitta* for each gut region.

Enzyme	Range	ρ	*P*
% dietary algae	5.66–100.00	−0.29	0.48
Pepsin (stomach)	6.24–17.72	0.35	0.36
Chitinase (stomach)	0.11–0.74	0.37	0.33
Aminopeptidase PC	0.17–0.51	−0.50	0.45
Aminopeptidase PI	0.79–8.75	−0.64	**0.05**
Aminopeptidase DI	0.30–0.80	−0.79	**0.01**
Trypsin PC	0.08–0.59	−0.90	0.08
Trypsin PI	0.19–1.06	−0.60	0.07
Trypsin DI	0.11–2.00	−0.60	0.07
Amylase PC	1.43–4.69	−0.40	0.52
Amylase PI	3.21–48.79	−0.64	**0.05**
Amylase DI	N.D. - 4.61	0.58	0.58
Maltase PC	0.39–0.97	−0.30	0.68
Maltase PI	0.24–5.96	−0.08	0.84
Maltase DI	0.12–2.41	−0.73	0.02
NAGase PC	2.21–5.94	−0.70	0.23
NAGase PI	1.92–10.84	−0.01	1.00
NAGase DI	1.48–23.67	−0.32	0.41
Lipase PC	0.81–22.86	−0.60	0.35
Lipase PI	1.09–2.10	0.02	**0.02**
Lipase DI	0.07–5.79	−0.79	**0.01**

PC = pyloric caeca, PI = proximal intestine, DI = distal intestine.

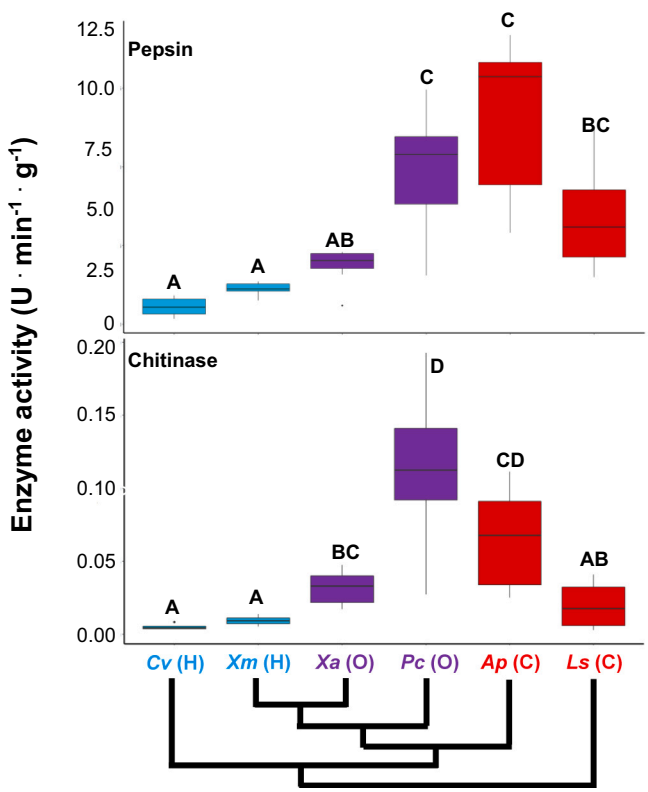

Fig. 3. Pepsin (top), and chitinase (bottom) activities in the stomachs of *Cebidichthys violaceus* (***Cv***), *Xiphister mucosus* (***Xm***), *Xiphister atropurpureus* (***Xa***), *Phytichthys chirus* (***Pc***), *Anoplarchus purpurescens* (***Ap***), and *Lumpenus sagitta* (***Ls***). Enzyme activities are International μmol product produced per minute, per gram of tissue. Box plots represent median values (bolded line in each box) with lower and upper quartiles bounding each box. Error bars are 95% confidence intervals and remaining dots are outliers. $n = 6$ for ***Cv***, ***Xm***, ***Xa***, and ***Ap***; $n = 9$ for ***Pc***; $n = 12$ for ***Ls***. **Herbivory (H)**, **Omnivory (O)**, and **Carnivory (C)** indicated accordingly. Interspecific comparisons made with ANOVA followed by Tukey's HSD for each enzyme individually. Boxes not sharing a letter are significantly different from each other for a given enzyme. Truncated phylogeny provided to view relatedness of studied taxa.

X. atropurpureus was significantly higher than all other species, except its sister taxon, the herbivorous *X. mucosus* ($F_{5,36} = 50.9$; $P = 0.01$; Fig. 4). In turn, amylase activity in *X. mucosus* was not different from that of *C. violaceus*. These highest amylase activities were all significantly higher than those in *P. chirus*, *A. purpurescens*, and *L. sagitta* (Fig. 4). For NAGase, *L. sagitta* had activity levels that were significantly higher than, and an order of magnitude higher than, all other species, which didn't differ from each other ($F_{5,36} = 31.38$; $P = 0.01$; Fig. 4).

3.4. Distal intestine microbial community analyses

Alpha diversity did not differ among the distal intestine content microbial communities found in *X. mucosus*, *X. atropurpureus*, *P. chirus*. *A. purpurescens*, and *L. sagitta* (Supplemental Fig. S3). Based on the PERMANOVA of the intestinal content community beta diversity ($P = 0.007$; $R^2 = 0.329$), *L. sagitta* had significantly different communities from *A. purpurescens* ($P = 0.066$) and *P. chirus* ($P = 0.033$), and in turn, *P. chirus* was different from both *Xiphister* taxa (vs *X. mucosus*, $P = 0.027$; vs *X. atropurpureus*, $P = 0.031$), and *A. purpurescens* ($P = 0.054$; Fig. 5). *L. sagitta* was not different from the two *Xiphister* taxa (vs *X. mucosus*, $P = 0.107$; vs *X. atropurpureus* $P = 0.106$), and the two *Xiphister* species were not different from each other ($P = 0.677$). Twenty nine bacterial taxa represented indicator species for *L. sagitta*, and were from six phyla of Campylobacterota, Pseudomonodota, Chloroflexota, Actinobacteriota, Bacillota, and Planctomycetota (Supplemental Table S3). Of those 29, nine are in the Pirellulaceae family within the Planctomycetota, and seven are in the Pseudomonodota representing three different families. Six were in the Chloroflexota, whereas the remainder were spread among the other phyla. *Phytichthys chirus* had 18 species with indicator status, almost entirely in the Pseudomonodota (Supplemental Table S3). The other fish taxa had four or fewer indicator species. Biplot analysis to determine significant taxa that are driving the variation in the non-metric multidimensional scaling plot similarly points to Pseudomonodota as explaining much of what separates *L. sagitta* and *P. chirus* from the other species in terms of the gut content microbiome (Supplemental Fig. S4).

The alpha diversity of the intestinal tissue microbial community did not vary significantly among the different species (Supplemental Fig. S5). The PERMANOVA ($P = 0.111$; $R^2 = 0.257$) suggested that none of the species differed statistically in terms of beta diversity among the intestinal tissue microbiome (Fig. 5). The vector analysis applied to the MDS plot for the intestinal tissue community pointed to several taxa within the Pseudomonodota and Bacillota as being important for *L. sagitta* (Supplemental Fig. S6). For the intestinal tissue community, a taxon in the genus *Vogesella*, which is part of Gammaproteobacteria within the Pseudomonodota, is explanatory for *L. sagitta* and *X. atropurpureus* (Supplemental Table S4). Perhaps the most interesting aspect of the analysis is that the intestinal tissue and contents have different bacterial communities, as is apparent in the MDS plot (Fig. 5). For each species, there was a statistical difference between the bacterial communities found in the intestinal tissue as compared to the intestinal contents: *A purpurescens* ($F_{1,9} = 2.97$, $P = 0.011$), *L. sagitta* ($F_{1,6} = 2.26$, $P = 0.032$), *P. chirus* ($F_{1,7} = 2.25$, $P = 0.034$), *X. atropurpureus* ($F_{1,7} = 3.25$, $P = 0.033$), *X. mucosus* ($F_{1,7} = 2.95$, $P = 0.025$). A core microbiota analysis revealed 17 core taxa shared among all five prickleback species, and 14 of those 17 are part of the Pseudomonodota (Supplemental Table S5). A stacked taxa barplot is shared as Supplemental Fig. S7, and the complete list of taxa and abundance levels is Supplemental Table S6.

4. Discussion

Lumpenus sagitta is known to consume considerable algal content in their diet (Tresierra-Aguilar, 1980). Hence, we collected a range of sizes of this species and made predictions about their gut structure and function relative to other well-studied carnivorous, omnivorous, and herbivorous fish species in the same family, Stichaeidae (Table 1; Fig. 1).

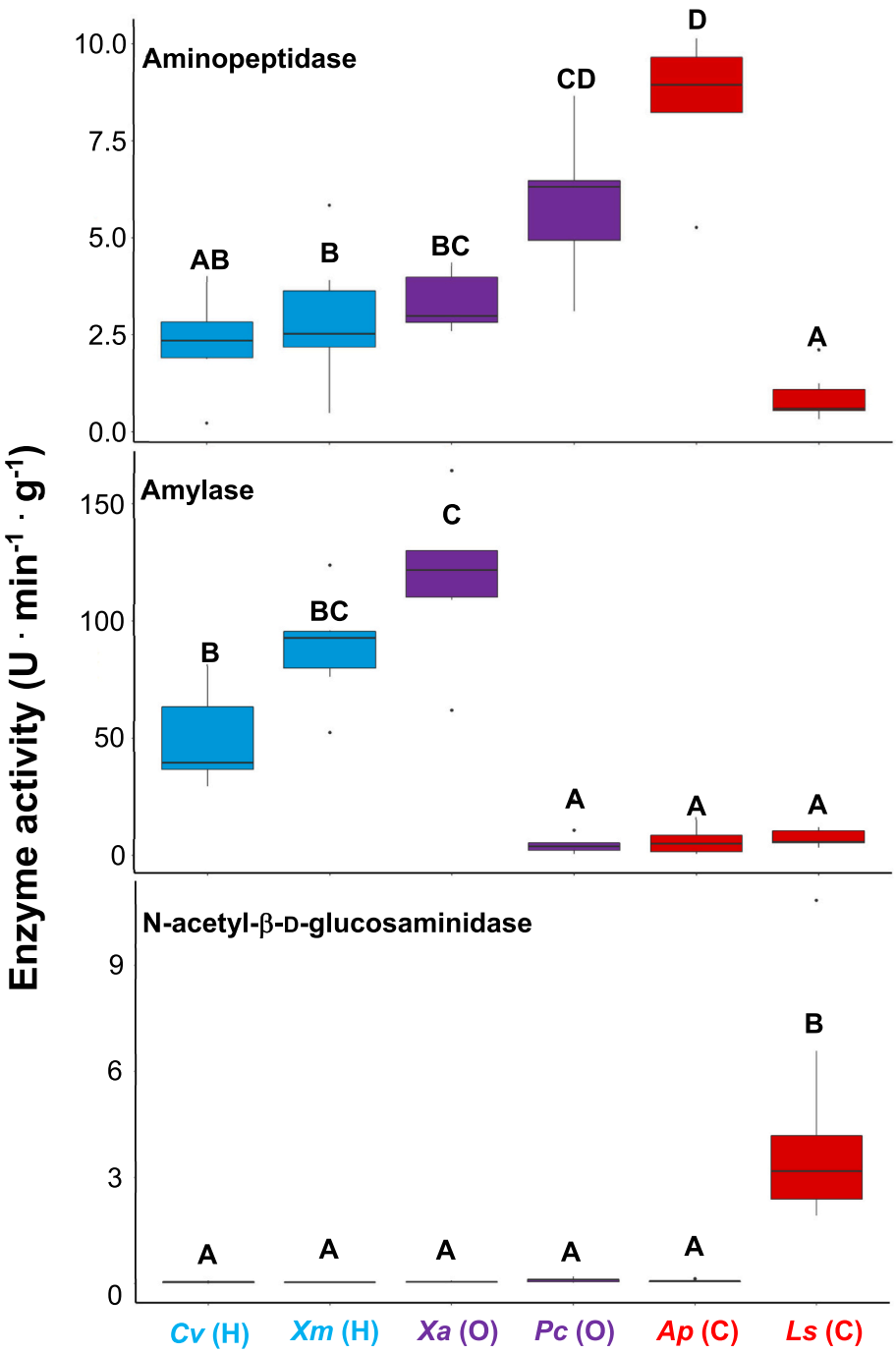

Fig. 4. Aminopeptidase (top), amylase (middle), and *N*-acetyl-β-D-glucosaminidase (bottom) activities in the proximal intestines of *Cebidichthys violaceus* (***Cv***), *Xiphister mucosus* (***Xm***), *Xiphister atropurpureus* (***Xa***), *Phytichthys chirus* (***Pc***), *Anoplarchus purpurescens* (***Ap***), and *Lumpenus sagitta* (***Ls***). Enzyme activities are μmol product produced per minute, per gram of tissue, except for *N*-acetyl-β-D-glucosaminidase, which is nmol product produced. Box plots represent median values (bolded line in each box) with lower and upper quartiles bounding each box. Error bars are 95% confidence intervals and remaining dots are outliers. $n = 6$ for ***Cv***, ***Xm***, ***Xa***, and ***Ap***; $n = 9$ for ***Pc***; $n = 12$ for ***Ls***. **Herbivory (H)**, **Omnivory (O)**, and **Carnivory (C)** indicated accordingly. Interspecific comparisons made with ANOVA followed by Tukey's HSD for each enzyme individually. Boxes not sharing a letter are significantly different from each other for a given enzyme. Data for all species other than ***Ls*** are from German et al. (2015).

We confirmed that *L. sagitta* does indeed consume algae, as it was the dominant item in their stomachs, at least from the collection period in June 2016. However, of all of the predictions made about this fish's ability to digest algae, only one was supported: relatively low aminopeptidase activity that decreased as the animals got larger. Other than that, *L. sagitta* possessed more of a carnivorous digestive physiology: a lack of pyloric caecal differential gene expression as the fish grew, a short gut that does not show positive allometry with body size, low amylolytic and maltasic activity that do not increase ontogenetically, low lipolytic activity with conflicting ontogenetic patterns depending on gut region, elevated pepsin and NAGase activities, and a microbiome that was enriched in taxa from phylum Pseudomonodota. All of these parameters set *L. sagitta* apart from most of the species we studied, but particularly from the herbivores and omnivores. Thus, although *L. sagitta* does consume algae, it doesn't appear that they target the carbohydrates in the algae, which would be the most abundant algal nutrient (Painter, 1983). Omnivores typically consume algae for the carbohydrates (Raubenheimer et al., 2005), and thus, have elevated amylolytic activity (German et al., 2015; Skea et al., 2007); this was not observed for *L. sagitta*.

In terms of gut length, animals that consume lower quality foods, like algal material or detritus, have higher intake, and higher intake means a longer gut is required to have adequate surface area for nutrient absorption, and to maintain material in the gut for some length of time to allow for sufficient digestion to occur (Davis et al., 2013; German and Horn, 2006; German et al., 2015; Leigh et al., 2018a; Sibly, 1981; Sibly and Calow, 1986; Wagner et al., 2009). Fishes that eat more animal material have lower intake and shorter guts (German et al., 2010a;

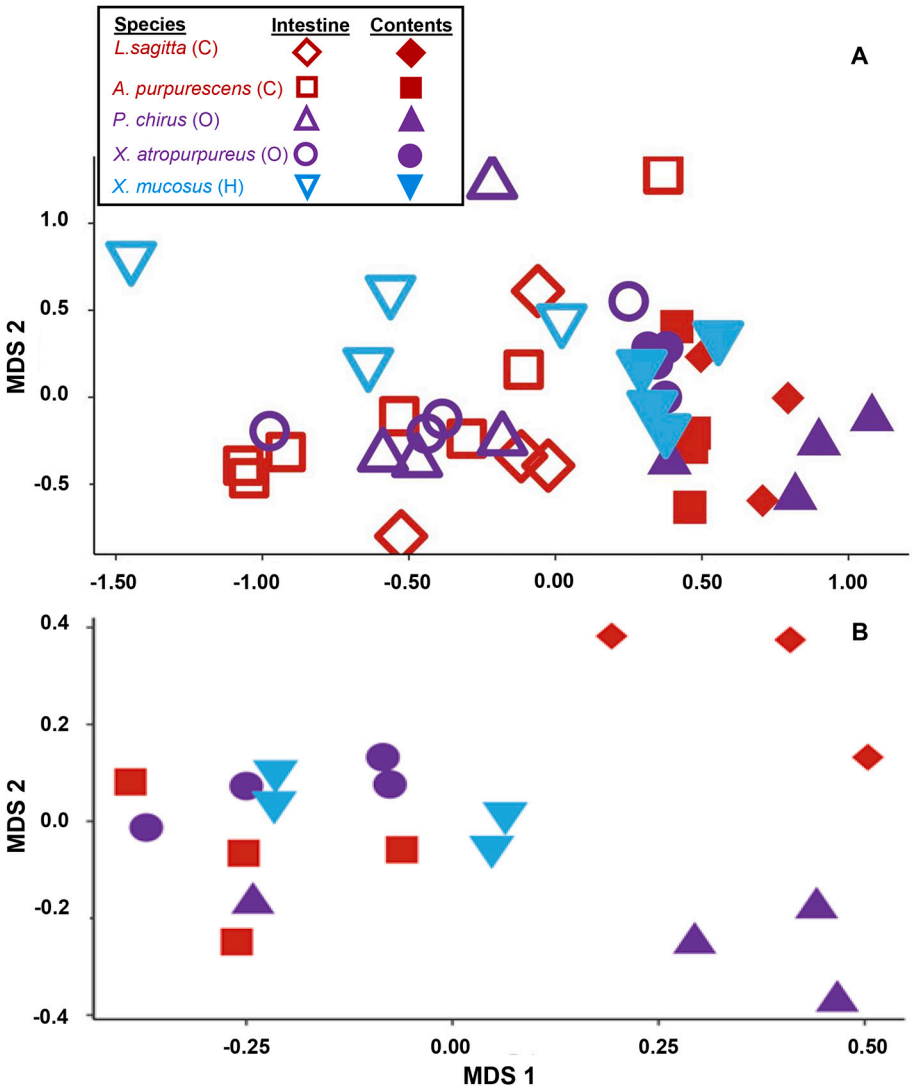

Fig. 5. Non-metric multidimensional scaling plots based on Bray-Curtis dissimilarity of the distal intestine bacterial communities of five prickleback species collected in the Salish Sea, Washington, USA. Proximity to other symbols indicates similarity. Plot A shows intestinal tissue and intestinal contents in the same plot (closed symbols are intestinal contents, whereas open symbols are intestinal tissue), while plot B shows just the intestinal content. **C** = carnivore, **O** = omnivore, **H** = herbivore.

Karasov and Douglas, 2013; Kramer and Bryant, 1995b; Stevens and Hume, 1995). Animals that undergo ontogenetic dietary shifts towards more consumption of plant material as they grow, also tend to have gut lengths that get disproportionately longer than their bodies as the fish get larger (i.e., positive allometry of gut size with body size; Davis et al., 2013; Kramer and Bryant, 1995a). This is true for the herbivores and omnivores examined here and in our previous work (German et al., 2014; German and Horn, 2006; German et al., 2015; Herrera et al., 2022). However, the allegedly omnivorous *L. sagitta* shows no change in gut size relative to body size as the animal gets larger (Fig. 2). They maintain a similar relative gut length throughout their lives, similar to the carnivorous *A. purpurescens*. We had larger size ranges for the other species, as *L. sagitta* reaches maximum recorded lengths of 513 mm SL (Eschmeyer et al., 1983), and our largest one was 213 mm SL. However, even with more limited size ranges, our previous analyses caught similar positive slopes of RGL with body size in pricklebacks that only held up as larger individuals were added (German et al., 2014; German and Horn, 2006), suggesting that the relationship of gut length with body length wouldn't likely change with larger individuals of *L. sagitta*. Hence, *L. sagitta* has a relatively short gut that stays a similar size relative to their body length throughout their lives, similar to other carnivorous fishes (e.g., German et al., 2010a, 2014). Although some omnivorous fishes have guts of similar size relative to body size as seen in *L. sagitta* (e.g., *Hyporhamphus regularis ardelio*) (Day et al., 2011), gut length shows strong phylogenetic correlation (German and Horn, 2006; German et al., 2010a), and thus, in comparison to other pricklebacks, *L. sagitta* has a short gut that does not lengthen relative to its body length as the animal grows. Thus, support for the hypothesis that this fish would have positive allometry of gut size relative to body size was not supported.

Digestion is a chemical process and hydrolytic digestive enzymes degrade large polymers down to monomers or oligomers that are absorbable by the animal (German, 2011; Karasov and Douglas, 2013; Vonk and Western, 1984). Many studies have shown dietary effects on intestinal or pancreatic enzyme activities in proximate and ultimate contexts. However, few studies have examined dietary impacts on gastric enzyme activities (e.g., Chakrabarti et al., 1995; Drewe et al., 2004; German et al., 2004; Sabapathy and Teo, 1993). Here, we observed that those fishes that consume more animal material—which means more dietary protein than fishes consuming more algal material—had higher pepsin activity. Pepsin is an endoprotease that operates under the acidic conditions of the stomach, kicking off protein digestion in the gut (Andreeva, 1995; Mazumder et al., 2018; Miura et al., 2015; Narita et al., 2010; Navarro-Guillén et al., 2022; Newton et al., 2015; Vonk and Western, 1984). Protein digestion is important for all animals, and in general, there are not as strong relationships for proteolytic activity in an animal's gut, and the amount of protein in the diet, as there is for carbohydrase activities and carbohydrate in the diet (German et al., 2004; German et al., 2010a; German et al., 2015; Karasov and Douglas,

2013; Kohl et al., 2011; Skea et al., 2005, 2007). However, elevated pepsin activity in more carnivorous pricklebacks, and an increase in pepsin activity in response to a high-protein diet in the laboratory has been observed before (German et al., 2004), as have ontogenetic decreases in pepsin in a frugivorous fish as they transition to a fruit-rich diet (Drewe et al., 2004).

A match between dietary substrate intake and digestive enzyme activity to digest that substrate is known as the Adaptive Modulation Hypothesis (AMH; Karasov, 1992; Karasov and Martínez del Rio, 2007). The AMH has strong support for carbohydrases (mostly amylase and maltase), but less so for proteases in the intestine (Yawitz et al., 2022). This study adds to the support for the AMH for pepsin in the stomach specifically. Most studies of pepsins have focused on carnivorous animals only (Mazumder et al., 2018; Miura et al., 2015; Navarro-Guillén et al., 2022; Newton et al., 2015), with one exception of elevated gene copy number for pepsinogen genes in Orangutans (Narita et al., 2010). In terms of *L. sagitta*, its elevated pepsin activity doesn't support our hypothesis relating to digestive enzyme activities for an omnivorous stichaeid, and suggests more protein in its diet.

Chitinase is a carbohydrase that generates di- and oligosaccharides from the polysaccharide chitin. Chitin is commonly found in the exoskeletons of crustaceans and fungal cell walls, and with 10^{13} metric tons of chitin generated annually, it is one of the most common biopolymers globally, and particularly in marine waters (Barikani et al., 2014; Muzzarelli, 1999). Therefore, fishes should show chitinolytic ability, whether to disrupt exoskeletons or cell walls (Gutowska et al., 2004), or to actually gain oxidizable substrates from the monomeric *N*-acetyl-glucosamine that composes chitin (Alliot, 1967; Pérès et al., 1973; Vervaet, 2019). Chitinase activities have been shown to match with diet in herbivorous and carnivorous minnows (i.e., more chitinase in fishes consuming more insects) (German et al., 2010a), and in some marine fishes (Danulat, 1986; Goodrich and Morita, 1977; Lindsay, 1984; Vervaet, 2019). However, German et al. (2010a) is the only comparison of which we are aware of chitinase activities in closely related fish species with different diets, like we have done in this current study. Here, *P. chirus* consumes the most crustaceans among the studied pricklebacks (German et al., 2014) and has the highest chitinase activities. Even *A. purpurescens* and *X. atropurpureus* consume the next most crustaceans (German and Horn, 2006; German et al., 2015) and they too show elevated chitinolytic activity. Crustaceans make up a considerable proportion of the diet of *L. sagitta*, yet their gastric chitinase activity is low. Recent genomic work on *C. violaceus* (Heras et al., 2020; Wright et al., 2023), and transcriptomic work on the same species used in this study (Herrera et al., 2022) shows that chitinases are indeed in the genome and are expressed in the fishes, and thus, these activities are endogenous. Much like pepsin, more work is needed with diet switching experiments to discern how flexible chitinase activities are with proximate diet, but natural diet does impact chitinolytic activities in prickleback fishes. We found that *L. sagitta* had moderate chitinase activities, in between the herbivores and carnivores (Fig. 3).

The next enzyme in the chitinolytic cascade is *N*-acetyl-β-D-glucosaminidase (NAGase), which generates *N*-acetyl-glucosamine by hydrolyzing the disaccharide chitobiose, the product of chitinase digestion. *Phytichthys chirus* has the highest chitinase activities in its stomach, and elevated NAGase activity in its distal intestine contents (German et al., 2015), consistent with an animal that may be targeting *N*-acetyl-glucosamine for metabolic use (Vervaet, 2019). Fishes can absorb and use *N*-acetyl-glucosamine in metabolic pathways (Alliot, 1967; Pérès et al., 1973; Vervaet, 2019). What is intriguing about *L. sagitta* is that it has the highest NAGase activities in its intestinal tissues by two orders of magnitude (Fig. 4), and this is true for all gut regions (Supplemental Table S2). Although *L. sagitta* didn't have the highest chitinase activities in its stomach, the elevated NAGase strongly suggests that this species absorbs and uses *N*-acetyl-glucosamine metabolically (Alliot, 1967; Pérès et al., 1973; Vervaet, 2019) and doesn't just disrupt crustacean exoskeletons during the digestive process to access other nutrients (Gutowska et al., 2004). The elevated NAGase could also be a function of the intestinal microbiome of *L. sagitta*, but this wouldn't explain the elevated NAGase found throughout the *L. sagitta* gut (especially the pyloric caecal and proximal intestine regions), which suggests the activity is endogenous. Elevated NAGase is a unique characteristic of *L. sagitta* in this study, and further distances *L. sagitta* from the herbivores and omnivores in the Stichaeidae. Coupled to the relatively low trypsin and aminopeptidase activities in their guts, it is possible *L. sagitta* meets some nitrogen requirements via *N*-acetyl-glucosamine (Vervaet, 2019).

The other digestive enzymes followed known patterns observed with diet: elevated amylase activities in the fish consuming more plant material (i.e., more starch), and elevated aminopeptidase in the those consuming more animal material (i.e., more protein). The one exception is *L. sagitta*. Why this "omnivorous" species had low aminopeptidase activity in its gut (all gut regions) is unknown. Three prickleback species (*C. violaceus*, *X. atropurpureus*, and *A. purpurescens*) showed significant increases in aminopeptidase activity when consuming a high-protein animal diet in the lab (German et al., 2004). Perhaps *L. sagitta* had lower aminopeptidase activity due to some recent dietary changes since our enzyme measurements only captured a single moment in time, but trypsin activity was also low (Supplemental Table S2).

Four prickleback species show ontogenetic changes in diet and concomitant digestive enzyme activities: *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *P. chirus* consume more algae and increase the amylase activities in their guts as they grow (German et al., 2014; German and Horn, 2006; German et al., 2004). Amylase gene expression also increases with size (Kim et al., 2014). Hence, we examined whether *L. sagitta* showed any changes in digestive enzyme activities as they grew. Unlike the other species, *L. sagitta* showed a significant negative correlation between amylase activity and body size in the proximal intestine region, where this enzyme is most active. Moreover, no ontogenetic change in amylase gene expression was detected in the pyloric caeca, the same gut region used by Kim et al. (2014) for their examination of amylase gene expression; the pyloric caeca and mid intestine have strong pancreatic expression and amylase is a pancreatic enzyme (Heras et al., 2020; Herrera et al., 2022; Kim et al., 2014). Similar to *L. sagitta*, the carnivorous *A. purpurescens* showed no change in amylase gene expression ontogenetically (Kim et al., 2014).

Consistent with the low aminopeptidase activity in *L. sagitta* overall, the activity of this enzyme decreased as the fish grew. If *L. sagitta* is an omnivorous fish, it isn't clear why aminopeptidase activity decreased with increase in fish size. The substrate we used in our assays measures alanine aminopeptidase activity (Roncari and Zuber, 1969), which is an important enzyme that completes the final step generating individual amino acids from dipeptides so that the amino acids can be absorbed (Karasov and Douglas, 2013). Fishes do possess the PEPT1 transporter in their enterocyte membranes, which is capable of transporting di- and tripeptides in addition to individual amino acids (Hart et al., 2016; He et al., 2022; Verri et al., 2017), and perhaps *L. sagitta* doesn't rely on amino acid absorption as much as absorbing larger peptides. Or, it is possible that *L. sagitta* expresses a different aminopeptidase along its brush border. Fishes possess five different alanyl aminopeptidase genes in their genomes: *anpepa, anpepb*, *anpepN*, *anpepEY*, and *anpep* EY-like (Heras et al., 2020), all of which show strong gut expression in pricklebacks (Heras et al., 2020; Herrera et al., 2022). Perhaps *L. sagitta* relies more on a different aminopeptidase, like Leucyl Aminopeptidase, which would be measured with a different substrate than an alanine conjugate, like we used. For instance, in abalone, some species (e.g., *Haliotis iris*) don't have measurable alanyl aminopeptidase, yet they have elevated Leucyl Aminopeptidase activity in comparison to its congener *H. rufescens* (Frederick et al., 2022). Further work is needed on aminopeptidases in pricklebacks and beyond (Heras et al., 2020).

An alternative to the Adaptive Modulation Hypothesis is known as the Nutrient Balancing Hypothesis (Clissold et al., 2010; Heras et al., 2020), which states that if a nutrient is limiting (e.g., essential fatty

acids), then enzymatic activity against that substrate may increase when the nutrient is in short supply in the diet. In pricklebacks, and fishes more generally (Leigh et al., 2018a), lipase activities—especially across the entire gut—are elevated in those species consuming more plant material (German et al., 2004; German et al., 2015) and the herbivores may have more copies of the carboxyl ester lipase gene in their genomes (Heras et al., 2020), even though most algae is low in lipid content, particularly those consumed by pricklebacks (Neighbors and Horn, 1991). The assay substrate we used measures carboxyl ester lipase (also known as bile-salt activated lipase), which is the main digestive lipase in fishes since they don't possess a pancreatic, co-lipase system, like mammals (Murray et al., 2003; Olsen and Ringø, 1997; Sæle et al., 2010; Tang et al., 2022). If *L. sagitta* was targeting lipids in algae (or even animal material), we would expect more elevated lipolytic activities in their guts. Moreover, lipase activity showed different correlations with size in different gut regions of *L. sagitta*: an increase in the proximal intestine, but a decrease in the distal. Ontogenetic increases in lipolytic activities were observed in *C. violaceus*, *X. mucosus*, and *X. atropurpureus*, but not *A. purpurescens* or *P. chirus* (German et al., 2014; German et al., 2004). Even with the ontogenetic changes in lipolytic activity in its proximal intestine, *L. sagitta* doesn't have elevated lipase activities in their guts (Supplemental Table S2).

An animal's intestinal microbiome can play many roles in the host's physiology (Clements et al., 2014; Egerton et al., 2018; Llewellyn et al., 2014; Pardesi et al., 2022; Stevenson et al., 2022; Sullam et al., 2012). Some patterns of microbial phyla are becoming clearer as more 16S sequencing is performed. For instance, Pseudomonodota is the most common phylum in fish guts (Egerton et al., 2018; Llewellyn et al., 2014; Stevenson et al., 2022; Sullam et al., 2012), and is more enriched in carnivorous fishes (Egerton et al., 2018; Liu et al., 2022), whereas Bacteriodota and Bacillota are more enriched in herbivores (Egerton et al., 2018; Liu et al., 2022; Stevenson et al., 2022; Pardesi et al., 2022). Certainly, Pseudomonodota dominate the gut content samples in this study (Supplemental Fig. S7). One of the goals of this investigation was to examine how the enteric microbiomes of prickleback fishes varied among species with different diets, with an emphasis on *L. sagitta* as one of the least studied prickleback species. From our multivariate analyses, it is clear that the intestinal microbiome of *L. sagitta*, especially that in their distal intestine contents, is different from other pricklebacks (Fig. 5). There are also clear indicator species in the phyla Planctomycetota and Pseudomonodota that set *L. sagitta* apart (Supplemental Table S3; Supplemental Fig. S4).

Members of the Planctomycetota are found in many environments, including soils, various waterbodies, and in guts, including fish and human guts (Baniel et al., 2021; Cayrou et al., 2013; Chen et al., 2020; Elshahed et al., 2007; Fregulia et al., 2022; Gardiner et al., 2020; Gullian-Klanian et al., 2023; Liu et al., 2022; van Kessel et al., 2011). Specifically, the Pirellulaceae (the most common family in the Planctomycetota found in *L. sagitta*) are found in gut environments and some taxa have amylolytic capabilities (Baniel et al., 2021; Fregulia et al., 2022; Gardiner et al., 2020). Thus, they are known to play some role within the gut, although specifics remain unresolved. The Pseudomonodota are among the most abundant bacteria in marine systems, with the Rhodobacteraceae comprising many taxa abundant in algal biofilms and in guts (Pohlner et al., 2019), including in *L. sagitta* in this study. A member of the genus *Mesorhizobium* was also an indicator species in *L. sagitta*, and these taxa could be involved in nitrogen metabolism and interspecific signaling (Krick et al., 2007). It stands out that *L. sagitta* was not more similar to those fishes that consume more algae in their family, further strengthening the argument that these fish are not omnivorous from the digestive standpoint. Interestingly, in the gut mucosal community, *L. sagitta* and *X. atropurpureus* share an indicator species in the genus *Vogesella* (Pseudomonodota), which could engage in many carbohydrate degradation pathways (Rameshkumar et al., 2016). Because *L. sagitta* likely doesn't digest algal carbohydrates with great efficiency on account of its low amylolytic capabilities, more algal starches may make it to the hindgut where microbial pathways digest it. We intend to explore more of the detail of the microbial communities in the other fish species in a subsequent study and will limit our discussion of microbial taxa to those that are abundant in *L. sagitta*, but it is worth noting that the herbivorous *X. mucosus* had a higher proportion of Oscillospiraceae, Rikenellaceae, and Lachnopiraceae in its gut (Supplemental Fig. S6; Liu et al., 2022; Stevenson et al., 2022), and these taxa were lacking in *L. sagitta*. This is offered with the caveat that *X. mucosus* does not appear to be reliant on enteric microbes to digest its algal diet (German et al., 2015), yet has members of its microbiome that may degrade algal components.

More generally, our results reveal stark differences in the microbial communities of the gut contents and those in the intestinal tissue (along the mucosal lining). Although this is not a new finding (e.g., Stevenson et al., 2022), it is striking how across multiple sympatric species with different diets, the tissues and contents cluster completely separately in multivariate space and are statistically different for each species. The gut lumen and intestinal tissue provide different selective environments with potential differences in chemistry (Cremin et al., 2023; Donaldson et al., 2016; Malmuthuge et al., 2012; Tropini et al., 2017), including the concentration of oxygen (Rivera-Chávez et al., 2017; Zheng et al., 2015). Those taxa associated with the gut contents are likely directly engaged with digestion of this material (Hanning and Diaz-Sanchez, 2015; Stevenson et al., 2022; Tarnecki et al., 2017), whereas those along the mucosal lining of the intestine can be engaged with the fish immune system and other processes, including maintenance of the mucus layer itself (Hanning and Diaz-Sanchez, 2015; Karkman et al., 2017; Sommer et al., 2017). Further work is needed to characterize the biochemical pathways that are active in these two areas in pricklebacks, but our work reinforces that the contents (luminal) and tissue (mucosal) communities should be analyzed separately to potentially discern their roles. We focused on the hindgut, but there are also different microbial communities along the length of the intestinal environment (Sparagon et al., 2022; Stevenson et al., 2022). Seventeen bacterial taxa were identified as "core microbiota" shared with 100% match among all five fish host species (Supplemental Table S5). Consistent with other fishes (Egerton et al., 2018; Llewellyn et al., 2014; Liu et al., 2022; Stevenson et al., 2022; Sullam et al., 2012), they were dominated by members of the Pseudomonodota and should be explored in more detail.

In conclusion, we went beyond gut content analyses to discern what *Lumpenus sagitta* would be capable of digesting from their allegedly omnivorous diet. Based on a rich dataset on other members of the same family (Stichaeidae) that do digest algae to varying degrees, we made specific predictions on what we expected to see in *L. sagitta* if they were indeed capable of algal digestion. Nearly none of our predictions were supported. With a short gut and no positive allometry between gut size and body size, it is unlikely that *L. sagitta* has the high intake its algivorous relatives do. The lack of elevated amylase activity, which is consistently high, not only among stichaeids that consume algae, but among herbivorous and omnivorous fishes, and animals more broadly (German et al., 2016), suggests that *L. sagitta* cannot efficiently digest algal starches. *Lumpenus sagitta* does indeed have elevated *N*-acetyl-β-D-glucosaminidase activities, suggestive of chitobiose digestion, with this coming from crustacean digestion, and an enteric microbial community that doesn't resemble those of the more algivorous members of its family. Hence, in concert with usually more elevated $\delta^{15}N$ values in *Lumpenus* (Tamelander et al., 2006; Hindell et al., 2012), we are left concluding that although *L. sagitta* ingests algae, it is more likely digesting epibionts growing on the algae, and invertebrates coming in with the algae, than the algae itself. Clearly, digestibility studies (e.g., Raubenheimer et al., 2005) are needed to finally confirm this, but digestion is a chemical process, and if the agents of that process aren't present in the gut, then it isn't clear how the process can happen. Our study shows the power of including multiple measures of digestive performance and that we need to move beyond gut content analyses to discern the diet and potential trophic role of an animal (Choat and

Clements, 1998; Clements et al., 2017; German, 2009; German and Bittong, 2009; Leigh et al., 2018b). Moreover, to better understand the natural world, and fishes in particular, we must expand the species we study to appreciate the diversity nature beholds (Clark et al., 2023). Although there are clearly diverse digestive strategies among fishes more broadly (e.g., Horn, 1989; Clements and Choat, 1995; Choat and Clements, 1998; Clements et al., 2017), our approach of using closely related, sympatric species overcomes many of the habitat and phylogenetic differences among study subjects that have obscured the general principles relating to fish digestion (e.g., Chakrabarti et al., 1995), yet, at the same time, we recognize that broader phylogenetic breadth can indeed uncover other aspects of digestion not considered here (e.g., Horn, 1989; Choat and Clements, 1998; Clements et al., 2017).

Funding

This work was funded by NSF Grant IOS-1355224 (PI: D.P. German), UCI DECADE Mentor Funds (D.P. German), FHL REU Program (NSF DBI-1852096). Further funding from UCI Competitive Edge was awarded to D.R. Rankins and N.Z. Hood. This study was supported by the Ridge to Reef NSF Research Traineeship, award DGE-1735040, and the American Physiological Society Porter Physiology Development Fellowship (both to MJH).

Data accessibility

The datasets presented in this study can be found in online repositories, accession number for the Bioproject is PRJNA947617, where the Biosamples and SRA files are located. The microbiome data have accession number PRJNA949661. All other data are located at German Lab:: Instruction and outreach (uci.edu).

Ethics statements

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.

Author contribution

M.J.H, J.H., and D.P.G. collected the samples, performed dissections, and morphometric measurements. M.J.H. prepared the samples for molecular work (transcriptomics and microbiome) and performed the sequencing. N.Z.H. and D.R.R. prepared the homogenates for enzyme assays. D.R.R. and M.J.H. did the bioinformatic analyses for transcriptomics, M.J.H. did the microbiome analyses. D.R.R., N.Z.H., M.P.C., and A.C. performed the enzyme assays. D.R.R. performed all statistical analyses. All authors contributed to the writing process.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Microbiome and Transcriptomic data is published on NCBI and projects codes are included in the manuscript. Additional data will be published at https://german.bio.uci.edu/Supplements.html

Acknowledgements

The authors acknowledge and honor the original inhabitants of the regions and sacred land where the authors live and work. Much of this work was done on the occupied and unceded land of the Acjachemen and Tongva Nations that inhabited the region before the founding of the state and that continue to live on these lands where University of California, Irvine is located. And much of this field work was done on the ancestral lands and waters of the Coast Salish Nations, where Friday Harbor Laboratories is located. We are grateful to the German lab members for their support and feedback throughout this project. We thank J. Emerson, K. Connor and N. Aguilar-Roca for guidance and feedback on RNA-seq and physiological techniques and analysis. The comparative physiology group at UCI for feedback on this project. The Friday Harbor Laboratories personnel and NSF REU BEACON mentors, especially L. Crummett and A. Summers, for use of laboratory tools and space, and V. Iyengar, for support and guidance. The 2016 FHL Fish Functional Morphology Class for aid in collection of specimens, and fellow REU trainees for support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2023.111508.

References

Alliot, E., 1967. Absorption intestinale de l' N-acetyl-glucosamine chez la petite rousette (*Scylliorhinus canicula*). Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales 161, 2544–5546.
Andreeva, N.S., 1995. Why and for what reason is pepsin stable and active at pH 2? Mol. Biol. 28, 869–873.
Anson, M.L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. Physiol. 22, 79–89.
Baldo, L., Pretus, J.L., Riera, J.L., Musilova, Z., Bitja Nyom, A.R., Salzburger, W., 2017. Convergence of gut microbiotas in the adaptive radiations of African cichlid fishes. ISME J. 11, 1975–1987.
Baldo, L., Tavecchia, G., Rotger, A., Igual, J.M., Riera, J.L., 2023. Insular holobionts: persistence and seasonal plasticity of the Balearic wall lizard (Podarcis lilfordi) gut microbiota. PeerJ 11, e14511.
Baniel, A., Amato, K.R., Beehner, J.C., Bergman, T.J., Mercer, A., Perlman, R.F., Petrullo, L., Reitsema, L., Sams, S., Lu, A., Snyder-Mackler, N., 2021. Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. Microbiome 9, 26.
Barikani, M., Oliaei, E., Seddiqi, H., Honarkar, H., 2014. Preparation and application of chitin and its derivatives: a review. Iran. Polym. J. 23, 307–326.
Bethea, D.M., Hale, L., Carlson, J.K., Cortés, E., Manire, C.A., Gelsleichter, J., 2007. Geographic and ontogenetic variation in the diet and daily ration of the bonnethead shark, *Sphyrna tiburo*, from the eastern Gulf of Mexico. Mar. Biol. 152, 1009–1020.
Bethea, D.M., Carlson, J.K., Hollensead, L.D., Papastamatiou, Y.P., Graham, B.S., 2011. A comparison of the foraging ecology and bioenergetics of the early life-stages of two sympatric hammerhead sharks. Bull. Mar. Sci. 87, 873–899.
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.
Boyle, K.S., Horn, M.H., 2006. Comparison of feeding guild structure and ecomorphology of intertidal fish assemblages from Central California and Central Chile. Mar. Ecol. Prog. Ser. 319, 65–84.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624.
Castro-Ruiz, D., Andree, K.B., Blondeau-Bidet, E., Fernández-Méndez, C., García-Dávila, C., Gisbert, E., Darias, M.J., 2021. Isolation, identification, and gene expression analysis of the main digestive enzymes during ontogeny of the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855). Aquaculture 543, 737031.
Cayrou, C., Sambe, B., Armougom, F., Raoult, D., Drancourt, M., 2013. Molecular diversity of the Planctomycetes in the human gut microbiota in France and Senegal. APMIS 121, 1082–1090.
Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R., Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. Comp. Biochem. Physiol. A 112, 167–177.
Chen, H., Li, C., Liu, T., Chen, S., Xiao, H., 2020. A metagenomic study of intestinal microbial diversity in relation to feeding habits of surface and cave-dwelling *Sinocyclocheilus* species. Microb. Ecol. 79, 299–311.
Choat, J.H., Clements, K.D., 1998. Vertebrate herbivores in marine and terrestrial environments: a nutritional ecology perspective. Annu. Rev. Ecol. Syst. 29, 375–403.
Choat, J.H., Clements, K.D., Robbins, W.D., 2002. The trophic status of herbivorous fishes on coral reefs. 1: dietary analyses. Mar. Biol. 140, 613–623.
Clark, C.J., Hutchinson, J.R., Garland, T., 2023. The inverse Krogh principle: all organisms are worthy of study. Physiol. Biochem. Zool. 96, 1–16.
Clements, K.D., Choat, J.H., 1995. Fermentation in tropical marine herbivorous fishes. Physiol. Biochem. Zool. 68, 355–378.
Clements, K.D., Angert, E.R., Montgomery, W.L., Choat, J.H., 2014. Intestinal microbiota in fishes: what's known and what's not. Mol. Ecol. 23, 1891–1898.

Clements, K.D., German, D.P., Piché, J., Tribollet, A., Choat, J.H., 2017. Integrating ecological roles and trophic diversification on coral reefs: multiple lines of evidence identify parrotfishes as microphages. Biol. J. Linn. Soc. 120, 729–751.
Clissold, F.J., Tedder, B.J., Conigrave, A.D., Simpson, S.J., 2010. The gastrointestinal tract as a nutrient-balancing organ. Proc. R. Soc. B: Biol. Sci. 277, 1751–1759.
Cremin, K., Duxbury, S.J.N., Rosko, J., Soyer, O.S., 2023. Formation and emergent dynamics of spatially organized microbial systems. Interf. Focus 13, 20220062.
Crossman, D.J., Choat, J.H., Clements, K.D., 2005. Nutritional ecology of nominally herbivorous fishes on coral reefs. Mar. Ecol. Prog. Ser. 296, 129–142.
Crummett, L.T., 2020. Acidification decreases microbial community diversity in the Salish Sea, a region with naturally high pCO2. PLoS One 15, e0241183.
Dahlqvist, A., 1968. Assay of intestinal disacharidases. Anal. Biochem. 22, 99–107.
Danulat, E., 1986. Role of bacteria with regard to chitin degradation in the digestive tract of cod *Gadus morhua*. Mar. Biol. 90, 335–343.
Davis, A.M., Unmack, P.J., Pusey, B.J., Pearson, R.G., Morgan, D.L., 2013. Ontogenetic development of intestinal length and relationships to diet in an Australasian fish family (Terapontidae). BMC Evol. Biol. 13, 1–16.
Day, R.D., German, D.P., Tibbetts, I.R., 2011. Why can't young fish eat plants? Neither digestive enzymes nor gut development preclude herbivory in the young of a stomachless marine herbivorous fish. Com. Biochem. Physiol. B 158, 23–29.
De Cáceres, M., Legendre, P., Wiser, S.K., Brotons, L., 2012. Using species combinations in indicator value analyses. Methods Ecol. Evol. 3, 973–982.
Donaldson, G.P., Lee, S.M., Mazmanian, S.K., 2016. Gut biogeography of the bacterial microbiota. Nat. Rev. Microbiol. 14, 20–32.
Drewe, K., Horn, M.H., Dickson, K.A., Gawlicka, A., 2004. Insectivore to frugivore: ontogenetic changes in gut morphology and digestive enzyme activity in the characid fish *Brycon guatemalensis* from Costa Rican rainforest streams. J. Fish Biol. 64, 890–902.
Duran, A., Adam, T.C., Palma, L., Moreno, S., Collado-Vides, L., Burkepile, D.E., 2019. Feeding behavior in Caribbean surgeonfishes varies across fish size, algal abundance, and habitat characteristics. Mar. Ecol. 40, e12561.
Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Front. Microbiol. 9.
Elshahed, M.S., Youssef, N.H., Luo, Q., Najar, F.Z., Roe, B.A., Sisk, T.M., Bühring, S.I., Hinrichs, K.U., Krumholz, L.R., 2007. Phylogenetic and metabolic diversity of *Planctomycetes* from anaerobic, sulfide- and sulfur-rich zodletone spring, Oklahoma. Appl. Environ. Microbiol. 73, 4707–4716.
Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.
Eschmeyer, W.N., Herald, E.S., Hammann, H., 1983. A Field Guide to Pacific Coast Fishes of North America. Houghton Mifflin Co., Boston, MA.
Finn, R.D., Clements, J., Eddy, S.R., 2011. HMMER web server: interactive sequence similarity searching. Nucleic Acids Res. 39, W29–W37.
Frederick, A.R., Lee, A.M., Wehrle, B.A., Catabay, C.C., Rankins, D.R., Clements, K.D., German, D.P., 2022. Abalone under moderate heat stress have elevated metabolic rates and changes to digestive enzyme activities. Comp. Biochem. Physiol. A 270, 111230.
Fregulia, P., Campos, M.M., Dias, R.J.P., Liu, J., Guo, W., Pereira, L.G.R., Machado, M.A., de Lima Reis Faza, D.R., Guan, L.L., Garnsworthy, P.C., Neves, A.L.A., 2022. Taxonomic and predicted functional signatures reveal linkages between the rumen microbiota and feed efficiency in dairy cattle raised in tropical areas. Front. Microbiol. 13.
Fris, M.B., Horn, M.H., 1993. Effects of diets of different protein content on food consumption, gut retention, protein conversion, and growth of *Cebidichthys violaceus* (Girard), an herbivorous fish of temperate zone marine waters. J. Exp. Mar. Biol. Ecol. 166, 185–202.
Gardiner, G.E., Metzler-Zebeli, B.U., Lawlor, P.G., 2020. Impact of intestinal microbiota on growth and feed efficiency in pigs: a review. Microorganisms 8, 1886.
Gawlicka, A., Horn, M.H., 2006. Trypsin gene expression by quantitative in situ hybridization in carnivorous and herbivorous prickleback fishes (Teleostei : Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Physiol. Biochem. Zool. 79, 120–132.
German, D.P., 2009. Inside the guts of wood-eating catfishes: can they digest wood? J. Comp. Physiol. B. 179, 1011–1023.
German, D.P., 2011. Digestive efficiency. In: Farrel, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, pp. 1596–1607.
German, D.P., Bittong, R.A., 2009. Digestive enzyme activities and gastrointestinal fermentation in wood-eating catfishes. J. Comp. Physiol. B. 179, 1025–1042.
German, D.P., Horn, M.H., 2006. Gut length and mass in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Mar. Biol. 148, 1123–1134.
German, D.P., Miles, R.D., 2010. Stable carbon and nitrogen incorporation in blood and fin tissue of the catfish *Pterygoplichthys disjunctivus* (Siluriformes, Loricariidae). Environ. Biol. Fish 89, 117–133.
German, D.P., Horn, M.H., Gawlicka, A., 2004. Digestive enzyme activities in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Physiol. Biochem. Zool. 77, 789–804.
German, D.P., Nagle, B.C., Villeda, J.M., Ruiz, A.M., Thomson, A.W., Contreras-Balderas, S., Evans, D.H., 2010a. Evolution of herbivory in a carnivorous clade of minnows (Teleostei: Cyprinidae): effects on gut size and digestive physiology. Physiol. Biochem. Zool. 83, 1–18.
German, D.P., Neuberger, D.T., Callahan, M.N., Lizardo, N.R., Evans, D.H., 2010b. Feast to famine: the effects of dietary quality and quantity on the gut structure and function of a detritivorous catfish (Teleostei: Loricariidae). Comp. Biochem. Physiol. A 155, 281–293.
German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387–1397.
German, D.P., Gawlicka, A.K., Horn, M.H., 2014. Evolution of ontogenetic dietary shifts and associated gut features in prickleback fishes (Teleostei: Stichaeidae). Com. Biochem. Physiol. B 168, 12–18.
German, D.P., Sung, A., Jhaveri, P.K., Agnihotri, A., 2015. More than one way to be an herbivore: convergent evolution of herbivory using different digestive strategies in prickleback fishes (family Stichaeidae). Zoology 118, 161–170.
German, D.P., Foti, D.M., Heras, J., Amerkhanian, H., Lockwood, B.L., 2016. Elevated gene copy number does not always explain elevated amylase activities in fishes. Physiol. Biochem. Zool. 89, 277–293.
Goodrich, T.D., Morita, R.Y., 1977. Incidence and estimation of chitinase activity associated with marine fish and other estuarine samples. Mar. Biol. 41, 349–353.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.
Gullian-Klanian, M., Quintanilla-Mena, M., Hau, C.P., 2023. Influence of the biofloc bacterial community on the digestive activity of Nile tilapia (*Oreochromis niloticus*). Aquaculture 562, 738774.
Gutowska, M., Drazen, J., Robison, B., 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. Comp. Biochem. Physiol. A 139, 351–358.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., MacManes, M., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., LeDuc, R.D., Friedman, N., Regev, A., 2013. De novo transcript sequence reconstruction from RNA-seq using the trinity platform for reference generation and analysis. Nat. Protoc. 8, 1494–1512.
Hanning, I., Diaz-Sanchez, S., 2015. The functionality of the gastrointestinal microbiome in non-human animals. Microbiome 3, 51.
Hart, H.R., Evans, A.N., Gelsleichter, J., Ahearn, G.A., 2016. Molecular identification and functional characteristics of peptide transporters in the bonnethead shark (*Sphyrna tiburo*). J. Comp. Physiol. B. 186, 855–866.
He, Z., Cai, Y., Yang, M., Liu, N., Zeng, Z., Li, X., Zhou, X., Liu, S., Liu, Z., 2022. Transcription factor CDX2 directly regulates the expression of Ctenopharyngodon idellus intestinal PepT1 to mediate the transportation of oligopeptide. Aquacult. Rep. 24, 101148.
Heras, J., Chakraborty, M., Emerson, J.J., German, D.P., 2020. Genomic and biochemical evidence of dietary adaptation in a marine herbivorous fish. Proc. R. Soc. B Biol. Sci. 287, 20192327.
Herrera, M.J., Heras, J., German, D.P., 2022. Comparative transcriptomics reveal tissue level specialization towards diet in prickleback fishes. J. Comp. Physiol. B. 192, 275–295.
Hindell, M.A., Lydersen, C., Hop, H., Kovacs, K.M., 2012. Pre-partum diet of adult female bearded seals in years of contrasting ice conditions. PLoS One 7, e38307.
Horn, M.H., 1989. Biology of marine herbivorous fishes. Oceanogr. Mar. Biol. Annu. Rev. 27, 167–272.
Horn, M.H., Neighbors, M.A., Murray, S.N., 1986. Herbivore responses to a seasonally fluctuating food supply: growth potential of two temperate intertidal fishes based on the protein and energy assimilated from their macroalgal diets. J. Exp. Mar. Biol. Ecol. 103, 217–234.
Jeuniaux, C., 1966. Chitinases. In: Neufeld, E.F., Ginsburg, V. (Eds.), Methods in Enzymology, Academic Press, 8. New York, NY, pp. 644–650.
Karasov, W.H., 1992. Tests of the adaptive modulation hypothesis for dietary control of intestinal nutrient transport. Am. J. Phys. 263, R496–R502.
Karasov, W.H., Douglas, A.E., 2013. Comparative digestive physiology. Comp. Physiol. 3, 741–783.
Karasov, W.H., Hume, I.D., 1997. Vertebrate gastrointestinal system. In: Dantzler, W.H. (Ed.), Handbook of Comparative Physiology. American Physiological Society, Bethseda, MD, USA, pp. 407–480.
Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ USA.
Karkman, A., Lehtimäki, J., Ruokolainen, L., 2017. The ecology of human microbiota: dynamics and diversity in health and disease. Ann. N. Y. Acad. Sci. 1399, 78–92.
Kim, K.H., Horn, M.H., Sosa, A.E., German, D.P., 2014. Sequence and expression of an α-amylase gene in four related species of prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and species-level effects. J. Comp. Physiol. B. 184, 221–234.
Kohl, K.D., Brzek, P., Caviedes-Vidal, E., Karasov, W.H., 2011. Pancreatic and intestinal carbohydrases are matched to dietary starch level in wild passerine birds. Physiol. Biochem. Zool. 84, 195–203.
Kramer, D.L., Bryant, M.J., 1995a. Intestine length in the fishes of a tropical stream: 1. Ontogenetic allometry. Environ. Biol. Fish. 42, 115–127.
Kramer, D.L., Bryant, M.J., 1995b. Intestine length in the fishes of a tropical stream: 2. Relationships to diet - the long and the short of a convoluted issue. Environ. Biol. Fish 42, 129–141.
Krick, A., Kehraus, S., Eberl, L., Riedel, K., Anke, H., Kaesler, I., Graeber, I., Szewzyk, U., König, G.M., 2007. A marine Mesorhizobium sp. produces structurally novel long-chain N-acyl-L-homoserine lactones. Appl. Environ. Microbiol. 73, 3587–3594.
Leigh, S.C., Nguyen-Phuc, B.Q., German, D.P., 2018a. The effects of protein and fiber content on gut structure and function in zebrafish (*Danio rerio*). J. Comp. Physiol. B. 188, 237–253.
Leigh, S.C., Papastamatiou, Y.P., German, D.P., 2018b. Seagrass digestion by a notorious "carnivore". Proc. R. Soc. B Biol. Sci. 285, 20181583.

Leigh, S.C., Papastamatiou, Y.P., German, D.P., 2021. Gut microbial diversity and digestive function of an omnivorous shark. Mar. Biol. 168, 55.
Leigh, S.C., Catabay, C., German, D.P., 2022. Sustained changes in digestive physiology and microbiome across sequential generations of zebrafish fed different diets. Comp. Biochem. Physiol. A 273, 111285.
Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinform. 12, 323.
Lindsay, G.J.H., 1984. Distribution and function of digestive tract chitinolytic enzymes in fish. J. Fish Biol. 24, 529–536.
Liu, Y., Li, X., Li, Y., Li, J., Zhu, S., 2022. Gut microbiomes of cyprinid fish exhibit host-species symbiosis along gut trait and diet. Front. Microbiol. 13.
Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5.
Lujan, N.K., German, D.P., Winemiller, K.O., 2011. Do wood grazing fishes partition their niche? Morphological and isotopic evidence for trophic segregation in Neotropical Loricariidae. Funct. Ecol. 25, 1327–1338.
Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P.J., Baurhoo, B., Zhao, X., Guan, L. L., 2012. Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiol. Ecol. 79, 337–347.
Mazumder, S.K., Das, S.K., Rahim, S.M., Ghaffar, M.A., 2018. Temperature and diet effect on the pepsin enzyme activities, digestive somatic index and relative gut length of Malabar blood snapper (Lutjanus malabaricus Bloch & Schneider, 1801). Aquacult. Rep. 9, 1–9.
McCauley, M., German, D.P., Lujan, N.K., Jackson, C.R., 2020. Gut microbiomes of sympatric Amazonian wood-eating catfishes (Loricariidae) reflect host identity but little role for in wood digestion. Ecol. Evolut. 10, 7117–7128.
Miura, Y., Kageyama, T., Moriyama, A., 2015. Pepsinogens and pepsins from largemouth bass, *Micropterus salmoides*: purification and characterization with special reference to high proteolytic activities of bass enzymes. Com. Biochem. Physiol. B 183, 42–48.
Murray, H., Gallant, J., Perez-Casanova, J., Johnson, S., Douglas, S., 2003. Ontogeny of lipase expression in winter flounder. J. Fish Biol. 62, 816–833.
Muzzarelli, R.A.A., 1999. Native, industrial, and fossil chitins. In: Jolles, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhauser Verlag, Basel, Switzerland, pp. 1–6.
Narita, Y., Oda, S., Takenaka, O., Kageyama, T., 2010. Lineage-specific duplication and loss of pepsinogen genes in hominoid evolution. J. Mol. Evol. 70, 313–324.
Navarro-Guillén, C., Yúfera, M., Perera, E., 2022. Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, *Seriola dumerili*. Front. Mar. Sci. 9, 960746.
Neighbors, M.A., Horn, M.H., 1991. Nutritional quality of macrophytes eaten and not eaten by two temperate-zone herbivorous fishes: a multivariate comparison. Mar. Biol. 108, 471–476.
Nelson, J.A., Wubah, D., Whitmer, M., Johnson, E., Stewart, D., 1999. Wood-eating catfishes of the genus *Panaque*: gut microflora and cellulolytic enzyme activities. J. Fish Biol. 54, 1069–1082.
Newton, K.C., Wraith, J., Dickson, K.A., 2015. Digestive enzyme activities are higher in the shortfin mako shark, *Isurus oxyrinchus*, than in ectothermic sharks as a result of visceral endothermy. Fish Physiol. Biochem. 41, 887–898.
Nicholson, G.M., Clements, K.D., 2020. Resolving resource partitioning in parrotfishes (Scarini) using microhistology of feeding substrata. Coral Reefs 39, 1313–1327.
Nicholson, G.M., Clements, K.D., 2021. Ecomorphological divergence and trophic resource partitioning in 15 syntopic indo-Pacific parrotfishes (Labridae: Scarini). Biol. J. Linn. Soc. 132, 590–611.
Nie, Y.G., Wei, F.W., Zhou, W.L., Hu, Y.B., Senior, A.M., Wu, Q., Yan, L., Raubenheimer, D., 2019. Giant pandas are macronutritional carnivores. Curr. Biol. 29, 1677–1682.e1672.
Olsen, R.E., Ringø, E., 1997. Lipid digestibility in fish: a review. Recent Res. Develop. Lipid Res. 1, 199–264.
Painter, T.J., 1983. Algal polysaccharides. In: Aspinall, G.O. (Ed.), The Polysaccharides, 2. Academic Press Inc., New York, NY, pp. 196–285.
Pardesi, B., Roberton, A.M., Lee, K.C., Angert, E.R., Rosendale, D.I., Boycheva, S., White, W.L., Clements, K.D., 2022. Distinct microbiota composition and fermentation products indicate functional compartmentalization in the hindgut of a marine herbivorous fish. Mol. Ecol. 31, 2494–2509.
Pérès, G., Bogé, G., Colin, D.A., Rigal, A., 1973. Effets de la température sur les processus digestifs des poissons, activités enzymatiques et absorption intestinale. Revue des Travaux de l'Institut Peches Maritimes 37, 223–232.
Pohlner, M., Dlugosch, L., Wemheuer, B., Mills, H., Engelen, B., Reese, B.K., 2019. The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: a molecular approach to link their distribution to environmental conditions. Front. Microbiol. 10.
Punta, M., Coggill, P.C., Eberhardt, R.Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E.L., Eddy, S. R., Bateman, A., Finn, R.D., 2012. The Pfam protein families database. Nucleic Acids Res. 40, D290–D301.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596.
Rameshkumar, N., Lang, E., Tanaka, N., 2016. Description of *Vogesella oryzae* sp. nov., isolated from the rhizosphere of saline tolerant pokkali rice. Syst. Appl. Microbiol. 39, 20–24.
Raubenheimer, D., Zemke-White, W.L., Phillips, R.J., Clements, K.D., 2005. Algal macronutrients and food selectivity by the omnivorous marine fish *Girella tricuspidata*. Ecology 86, 2601–2610.
Reissig, J.L., Strominger, J.L., Leloir, L.F., 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217, 959–966.
Rivera-Chávez, F., Lopez, C.A., Bäumler, A.J., 2017. Oxygen as a driver of gut dysbiosis. Free Radic. Biol. Med. 105, 93–101.
Roberts, A., Pachter, L., 2013. Streaming fragment assignment for real-time analysis of sequencing experiments. Nat. Methods 10, 71–73.
Roncari, G., Zuber, H., 1969. Thermophilic aminopeptidases from *Bacillus stearothermophilus*. I. Isolation, specificity, and general properties of the thermostable aminopeptidase I. Int. J. Protein Res. 1, 45–61.
Sabapathy, U., Teo, L.H., 1993. A quantitative study of some digestive enzymes in the rabbitfish, *Siganus canaliculatus* and the sea bass, *Lates calcarifer*. J. Fish Biol. 42, 595–602.
Sæle, Ø., Nordgreen, A., Olsvik, P.A., Hamre, K., 2010. Characterization and expression of digestive neutral lipases during ontogeny of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. A 157, 252–259.
Schaefer, S., Stewart, D., 1993. Systematics of the *Panaque dentex* species group (Siluriformes: Loricariidae), wood-eating armored catfishes from tropical South America. Ichthyol. Explorat. Freshwaters 4, 309–342.
Sibly, R.M., 1981. Strategies of digestion and defecation. In: Townsend, C.R., Calow, P. (Eds.), Physiological Ecology: An Evolutionary Approach to Resource Use. Sinauer Associates, Sunderland, pp. 109–139.
Sibly, R.M., Calow, P., 1986. Physiological Ecology of Animals, an Evolutionary Approach. Blackwell Scientific Publications, Oxford, England.
Skea, G., Mountfort, D., Clements, K.D., 2005. Gut carbohydrases from the New Zealand marine herbivorous fishes *Kyphosus sydneyanus* (Kyphosidae), *Aplodactylus arctidens* (Aplodactylidae), and *Odax pullus* (Labridae). Com. Biochem. Physiol. B 140, 259–269.
Skea, G., Mountfort, D., Clements, K.D., 2007. Contrasting digestive strategies in four New Zealand herbivorous fishes as reflected by carbohydrase activity profiles. Com. Biochem. Physiol. B 146, 63–70.
Sommer, F., Anderson, J.M., Bharti, R., Raes, J., Rosenstiel, P., 2017. The resilience of the intestinal microbiota influences health and disease. Nat. Rev. Microbiol. 15, 630–638.
Sparagon, W.J., Gentry, E.C., Minich, J.J., Vollbrecht, L., Laurens, L.M.L., Allen, E.E., Sims, N.A., Dorrestein, P.C., Kelly, L.W., Nelson, C.E., 2022. Fine scale transitions of the microbiota and metabolome along the gastrointestinal tract of herbivorous fishes. Animal Microbiome 4, 33.
Stevens, C.E., Hume, I.D., 1995. Comparative Physiology of the Vertebrate Digestive System, Second ed. Press Syndicate of the University of Cambridge, Melbourne, Australia.
Stevenson, S.J.R., Lee, K.C., Handley, K.M., Angert, E.R., White, W.L., Clements, K.D., 2022. Substrate degradation pathways, conserved functions and community composition of the hindgut microbiota in the herbivorous marine fish *Kyphosus sydneyanus*. Comp. Biochem. Physiol. A 272, 111283.
Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S., Russell, J.A., 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol. Ecol. 21, 3363–3378.
Tamelander, T., Renaud, P.E., Hop, H., Carroll, M.L., Ambrose, W.G.J., Hobson, K.A., 2006. Trophic relationships and pelagic–benthic coupling during summer in the Barents Sea marginal ice zone, revealed by stable carbon and nitrogen isotope measurements. Mar. Ecol. Prog. Ser. 310, 33–46.
Tang, S.-L., Liang, X.-F., He, S., Li, L., Alam, M.S., Wu, J., 2022. Comparative study of the molecular characterization, evolution, and structure modeling of digestive lipase genes reveals the different evolutionary selection between mammals and fishes. Front. Genet. 13.
Tarnecki, A.M., Burgos, F.A., Ray, C.L., Arias, C.R., 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. J. Appl. Microbiol. 123, 2–17.
Tengjaroenkul, B., Smith, B., Caceci, T., Smith, S.A., 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture 182, 317–327.
Tresierra-Aguilar, A., 1980. Life History of the Snake Prickleback, *Lumpenus sagitta* Wilimovsky, 1956, MS Thesis. Oregon State University, Corvalis, OR, Fisheries and Wildlife, p. 75.
Tropini, C., Earle, K.A., Huang, K.C., Sonnenburg, J.L., 2017. The gut microbiome: connecting spatial organization to function. Cell Host Microbe 21, 433–442.
van Kessel, M., Dutilh, B., Neveling, K., Kwint, M., Veltman, J., Flik, G., Jetten, M., Klaren, P., Op den Camp, H., 2011. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). AMB Express 1, 41.
Verri, T., Barca, A., Pisani, P., Piccinni, B., Storelli, C., Romano, A., 2017. Di- and tripeptide transport in vertebrates: the contribution of teleost fish models. J. Comp. Physiol. B. 187, 395–462.
Vervaet, A., 2019. Assessing the Presence of Chitinases in the Digestive Tract and their Relationship to Diet and Morphology in Freshwater Fish, PhD Dissertation. Ghent University, Ghent, Belgium, Veterinary Medicine, p. 67.
Vonk, H.J., Western, J.R.H., 1984. Comparative Biochemistry and Physiology of Enzymatic Digestion. Academic Press, London.
Wagner, C.E., McIntyre, P.B., Buels, K.S., Gilbert, D.M., Michel, E., 2009. Diet predicts intestine length in Lake Tanganyika's cichlid fishes. Funct. Ecol. 23, 1122–1131.
Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R., 2016. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 1 e00009-00015.
Wright, D.B., Escalona, M., Marimuthu, M.P.A., Sahasrabudhe, R., Nguyen, O., Sacco, S., Beraut, E., Toffelmier, E., Miller, C., Shaffer, H.B., Bernardi, G., German, D.P., 2023.

Reference genome of the monkeyface Prickleback, *Cebidichthys violaceus*. J. Hered. 114, 52–59.
Yawitz, T.A., Barts, N., Kohl, K.D., 2022. Comparative digestive morphology and physiology of five species of Peromyscus under controlled environment and diet. Comp. Biochem. Physiol. A 271, 111265.
Zheng, L., Kelly, C.J., Colgan, S.P., 2015. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A review in the theme: cellular responses to hypoxia. Am. J. Phys. Cell Phys. 309, C350–C360.