More than one way to be an herbivore: convergent evolution of herbivory using different digestive strategies in prickleback fishes (Stichaeidae)

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ARTICLE INFO

ABSTRACT

In fishes, the evolution of herbivory has occured within a spectrum of digestive strategies, with two extremes on opposite ends: (i) a rate-maximization strategy characterized by high intake, rapid through-put of food through the gut, and little reliance on microbial digestion or (ii) a yield-maximization strategy characterized by measured intake, slower transit of food through the gut, and more of a reliance on microbial digestion in the hindgut. One of these strategies tends to be favored within a given clade of fishes. Here, we tested the hypothesis that rate or yield digestive strategies can arise in convergently evolved herbivores within a given lineage. In the family Stichaeidae, convergent evolution of herbivory occured in *Cebidichthys violaceus* and *Xiphister mucosus*, and despite nearly identical diets, these two species have different digestive physiologies. We found that *C. violaceus* has more digesta in its distal intestine than other gut regions, has comparatively high concentrations (>11 mM) of short-chain fatty acids (SCFA, the endpoints of microbial fermentation) in its distal intestine, and a spike in β-glucosidase activity in this gut region, findings that, when coupled to long retention times (>20 h) of food in the guts of *C. violaceus*, suggest a yield-maximizing strategy in this species. *X. mucosus* showed none of these features and was more similar to its sister taxon, the omnivorous *Xiphister atropurpureus*, in terms of digestive enzyme activities, gut content partitioning, and concentrations of SCFA in their distal intestines. We also contrasted these herbivores and omnivores with other sympatric stichaeid fishes, *Phytichthys chirus* (omnivore) and *Anoplarchus purpurescens* (carnivore), each of which had digestive physiologies consistent with the consumption of animal material. This study shows that rate- and yield-maximizing strategies can evolve in closely related fishes and suggests that resource partitioning can play out on the level of digestive physiology in sympatric, closely related herbivores.

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1. Introduction

What an animal eats and how it digests its food directly affects that animal's role in its biotic community (Karasov and Martínez del Rio, 2007; Karasov, 2011). Yet, beyond gut content and ecomorphological analyses, animal nutritional physiology is often ignored. In particular, fish nutritional ecology and physiology remain woefully understudied, which has created mismatches in the states of fish vs. terrestrial vertebrate nutritional ecology and physiology (Choat and Clements, 1998; Clements et al., 2009, 2014). Recently, studies began to shed light on fish dietary adaptations, revealing that dietary specialization in the largest vertebrate class exists on the levels of the community (Lujan et al., 2011), ecomorphology (Ferry-Graham et al., 2010; Wainwright et al., 2012), on down to individual species' digestive biochemistry and metabolism (Crossman et al., 2005; Skea et al., 2005, 2007; Willmott et al., 2005; German et al., 2010a, 2014). Moreover, phylogenetically informed studies of fishes show common patterns of diversification of gut morphology and digestive enzyme activities, namely, longer guts and elevated amylase (a carbohydrase) activities in herbivorous fishes, and shorter guts with elevated aminopeptidase (a protease) activities in carnivores (German et al., 2004, 2010a; German and Horn, 2006; German, 2009a; Day et al., 2011). Omnivores, if included, can occupy positions in the middle of these spectra, although there are exceptions (German et al., 2014).

Beyond these common patterns of gut size and digestive enzyme activities, there exists a spectrum of digestive strategies for fishes consuming herbivorous and detritivorous diets that fit within a

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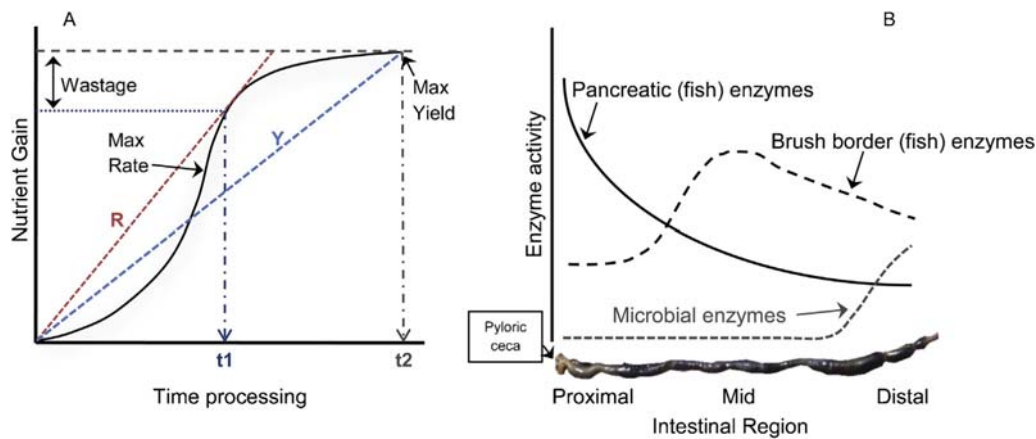 *E-mail address:* dgerman@uci.edu (D.P. German).

Fig. 1. (A) Cumulative nutrient gained (solid black line) by a fish as a function of time spent processing a meal (modified from Clements and Raubenheimer, 2006). The slope of the black line labeled "Max Rate" is the maximum rate at which the nutrient can be absorbed from the meal. A rate-maximizing strategy is characterized by a line tangential to the curve (red line "R"), with defecation of gut contents occurring at time 1 (t1). A portion of the nutrient consumed is lost in the feces ("Wastage"), but at t1 the animal can take a new meal. This is the rate-maximizing strategy with high intake. Maximum yield (blue line "Y") is attained by extending processing time to time 2 (t2); however, this is done at the price of a reduced digestive rate. In herbivores, this strategy tends to involve longer retention times of food in the gut and microbial fermentation. (B) Potential patterns of digestive enzyme activities along the stichaeid gut. Pancreatic enzymes are produced in the acinar cells, which, in stichaeids, are located in the pyloric ceca (Kim et al., 2014) that are attached to the proximal intestine. Thus, activities of pancreatic digestive enzymes would be expected to decrease along the intestine. Brush border enzymes tend to peak in the mid-intestine of many fishes. However, microbially produced enzymes peak in the distal intestine contents of fish utilizing a yield-maximizing strategy (see Skea et al., 2005). Fish adopting a rate-maximizing strategy would not show a spike in microbial digestive enzymes in their distal intestines (see German, 2009a,b; German and Bittong, 2009).

"rate vs. yield" theoretical framework (Sibly, 1981; Clements and Raubenheimer, 2006) (Fig. 1). Rate maximizers tend to have high intake of algae and/or detritus, rapid transit of food through the gut, and little microbial fermentation occurring in their digestive tracts (Crossman et al., 2005; German, 2009a,b; German and Bittong, 2009; Clements et al., 2014). Rate-maximizing fishes assimilate only those components of their food that are easily digestible by endogenous digestive enzymes (e.g., soluble carbohydrates, protein), passing less tractable components (e.g., cell wall constituents) in their feces (Crossman et al., 2005; German, 2009b). Rate maximizers compensate for fecal loss of nutrients (wastage) with high intake (Fig. 1). On the other end, yield maximizers are represented by herbivores that ingest algae, but these fishes tend to have slower transit of digesta through their alimentary canals (Clements and Rees, 1998; Choat et al., 2004) and well-developed microbial fermentation in their hindguts (Mountfort et al., 2002; Crossman et al., 2005), thereby allowing such fishes access to nutrients (e.g., mannitol; White et al., 2010) that might otherwise be indigestible to the fish via endogenous digestive mechanisms. Carnivores tend to fit more within a yield-maximizing strategy since they have a relatively low intake of food (with high protein content) and slower digesta transit, albeit carnivores are less reliant on microbial fermentation to meet their energetic demands (Stevens and Hume, 1998; German, 2009a; Clements et al., 2014).

A rate or yield strategy tends to be well-represented within a given phylogeny of fishes. For instance, herbivores in separate clades (i.e., convergent evolution of herbivory) within the family Cyprinidae tend to be rate maximizers, with long, thin-walled guts, rapid gut transit, and little microbial fermentation (German, 2009a; German et al., 2010a). The same can be said of herbivorous and detritivorous armored catfishes in the family Loricariidae (even those consuming wood) (German, 2009b; German and Bittong, 2009; Lujan et al., 2011, 2015), whereas herbivorous fishes in the genus *Kyphosus* all tend to be yield maximizers with high concentrations of short-chain fatty acids (SCFA) – the end points of microbial fermentation – in their hindguts (Clements and Choat, 1997; Mountfort et al., 2002; Crossman et al., 2005). Rate- *and* yield-maximizing strategies have evolved for herbivorous fishes (e.g., *Ctenochaetus striatus* vs. *Naso unicornis*) within the family

Acanthuridae (Crossman et al., 2005); however, these are fairly distantly related taxa within the phylogeny (Sorenson et al., 2013). It remains unknown how rate- and yield-maximizing strategies would manifest within the broader patterns of gut size and digestive enzyme activities known to represent adaptive diversification towards diet within closely related fishes that have converged on similar diets (Skea et al., 2005, 2007; German, 2009a; German et al., 2010a, 2014).

In the present study, we examined gut size and function in prickleback fishes (family Stichaeidae) to observe whether patterns of rate- or yield-maximizing strategies prevail for closely related fishes with different diets. We, therefore, tested the hypothesis that only a single strategy could be represented within closely related taxa. Fishes in the stichaeid phylogeny have emerged as excellent systems for studying adaptations of the digestive tract for specific diets (Kim et al., 2014) (Fig. 2). With dietary diversity, convergent evolution of herbivory, and sister taxa with different diets (German et al., 2004, 2014; German and Horn, 2006), the stichaeid phylogeny (Chereshnev et al., 2013; Kim et al., 2014) may be one of the best groups in which to examine whether gut diversification occurs one way in closely related fishes. Thus, we examined gut length, gut content masses, digestive enzyme activities, and levels of microbial fermentation in five stichaeid species with different diets (Fig. 2).

Our study had four parts. First, we examined the stomach contents of *Cebidichthys violaceus* (herbivore), *Phytichthys chirus* (omnivore), *Xiphister mucosus* (herbivore), *Xiphister atropurpureus* (omnivore), and *Anoplarchus purpurescens* (carnivore) to confirm whether these species were consuming diets consistent with what is known for these species in the literature (Fig. 2; Boyle and Horn, 2006; German and Horn, 2006). Second, we compared gut length, gut masses, and gut content masses among the five species to observe whether they exhibited patterns consistent with a rate- or yield-maximizing strategy. Although both rate- and yield-maximizing fishes can have long digestive tracts (German, 2011), rate maximizers do not concentrate gut contents in one region of the gut, whereas yield maximizers tend to have heavier hind guts and more contents in their hindguts at any given time (Choat et al., 2004; Crossman et al., 2005; German, 2009a). Third, we examined patterns of digestive enzyme activities in the fishes'
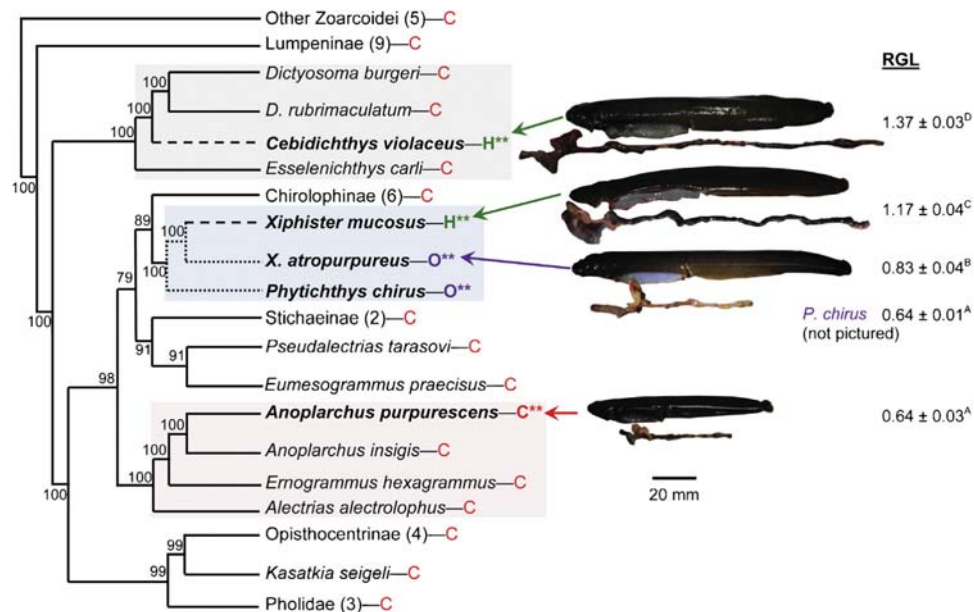
Fig. 2. Phylogenetic relationships of the family Stichaeidae based on 2100 bp of *cytb*, *16s*, and *tomo4c4* genes (Kim et al., 2014). Bayesian posterior probabilities are indicated on nodes. Species used in this study are in bold script and indicated with **. H: herbivory, O: omnivory, and C: carnivory. Evolution of herbivory (- - - -) and omnivory (.......) are shown. Numbers in parentheses show number of taxa evaluated at that branch. Boxes highlight alleged tribes within the polyphyletic subfamily Xiphisterinae, with Esselenichthyini (top), Xiphisterini (middle), and Alectriini (bottom) all highlighted (Kim et al., 2014). Esselenichthyini is recognized as Cebidichthyidae, and Xiphisterini as Xiphisteridae by Chereshnev et al. (2013). Relative gut length [RGL = gut length (mm)/standard length (mm)] values are shown for the studied fish species on right side. RGL varied significantly among the species ($F_{5,64}$ = 124.42; $P < 0.001$), and those values sharing a superscript letter are not significantly different.

intestines to see whether they matched with either a rate- or yield-maximizing strategy (Fig. 1). We measured the activity levels of the carbohydrases amylase, maltase, N-acetyl-β-D-glucosaminidase, and β-glucosidase, the proteases trypsin and aminopeptidase, and of lipase and observed how the activities of these enzymes changed moving down the gut, both in the fishes' tissues, and in gut contents that would represent microbially produced enzymes (Skea et al., 2005, 2007; German and Bittong, 2009) (Table 1 and Fig. 1). Finally, we measured the concentrations of SCFA present in the distal-most region of the fishes' intestines to confirm whether there were active fermentations occurring in any of the species, with the expectation that yield maximizers would have elevated levels of SCFA in their hindguts, whereas rate maximizers would not. Overall, our design allowed us to test whether a single digestive strategy – rate- vs. yield-maximizing – can arise within closely related fishes.

2. Materials and methods

2.1. Fish capture, tissue preparation, and gut content analyses

Six individuals each of *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *A. purpurescens* were collected by hand and dipnet in November 2011 at low tide from rocky intertidal habitats on the central California coast near Piedras Blancas (35.65° N, 121.24° W). Nine individuals of *P. chirus* were collected from rocky intertidal habitats at low tide on San Juan Island (Dead Man Bay; 48.50° N, 123.13° W) in July 2012. Although the range of *P. chirus* extends to California, they are more abundant in Washington (German et al., 2014). Thus, to get sufficient sample sizes, this species was collected in Washington.

Each fish was euthanized with an overdose of MS-222 (1 g l⁻¹ seawater), measured (standard length, SL ± 0.5 mm), weighed (body mass, BM ± 0.1 g) and dissected on a cutting board kept on ice (4 °C) within 4 h of collection. The fish sizes are presented in Table 2. Each digestive system was removed by cutting just anterior to the stomach and at the anus. The guts were gently uncoiled, measured (gut length, GL), and the stomachs excised. The stomachs were placed in individual centrifuge vials and frozen in liquid nitrogen for later use in gut content analyses (Boyle and Horn, 2006; German and Horn, 2006). The intestine was divided into three (*C. violaceus*, *X. mucosus*, and *X. atropurpureus*) or two (*P. chirus* and *A. purpurescens*) sections of equal length, designated as the proximal, mid, or distal intestine (Fig. 1; with *P. chirus* and *A. purpurescens* lacking a mid-intestine section).

Table 1
Digestive enzymes assayed in intestinal tissues and intestinal contents in prickleback fishes in the present study.

Enzyme	Synthesis[a]	Substrate	Dietary source	Expected pattern[b]	>Fraction[c]
Amylase	Pancreatic	Starch, α-glucans	Algae	Decrease	Tissue
Maltase	Brush border	Maltose	Algae	Middle spike	Tissue
β-Glucosidase	Microbial	β-Glucosides	Algae	Distal spike	Contents
N-acetyl-β-D-glucosaminidase	Brush border	N-acetyl-β-D-glucoaminides	Crustaceans	Middle spike	Tissue
Trypsin	Pancreatic	Protein	Algae, animals	Decrease	Tissue
Aminopeptidase	Brush border	Dipeptides	Algae, animals	Middle spike	Tissue
Lipase	Pancreatic	Lipid	Algae, animals	Decrease	Tissue

[a] Indicates where the enzyme is synthesized, either from fish (pancreatic or brush border) or from microbial sources.

[b] This column shows the expected patterns of activity along the gastrointestinal tracts of the fishes. For example, "decrease" means that the activity of this enzyme should decrease toward the distal intestine of the fish, whereas a "distal spike" means that the enzyme is expected to increase in activity in the distal intestine (see Fig. 1).

[c] Predictions of which assayed fractions will have higher activity of a particular enzyme. "Tissue" means that the activity of that enzyme is expected to be greater in the fishes' gut tissues than in the intestinal contents of a given gut region. "Contents" means that the activity of this microbially produced enzyme should be greater in the gut contents than in the gut tissue.

Table 2
Body sizes of prickleback fishes used for measurements of digestive enzyme activities. Values are mean ± SEM. Comparisons of body length and mass among species were made with ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$. Body size values for different species that share a superscript letter are not significantly different. $N = 6$ for *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *A. purpurescens*. $N = 9$ for *P. chirus*. H: herbivore, O: omnivore, and C: carnivore.

Species	Standard length (mm)	Body mass (g)
Cebidichthys violaceus (H)	152.82 ± 6.76^b	22.52 ± 3.13^b
Phytichthys chirus (O)	108.33 ± 6.92^a	5.67 ± 1.26^a
Xiphister mucosus (H)	160.85 ± 11.70^b	16.52 ± 4.11^b
Xiphister atropurpureus (O)	183.48 ± 6.51^b	19.77 ± 1.99^b
Anoplarchus purpurescens (C)	80.42 ± 1.76^a	2.63 ± 0.20^a
$F_{4,32}$	30.12	13.64
P	<0.001	<0.001

Each section was emptied of their contents by pushing with the blunt side of a razorblade, and the contents and intestinal tissues placed in separate centrifuge vials and frozen in liquid nitrogen (German and Bittong, 2009). Frozen samples were transported in liquid nitrogen or on dry ice to UC Irvine where they were stored at −80 °C until analyzed (within 6 months). Gut lengths and body lengths were used to calculate relative gut length [RGL = gut length (mm) × standard length (mm)$^{-1}$], and regional intestinal masses were used to calculate relative regional gut mass [RRGM = regional gut mass (g) × body mass (g)$^{-1}$]. Regional gut content masses were used to calculate relative regional gut content mass [RRGCM = regional gut content mass (g) × body mass (g)$^{-1}$]. The masses of the gut regions were added up for each individual fish to get a total gut mass for the calculation of relative total gut mass [RTGM = total gut mass (g) × body mass (g)$^{-1}$] (German and Horn, 2006).

Gut tissues or contents from each gut region from individual fish were weighed (regional gut or content mass ± 0.001 g) and homogenized following German and Bittong (2009). Intestinal contents were homogenized in 25 mM Tris–HCl, pH 7.5, whereas intestinal tissues were homogenized in 350 mM mannitol with 1 mM HEPES, pH 7.5. The supernatants of homogenates were collected and stored in small aliquots (100–200 μl) at −80 °C until just before use in spectrophotometric or fluorometric assays of digestive enzyme activities. The protein content of the homogenates was measured using bicinchoninic acid (Smith et al., 1985; German and Bittong, 2009).

Stomach contents were analyzed from all specimens following Boyle and Horn (2006). Contents were gently squeezed from the stomach tissue into a petri dish filled with deionized water, and under a dissecting microscope (AmScope, Irvine, CA, USA) 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 species. Following German et al. (2004), diets for each fish species were condensed to the average percent algal or animal material in the stomachs of the five fish species. For more detailed gut content analyses on the studied species, see German and Horn (2006), Boyle and Horn (2006), and German et al. (2014).

2.2. Assays of digestive enzyme activity

All assays were carried out at 15 °C in duplicate or triplicate using a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer equipped with a monochromator (BioTek, Winooski, VT, USA). All assay protocols generally followed methods detailed in German

and Bittong (2009), unless otherwise noted. All pH values listed for buffers were measured at room temperature (22 °C), and all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from the five species (German et al., 2004, 2014). Each enzyme activity was measured in each gut region of each individual fish, and control experiments with blanks consisting of substrate only and homogenate only (in buffer) were conducted simultaneously to account for endogenous substrate and/or product in the tissue homogenates and substrate solutions.

The α-amylase activity was measured using 1% potato starch dissolved in 25 mM Tris–HCl containing 1 mM CaCl$_2$. Previous work had shown that low concentrations of Tris are suitable for the measurement of amylase and maltase (German and Bittong, 2009). The α-amylase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per min) per gram wet weight of gut tissue.

Maltase activities were measured following Dahlqvist (1968), as described by German and Bittong (2009). We used 112 mM maltose dissolved in 200 mM phosphate buffer, pH 7.5. The maltase activity was determined from a glucose standard curve and expressed in U (μmol glucose liberated per min) per gram wet weight of gut tissue.

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

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

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

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

The activity of each enzyme was regressed against the protein content of the homogenates to confirm that there were no significant correlations between the two variables. Because no significant correlations were observed, the data are not reported as U per mg protein. Total standardized gut enzymatic activity (TSGA) was calculated for each enzyme by multiplying the mass-specific activity for each section by the weight of the intestinal tissue for that section and then summing the results from each section to get a total gut activity (as U min^{-1}). This was done to quantify the digestive

capacity of the entire gut for each enzyme (Horn et al., 2006; Day et al., 2011).

2.3. Gut fluid preparation and gastrointestinal fermentation

Measurements of symbiotic fermentation activity were based on the methods of Pryor and Bjorndal (2005), as described in German and Bittong (2009). Fermentation activity was indicated by relative concentrations of SCFA in the fluid contents of the distal intestines of the fishes at the time of death. An additional 14 individuals each of *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *A. purpurescens* were collected in June 2013 and July 2014, as described above, and distal intestine contents were frozen in sterile centrifuge vials. Distal intestine gut content samples were weighed [gut content mass (GCM ± 0.001 g)], thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4 °C) at 16,000 × g for 10 min. The supernatant was then pipetted into a sterile centrifuge vial equipped with a 0.22 μm cellulose acetate filter (Costar Spin-X gamma sterilized centrifuge tube filters; Coming, NY, USA) and centrifuged under refrigeration at 13,000 × g for 5 min to remove particles from the fluid (including bacterial cells). The filtrates were collected and frozen until they were analyzed for SCFA and nutrient concentrations. Estimates of the levels of fermentation were not made for *P. chirus* due to limited sampling of this species.

Concentrations of SCFA in the gut fluid samples from each gut region were measured using gas chromatography. Samples were hand-injected into a Shimadzu GC-mini-2 gas chromatograph equipped with a flame ionization detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and attached to a Hewlett-Packard HP3392A integrator (Hewlett-Packard Co., Palo Alto, CA, USA). Two microliters of each sample were injected onto a 2 m-long stainless steel column (3.2 mm ID) packed with 10% SP-1000 and 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA, USA). An external standard containing 100 mg l^{-1} each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate was used for calibration. The SCFA concentrations are expressed as mM of gut fluid.

2.4. Statistical analyses

Prior to all significance tests, Levene's test for equal variance was performed and residuals vs. fits plots were examined to ensure the appropriateness of the data for parametric analyses. Where necessary, data were log-transformed prior to analysis. All tests were run using SPSS statistical software (version 20). Intraspecific comparisons of mass-specific enzymatic activities, regional relative gut mass, and regional relative gut content mass were made among intestinal regions with ANOVA followed by Tukey's HSD test with a family error rate of $P = 0.05$. Interspecific comparisons of SCFA concentrations from the distal intestines of the five species, as well as body lengths and masses were made with ANOVA. TSGA for each enzyme and relative gut length were compared among species with ANCOVA (using body length as a covariate) followed by Tukey's HSD test with a family error rate of $P = 0.05$. Heterogeneity of slopes was tested with the interaction term of species and body length, which was never significant in our analyses (confirming the appropriateness of ANCOVA). Interspecific comparisons of regional enzymatic activity were not made because of the lack of a mid-intestine in *P. chirus* and *A. purpurescens* (German, 2009a). Overall, our design allowed us to test whether prickleback fishes follow digestive strategies outlined in Fig. 1 and Table 1, and how these vary among the fishes with different diets.
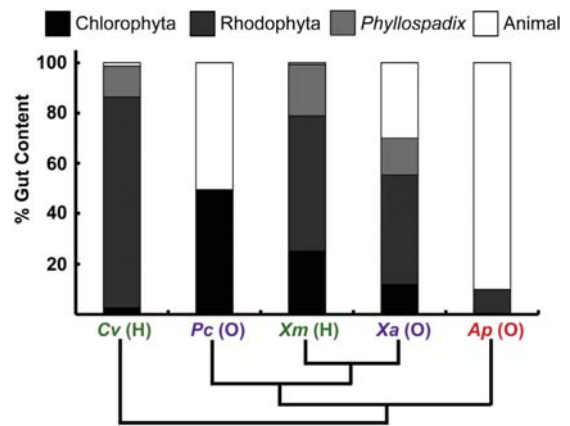

Fig. 3. Percentage of chlorophyte, rhodophyte, *Phyllospadix* (sea grass) and algal material in gut contents of wild-caught *Cebidichthys violaceus* (*Cv*), *Phytichthys chirus* (*Pc*), *Xiphister mucosus* (*Xm*), *Xiphister atropurpureus* (*Xa*), and *Anoplarchus purpurescens* (*Ap*); $n = 6$ for *Cv*, *Xm*, and *Xa*; $n = 7$ for *Ap*; and $n = 14$ for *Pc*. H: herbivory, O: omnivory, and C: carnivory. The clade shows a pruned tree for the analyzed taxa. See Fig. 2 for the full phylogeny.

3. Results

3.1. Diet, relative gut length, and gut content masses

C. violaceus (84%) and *X. mucosus* (54%) each consumed diets dominated by red algae (Rhodophyta), primarily the epiphytic species *Smithora naiadum*, but also including *Porphyra perforata* and *Mazzaella flaccida* (Fig. 3). *X. mucosus* (25%) consumed more of the green alga *Ulva lobata* (Chlorophyta) than did *C. violaceus* (3%), and each species consumed some of the sea grass *Phyllospadix* sp., probably in pursuit of the epiphytic *S. naiadum*, which grows on *Phyllospadix* blades. Each of these herbivorous species had about 1% of their gut content masses represented by amphipods. *X. atropurpureus* had a diet consisting of 43% Rhodophyta, 12% Chlorophyta, and 15% Phyllospadix material, the latter probably in pursuit of *S. naiadum*, which dominated the rhodophyte material in the guts of this species. *X. atropurpureus* had 30% animal material in their stomachs, dominated by polychaetes and crustaceans (Fig. 3). *P. chirus* showed equal consumption (50% each) of *Ulva lobata* and small crustaceans (amphipods, decapods, isopods), whereas *A. purpurescens* consumed 90% animal foods represented by various worm groups (Nemerta, Sipunculida, Polychaeta), and 10% of the green alga *Ulva lobata* (Fig. 3). Significant differences in relative gut length were detected among the five species (ANCOVA species: $F_{5,64} = 124.42$, $P < 0.001$; body length: $F_{1,55} = 2.48$, $P = 0.121$), with *C. violaceus* possessing the longest guts, followed by *X. mucosus*, *X. atropurpureus*, *P. chirus*, and *A. purpurescens*, in order, with the final two species not different from one another (Fig. 2).

The five species also showed differences in relative regional gut mass. *C. violaceus* possessed proximal and distal intestinal sections of equivalent relative mass, and each was significantly heavier ($F_{2,17} = 16.32$, $P < 0.001$) than the mid-intestine in this species (Fig. 4A). Both *Xiphister* taxa had heavier proximal intestinal segments than either their mid or distal intestines, which were not different from each other (*X. mucosus*: $F_{2,17} = 13.93$, $P < 0.001$; *X. atropurpureus*: $F_{2,17} = 19.52$, $P < 0.001$). *P. chirus* ($t = 1.09$, d.f. = 16, $P = 0.292$) and *A. purpurescens* ($t = 0.46$, d.f. = 10, $P = 0.657$) had proximal and distal intestine segments of equal mass (Fig. 4A). *C. violaceus* showed significant differences in relative regional gut content masses among gut regions ($F_{2,17} = 4.73$, $P = 0.026$), with significantly more contents in their distal intestine than either their proximal or mid-intestine (Fig. 4B). *X. mucosus* and *X. atropurpureus* showed no differences in relative regional gut content
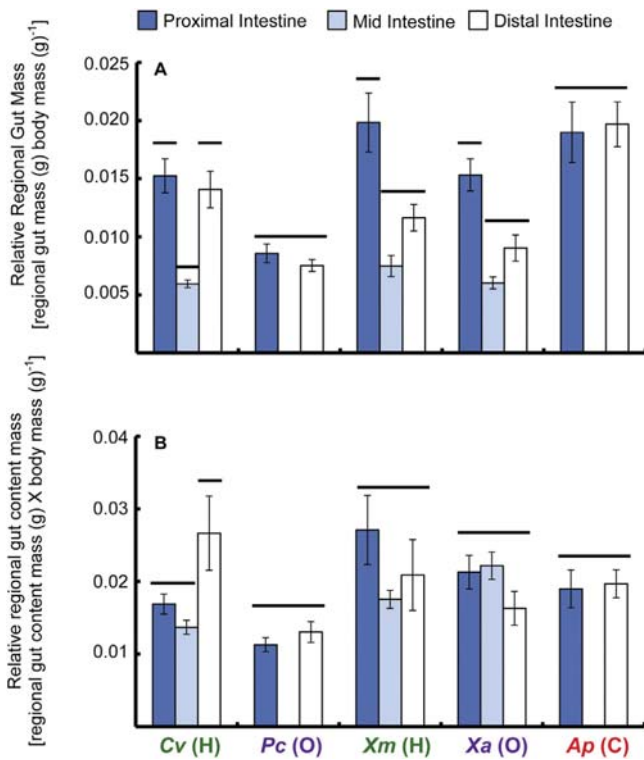
Fig. 4. (A) Relative regional gut mass, (B) relative regional gut content mass in *Cebidichthys violaceus* (*Cv*), *Phytichthys chirus* (*Pc*), *Xiphister mucosus* (*Xm*), *Xiphister atropurpureus* (*Xa*), and *Anoplarchus purpurescens* (*Ap*). Values are mean ± standard error. $n = 6$ for *Cv*, *Xm*, and *Xa*; $n = 7$ for *Ap*; and $n = 9$ for *Pc*. H: herbivory, O: omnivory, and C: carnivory. Intraspecific comparisons made with ANOVA, where lines of a different elevation for a given metric and species indicate significant differences among the gut regions for that species. Interspecific comparisons were not made. Note that there is no mid-intestine section for *Pc* and *Ap*.

Fig. 5. Amylase (top) and aminopeptidase (bottom) activities in different regions of the digestive tracts of *Cebidichthys violaceus* (*Cv*), *Phytichthys chirus* (*Pc*), *Xiphister mucosus* (*Xm*), *Xiphister atropurpureus* (*Xa*), and *Anoplarchus purpurescens* (*Ap*). Values are mean ± standard error. $n = 6$ for *Cv*, *Xm*, *Xa*, and *Ap*; and $n = 9$ for *Pc*. H: herbivory, O: omnivory, and C: carnivory. Intraspecific comparisons made with ANOVA, where lines of a different elevation for a given enzyme and species indicate significant differences among the gut regions for that species. Interspecific comparisons were not made. Note that there is no mid-intestine section for *Pc* and *Ap*.

masses among intestinal regions (*X. mucosus*: $F_{2,17} = 1.47$, $P = 0.262$; *X. atropurpureus*: $F_{2,17} = 2.12$, $P = 0.155$) (Fig. 4B). Neither *P. chirus* ($t = 1.02$, *d.f.* = 16, $P = 0.323$) nor *A. purpurescens* ($t = 0.22$, *d.f.* = 10, $P = 0.834$) showed differences in gut content masses among their proximal or distal intestines (Fig. 4B).

3.2. Digestive enzyme activities

C. violaceus showed significant differences in amylase activities among gut regions ($F_{3,23} = 12.34$, $P < 0.001$), with significantly higher activities in their pyloric ceca and proximal intestine than either their mid or distal intestine, which were not different from one another (Fig. 5). The same pattern was observed for amylase activity in *X. mucosus* ($F_{3,23} = 10.58$, $P < 0.001$) and *X. atropurpureus* ($F_{3,23} = 20.44$, $P < 0.001$). Differences in amylase activity were not detected among the gut regions of *P. chirus* ($F_{2,26} = 2.59$, $P = 0.096$) and *A. purpurescens* ($F_{2,17} = 0.34$, $P = 0.715$). In terms of TSGA, *X. atropurpureus* possessed significantly higher amylase activity than all other species (ANCOVA species: $F_{4,32} = 27.22$, $P < 0.001$; body length: $F_{1,27} = 12.34$, $P < 0.001$) except *X. mucosus*, which, in turn, was not different from *C. violaceus* (Fig. 6). *P. chirus* and *A. purpurescens* had the lowest amylase activities that were not different from one another (Fig. 6). Body size was a significant covariate, likely because of the differences in size among the species (Table 2), which reflects their natural differences in maximum body size (see German et al., 2014).

For aminopeptidase, *C. violaceus* showed significant differences in activity among gut regions ($F_{3,23} = 28.54$, $P < 0.001$), with significantly higher activity in their mid-intestine than in their other gut
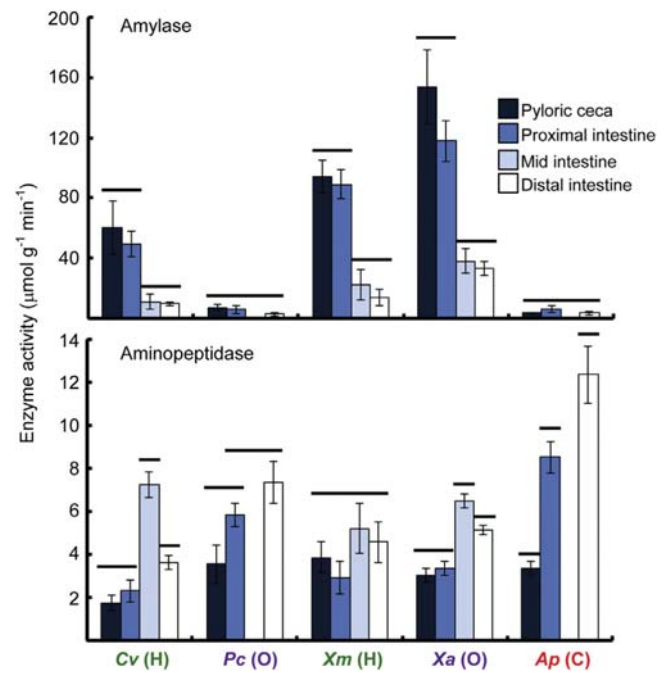
regions (Fig. 5). *C. violaceus* also had significantly greater activity in their distal intestine than their pyloric ceca or proximal intestine, which were not different from one another. An identical pattern of aminopeptidase activity was apparent in *X. atropurpureus* ($F_{3,23} = 28.25$, $P < 0.001$), whereas *X. mucosus* showed a similar pattern, but the differences in activity were not different ($F_{3,23} = 1.14$, $P = 0.356$) among intestinal regions. *P. chirus* showed an increasing pattern of aminopeptidase activity moving distally along the intestine, with their distal intestine showing significantly greater activity ($F_{2,26} = 5.34$, $P = 0.012$) than their pyloric ceca. Aminopeptidase activity in the *P. chirus* proximal intestine was not significantly different from the activity of this enzyme in their pyloric ceca or distal intestine (Fig. 5). *A. purpurescens* also showed increasing activity moving distally along the intestine, with the activity in each intestinal region significantly greater ($F_{2,17} = 25.43$, $P < 0.001$) than in the one before. In terms of TSGA, *C. violaceus*, *X. mucosus*, and *X. atropurpureus* showed no differences among each other in aminopeptidase activity, but each of these species possessed greater aminopeptidase activity than *P. chirus* or *A. purpurescens*, which were not different from one another (ANCOVA species: $F_{4,32} = 13.34$, $P < 0.001$; body length: $F_{1,27} = 18.04$, $P < 0.001$) (Fig. 6). Overall, the patterns of TSGA for amylase and aminopeptidase generally follow those seen for relative total gut mass and body size (see Table 2 and the supplementary Fig. S1 in the online Appendix). Thus, the fishes with the larger gut masses (i.e., those that consume the most algae, *C. violaceus*, *X. mucosus*, and *X. atropurpuresus*) generally possessed greater TSGA, although the differences in amylase activity (Fig. 6) far exceed those for relative total gut mass (Fig. S1).

β-Glucosidase activities varied significantly along the digestive tract of *C. violaceus* ($F_{4,20} = 4.95$, $P = 0.009$), peaking in the distal intestine gut contents, which had significantly greater
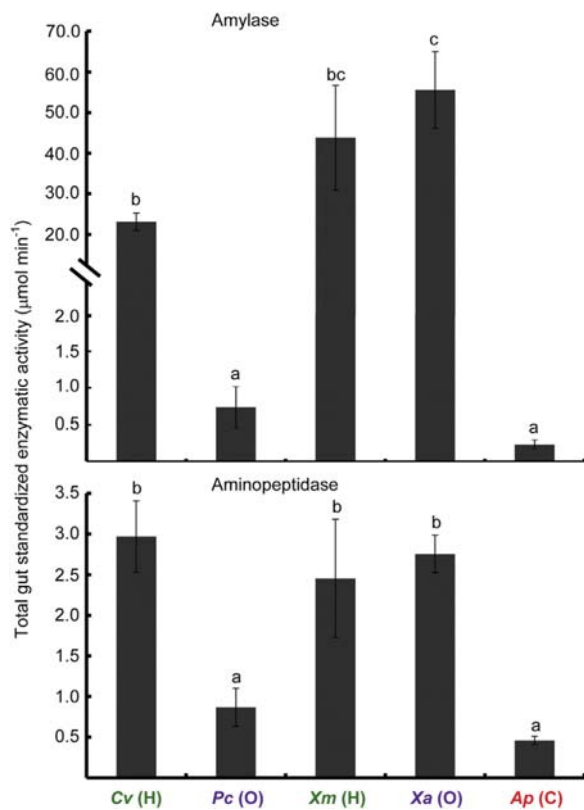
Fig. 6. Total gut standardized enzymatic activity of amylase (top) and aminopeptidase (bottom) in the digestive tracts of *Cebidichthys violaceus* (*Cv*), *Phytichthys chirus* (*Pc*), *Xiphister mucosus* (*Xm*), *Xiphister atropurpureus* (*Xa*), and *Anoplarchus purpurescens* (*Ap*). Values are mean ± standard error. $n = 6$ for *Cv*, *Xm*, *Xa*, and *Ap*; and $n = 9$ for *Pc*. H: herbivory, O: omnivory, and C: carnivory. Interspecific comparisons of enzyme activity were made with ANOVA, where bars for a specific enzyme that share a letter are not significantly different.

Fig. 7. β-Glucosidase activities in different gut regions of the herbivorous fishes *Cebidichthys violaceus* and *Xiphister mucosus*. Values are mean ± standard error. Intraspecific comparisons made with ANOVA, where bars representing different gut regions for a single species sharing a letter are not significantly different. Interspecific comparisons were not made. "Contents" indicate activities measured in the gut contents from the different gut regions, whereas the intestinal fractions are the fishes' gut wall tissue. Abbreviations: DI, distal intestine; MI, mid-intestine; PI, proximal intestine.

β-glucosidase activity than all other gut regions except the mid-intestine (Fig. 7). No significant differences in β-glucosidase activity were detected among gut regions (or among gut tissue and gut contents) of *X. mucosus* ($F_{4,16} = 1.84$, $P = 0.186$). Activities of this enzyme were not readily detectable in the guts of the other studied species.

Activity levels of maltase, N-acetyl-β-D-glucosaminidase, trypsin, and lipase in the five prickleback species are shown in the supplementary Figs. S2 and S3. Generally, these enzymes follow the predicted patterns for pancreatic (trypsin) or brush border (maltase, N-acetyl-β-D-glucosaminidase) enzymes outlined in Fig. 1 and Table 1, although lipase showed no discernable pattern. One interesting departure is a spike in N-acetyl-β-D-glucosaminidase in the distal intestine of *P. chirus* (see supplementary Fig. S2). TSGA for these enzymes follows a pattern similar to those for amylase, aminopeptidase, and RGM (see supplementary Fig. S3). Enzymatic activities in the gut contents of the fish were generally an order of magnitude lower than those in the fishes' tissues, and few enzymes showed any pattern moving along the intestine in any of the studied species (see supplementary Table S1 in the online Appendix). Two notable observations are significant increases in amylase activities in the mid and distal intestine contents of the two species of *Xiphister* that were not seen in the other taxa, and elevated (more than an order of magnitude in comparison to the other species) N-acetyl-β-D-glucosaminidase activities in the gut contents of *P. chirus* (Table S1).
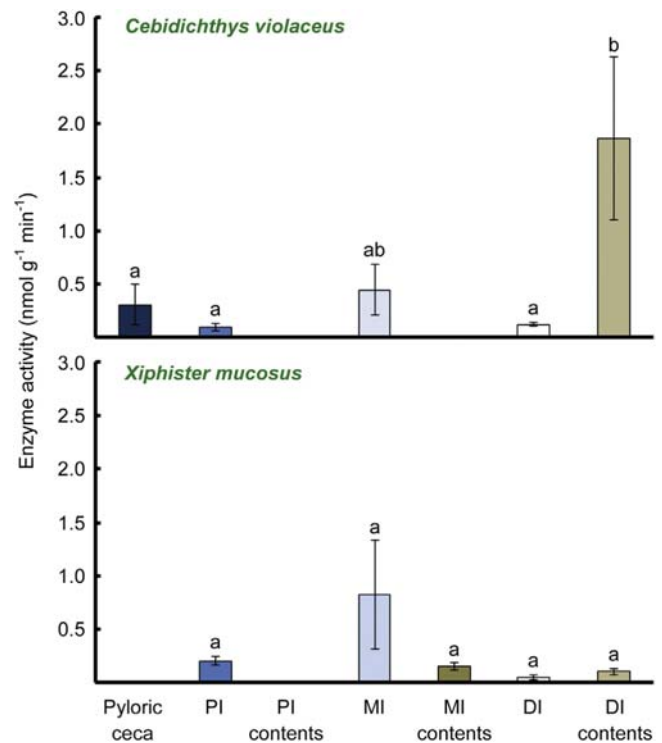
3.3. Gastrointestinal fermentation

C. violaceus possessed significantly greater SCFA concentrations ($F_{3,33} = 127.92$, $P < 0.001$) in their distal intestines than *X. mucosus*, *X. atropurpureus*, and *A. purpurescens* (Table 3). *C. violaceus* also had proportionately less acetate ($F_{3,33} = 8.63$, $P < 0.001$) and proportionately more propionate ($F_{3,33} = 18.61$, $P < 0.001$) in their hindguts than the other species, which did not differ in this respect (Table 3).

Table 3
Total short-chain fatty acid (SCFA) concentrations and ratios of acetate:propionate:butyrate in the distal intestines of four prickleback species with different diets. Values are mean ± SEM. Comparisons of SCFA concentrations among species were made with ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$. SCFA values for different species that share a superscript letter are not significantly different. $n = 12$ for *C. violaceus*, $n = 7$ for *X. mucosus*, $n = 8$ for *X. atropurpureus*, and $n = 6$ for *A. purpurescens*. H: herbivore, O: omnivore, and C: carnivore.

Species	Total SCFA (mM)	Ratio[a]
Cebidichthys violaceus (H)	11.68 ± 0.51[b]	53:32:11
Xiphister mucosus (H)	1.83 ± 0.39[a]	76:17:5
Xiphister atropurpureus (O)	2.76 ± 0.34[a]	77:10:13
Anoplarchus purpurescens (C)	2.72 ± 0.39[a]	78:6:12
$F_{3,33}$	127.92	
P	<0.001	

[a] If sum does not equal 100, the remainder is composed of isobutyrate, isovalerate, or valerate in small quantities. Acetate, propionate, and butyrate are the main SCFA present, composing 96–100% of total SCFA. *C. violaceus* also had proportionately less acetate ($F_{3,33} = 8.63$, $P < 0.001$) and proportionately more propionate ($F_{3,33} = 18.61$, $P < 0.001$) in their distal intestines than the other species, which did not differ from each other. There were no differences in butyrate proportions among species ($F_{3,33} = 2.01$, $P = 0.134$).

No significant differences in butyrate proportion were detected ($F_{3,33} = 2.01$, $P = 0.134$). Consistent with its carnivorous diet, *A. purpurescens* had measureable isovalerate levels (0.25 ± 0.08 mM) in four out of six tested individuals, whereas none of the other species had measurable quantities of isovalerate in more than two individuals.

4. Discussion

The results of this study show that sympatric, closely related animals that convergently evolved similar diets can specialize in those diets in different ways. Based on gut size, locations of gut contents, digestive enzyme activity patterns, and levels of SCFAs in the intestines of the fish, it is clear that *C. violaceus* fits better within a yield-maximization strategy with some reliance on microbial digestion, whereas *X. mucosus* is more of a rate maximizer with a stronger reliance on endogenous digestive processes. The two omnivores in this study also showed some differences based on how much algae they consumed: *X. atropurpureus*, which consumed about 70% algal material, appears to better fit a rate-maximization pattern (like its sister taxon, *X. mucosus*), whereas *P. chirus*, which consumed about 50% algal material, may be more of a yield maximizer (with little microbial fermentation), as is typical of carnivores. Overall, it appears that there is more than one solution to the problem of digesting algae, and this is clearly displayed within the stichaeid lineage. What sets this example apart from other examples of sympatric herbivore diversification (e.g., the ruminant diversification hypothesis; Hofmann, 1989) is that both *C. violaceus* and *X. mucosus* are "browsers", consuming the same thalate algal taxa (Horn et al., 1986; Boyle and Horn, 2006; German and Horn, 2006), as opposed to one being a browser and one being a "grazer" (i.e., consuming filamentous, or turfing algae; Choat et al., 2004), thereby representing niche partitioning through the consumption of different resources. Thus, here, *C. violaceus* and *X. mucosus* represent two browsers consuming the same resources (Saba, 2004; Boyle and Horn, 2006; German and Horn, 2006), but utilizing them in different ways.

The three most striking results in this study setting *C. violaceus* apart from the other taxa all have to do with their distal intestines: (i) individuals of this species have heavier distal intestine regional gut mass (Fig. 4A), and greater amounts of relative gut content mass in their distal intestines (Fig. 4B), (ii) they have greater concentrations of SCFA in their distal intestines (Table 3), and (iii) they have a spike in β-glucosidase activity in this gut region (Fig. 7). Each of these results is consistent with elevated microbial activity in the distal intestine of *C. violaceus* (Mountfort et al., 2002; Skea et al., 2005, 2007). Animals that take a yield-maximizing strategy to digestion tend to share these three characteristics, as well (Stevens and Hume, 1998; Karasov and Martínez del Rio, 2007). Although SCFA concentrations in the *C. violaceus* hindgut are lower (by about 2/3) than those seen in other fishes with elevated levels of microbial fermentation (Clements et al., 1994; Mountfort et al., 2002), *C. violaceus* are benthic, and considered "sluggish" (Ralston and Horn, 1986; Horn and Messer, 1992) in comparison to roving reef taxa like *Kyphosus sydneyanus* or *Odax pullus* (Clements et al., 2014), and, therefore, may have lower metabolic needs for fermentation than active taxa. However, in comparison to sympatric, closely related, stichaeid species with similar life styles, *C. violaceus* stands out, especially when contrasted with *X. mucosus*. Furthermore, individuals of *C. violaceus* can exhibit higher levels of SCFA (~29 mM) in their hindguts than those found in the present study (Clements et al., 2014). The elevated proportion of propionate (30% of total SCFA) seen in *C. violaceus* is suggestive of the fermentation of soluble components of their algal diet (Stevens and Hume, 1998). Thus, although there is a spike in the activity of a cellulolytic enzyme

(β-glucosidase) in the *C. violaceus* hindgut, the microbes in this gut region appear to be generating SCFA from soluble components of green and red algae, not too dissimilar from soluble constituents of algae (i.e., mannitol; White et al., 2010) fermented in kyphosids and odacines (Mountfort et al., 2002).

Another hallmark of a yield-maximizing strategy is long retention times of food in the gut. *C. violaceus* is known to have gut transit times of approximately 20 h on an algal diet (Fris and Horn, 1993), but times exceeding 50 h have also been documented for this species (Urquhart, 1984). Transit times exceeding 20 h are consistent with other fishes that utilize a yield-maximizing strategy and hold digesta in their hindguts (Clements and Rees, 1998; Choat et al., 2004). Moreover, the distal intestine contents of *C. violaceus* contain more fluid and smaller particles than those of the other stichaeid species (German, pers. obs.), which made it simpler to extract distal intestine fluid from *C. violaceus* than the other taxa. Although not completely discernable in Fig. 2, the distal intestine of *C. violaceus* is commonly expanded with fluid and digesta just before the rectal sphincter. Non-ruminant animals that are reliant on microbial fermentation tend to retain fluid and smaller particles in their hindguts, where microbes aid in the digestive process (Vispo and Hume, 1995; Stevens and Hume, 1998; Felicetti et al., 2000). Digesta transit times are unknown for the other stichaeid species, including *X. mucosus*, but all of the other pieces of the puzzle suggest that transit time is shorter in *X. mucosus* and *X. atropurpureus* than in *C. violaceus*; this needs to be confirmed in future investigations. Carnivores and omnivores that consume a considerable amount of animal material can also have longer retention of food in their guts, but this is because they eat proteinaceous foods less frequently as opposed to a reliance on microbial fermentation in their hindguts (Table 3) (German, 2009a; Clements et al., 2014).

Spikes in the activity levels of microbially produced digestive enzymes are commonly observed in the hindguts of fishes reliant on microbial fermentation. For instance, *Kyphosus sydneyanus* shows peaks of enzymatic activities against laminarin, carrageenan, and alginate (storage and structural polysaccharides of algae; Painter, 1983) in its distal intestine (Skea et al., 2005). In the present study, *C. violaceus* showed a peak of β-glucosidase activity in its distal intestine (Fig. 7), and the source of the β-glucosides for this enzyme would be cell wall constituents (e.g., cellulose degradation products) of green algae and seagrass (Painter, 1983). Moreover, herbivorous and omnivorous fishes that tend to be more reliant on endogenous digestive mechanisms tend to have higher amylase activities than those herbivores that are reliant on microbial digestion (Skea et al., 2005, 2007; German and Bittong, 2009; German et al., 2010a), a pattern that is mirrored by the elevated amylase activities in the *Xiphister* taxa in comparison to *C. violaceus* in this, and previous studies (Chan et al., 2004; German et al., 2004).

Beyond the differences between *C. violaceus* and the other species, most of the other patterns of enzymatic activities matched the expectations outlined in Table 1 and Fig. 1. Amylase and trypsin activities decreased moving distally along the intestine, which are common observations for these two pancreatic enzymes (Skea et al., 2005; German, 2009a; German and Bittong, 2009; German et al., 2010b). The taxa eating more algal material (and thus, more starch) had the highest amylase activities, a common pattern seen in fishes (German et al., 2010a), birds (Kohl et al., 2011), canids (Axelsson et al., 2013), and even humans (Perry et al., 2007). The spike in amylase activity in the mid-intestine contents of the *Xiphister* taxa is likely of endogenous (i.e., pancreatic) origin given the elevated amylase activities in the tissues of these species. Aminopeptidase activities tended to peak in the mid-intestines of the herbivorous and omnivorous taxa, whereas *P. chirus* and *A. purpurescens* showed increases in the activity of this enzyme in their distal intestines, a pattern also seen in comparisons of other herbivores and carnivores (German, 2009a; Day et al., 2011). Given the

higher protein content in animal material, this enzyme is important in the acquisition of amino acids from a carnivorous diet that is low in carbohydrates; amino acids play an important role in a carnivore's metabolism (beyond tissue maintenance) because of the limited carbohydrates available in their high-protein diets to be used explicitly for ATP production (Karasov and Martínez del Rio, 2007). Maltase and lipase showed varying activity patterns, which are not unknown for these enzymes (German, 2009a; German and Bittong, 2009; German et al., 2010b; Day et al., 2011), but make interpretations of activity difficult for this carbohydrase and lipolytic enzyme, respectively.

N-acetyl-β-ᴅ-glucosaminidase, which digests the breakdown products of chitin, generating the absorbable N-acetyl-glucosamine, generally followed patterns predicted for brush border enzymes (Fig. 1). However, *P. chirus* showed a clear spike in the activity of this enzyme in their distal intestines, and had elevated activities of this enzyme in their gut contents. Chitinase activities are elevated in carnivorous fishes that consume insect larvae and crustaceans (Goodrich and Morita, 1977; Danulat, 1986; Gutowska et al., 2004; German et al., 2010a), diets rich in chitin, and the evolution of chitinase is correlated with the evolution of a carnivorous diet in fishes (German et al., 2010a). Such patterns have not been explicitly demonstrated for N-acetyl-β-ᴅ-glucosaminidase in fishes, but clear positive correlations between N-acetyl-β-ᴅ-glucosaminidase activities and substrate concentration have been demonstrated for microbial decomposers (Allison et al., 2014). Some fishes do show the ability to use chitin as a source of nutrition (Alliot, 1967; Peres et al., 1973), absorbing N-acetyl-glucosamine across the intestinal epithelium faster than glucose (Alliot, 1967). Gutowska et al. (2004) showed that carnivorous fishes with shorter guts had higher chitinase activities than other sympatric taxa from Monterey Bay, CA. *P. chirus* has shorter guts than the other omnivorous and herbivorous taxa we studied (but not shorter than *A. purpurescens*), and has higher mass-specific N-acetyl-β-ᴅ-glucosaminidase activities than all the others. Thus, given that *P. chirus* consumed more crustaceans than the other taxa (including *A. purpurescens*; Fig. 3, and see Section 3.1), *P. chirus* may rely more on chitin as a source of nutrition than the other taxa. Nevertheless, the results of this study clearly set *P. chirus* apart from their close relatives in the genus *Xiphister*, and the physiology of *P. chirus* seems to reflect more of a carnivorous diet than a truly omnivorous one as seen in *X. atropurpureus*. Based on its place in the phylogeny (Fig. 2), *P. chirus* may represent a transition from carnivory to the herbivory and omnivory observed in the *Xiphister* taxa (German et al., 2014).

Fishes in the stichaeid phylogeny continue to be highly appropriate organisms for studying the evolution of dietary specialization in marine systems (Saba, 2004; Chereshnev et al., 2013; German et al., 2014; Kim et al., 2014) and for animals as a whole. The differences between *C. violaceus* and the species of *Xiphister* do not stop with their digestive strategies, as they have different amylase genetics, with *C. violaceus* possessing 12 copies of amylase genes in their genome, and a diversity of amylase isoforms, whereas the *Xiphister* species only have 4 copies of amylase genes with little variation among them (D.P. German and D.M. Foti, unpublished data). These genetic disparities may manifest in further differences in the digestive biochemistry of these species, amplifying the niche partitioning among these allegedly trophically similar taxa. Indeed, using C and N stable isotope analyses, Saba (2004) showed that *C. violaceus* and *X. mucosus* do not occupy the same trophic position in their shared environment, and because they have similar diets, this difference is likely driven by their different digestive strategies, which affects what nutrients are assimilated from the ingested foods.

In conclusion, it is clear that yield- *and* rate-maximizing strategies can evolve within closely related fishes. Although some other fish clades feature one strategy or the other (Clements and Choat, 1997; Mountfort et al., 2002; Crossman et al., 2005; German, 2009a,b; German and Bittong, 2009; German et al., 2010a; Lujan et al., 2015), this is not a fixed paradigm for all fishes, or for all animals (Hofmann, 1989; Crossman et al., 2005). This also suggests that *C. violaceus* and *X. mucosus* play different roles within their shared communities (Saba, 2004). These two herbivorous species may optimize protein or energy consumption at different times of the year from each other based on what algal species are available for consumption (Horn et al., 1986), and these differences may have underpinnings on the digestive level. Fishes that pursue yield- or rate-maximization strategies will have different contributions to ecosystem fluxes based on what, and how much, they eat, but also based on what they excrete back into the environment (e.g., Taylor et al., 2006), and yield and rate maximizers will excrete differently, both in terms of fecal and ionic contributions (Choat et al., 2004; Crossman et al., 2005; Karasov and Martínez del Rio, 2007). Thus, understanding the post-ingestive processes that occur within an animal is crucial to understanding the role that animal plays in its ecosystem (Choat and Clements, 1998; Clements et al., 2009, 2014).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.zool.2014.12.002.

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