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

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Abstract

In fishes, the evolution of herbivory has occurred within a spectrum of digestive strategies, with two extremes on opposite ends: 1) a Rate-maximization strategy characterized by high intake, rapid throughput of food through the gut, and little reliance on microbial digestion; or 2) 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 occurred 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 *X. 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.
1. Introduction

What an animal eats and how it digests its food directly affects that animal’s role in its biotic community (Karasov, 2011; Karasov and Martínez del Rio, 2007). Yet, beyond gut content and ecomorphological analyses, animal nutritional physiology is often ignored. In particular, fish nutritional ecology and physiology remains 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., 2014; Clements et al., 2009). 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), to ecomorphology (Ferry-Graham et al., 2010; Wainwright et al., 2012), on down to individual species digestive biochemistry and metabolism (Crossman et al., 2005; German et al., 2014; German et al., 2010a; Skea et al., 2005, 2007; Wilmott et al., 2005). 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 (Day et al., 2011; German, 2009a; German and Horn, 2006; German et al., 2004; German et al., 2010a). 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 “Rate vs Yield” theoretical framework (Fig. 1; Clements and Raubenheimer, 2006; Sibly, 1981). 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.
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 (Choat et al., 2004; Clements and Rees, 1998), and well-developed microbial fermentation in their hindguts (Crossman et al., 2005; Mountfort et al., 2002), 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 lower intake of proteinaceous food and slower digesta transit, albeit carnivores are less reliant on microbial fermentation to meet their energetic demands (Clements et al., 2014; German, 2009a; Stevens and Hume, 1998).

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; Lujan et al., 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; Crossman et al., 2005; Mountfort et
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 (German, 2009a; German et al., 2014; German et al., 2010a; Skea et al., 2005, 2007).

In this 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 (Fig. 2; Kim et al., 2014). With dietary diversity, convergent evolution of herbivory, and sister-taxa with different diets (German et al., 2014; German and Horn, 2006; German et al., 2004), 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 within 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), *X. 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’ 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 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 microbiially-produced enzymes (Table 1; Fig. 1;
German and Bittong, 2009; Skea et al., 2005, 2007). 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 a Yield-maximizer
would have elevated levels of SCFA in their hindguts, whereas a Rate-maximizer 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 Cebidichthys violaceus, Xiphister mucosus, X. atropurpureus, and
Anoplarchuhs 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 *Phytoichthys 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 (SL ± 0.5 mm), weighed (body mass, BM ± 0.1 g;) and dissected on a cutting board kept on ice (4°C) within 4h 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). 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 six months). Gut lengths and body lengths were used to calculate relative gut length [RGL = gut length (mm) X standard length (mm)⁻¹], and regional intestinal masses were used to calculate relative regional gut mass [RRGM = regional gut mass (g) X body mass (g)⁻¹]. Regional gut content masses were used to calculate relative regional gut content mass [RRGCM = regional gut content mass (g) X 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 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 (German and Bittong, 2009; Smith et al., 1985).

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) 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). 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 Chemical (St. Louis). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from the five species (German et al., 2014; German et al., 2004). Each enzyme activity was measured in each gut region of each individual fish, and blanks consisting of substrate only and homogenate only (in buffer) were conducted simultaneously to account for endogenous substrate and/or product in the tissue homogenates and substrate solutions.

α-amylase activity was measured using 1% potato starch dissolved in 25 mM Tris-HCl containing 1 mM CaCl$_2$. 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 minute) 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 minute) per gram wet weight of gut tissue.

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 minutes. Following incubation, 2.5 µL of 1 M NaOH was added to each microplate well, and the fluorescence read immediately at 365
nm excitation and 450 nm emission. Each plate included a standard curve of the product (4-
methylumbelliferone), substrate controls, and homogenate controls, and enzymatic activity
(µmol product released per minute per gram wet weight tissue) was calculated from the MUB
standard curve.

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

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

Lipase (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 minute) 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 Gut Standardized 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 (Day et al., 2011; Horn et al., 2006).

2.3 Gut fluid preparation and gastrointestinal fermentation

Measurements of symbiotic fermentation activity were based on the methods of Pryor and Bjomdal (2005), as described in German and Bittong (2009). Fermentation activity was indicated by relative concentrations of short-chain fatty acids (SCFA) in the fluid contents of the distal intestines of the fishes at the time of death. An additional fourteen 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 x g for 10 min. The supernatant was then pipetted into a sterile centrifuge vial equipped with a 0.22 µm cellulose acetate filter (Costar Spin-X gamma sterilized centrifuge tube filters, Coming, NY) and centrifuged under refrigeration at 13,000 x g for 5 min to remove particles from the fluid (including bacterial cells). The filtrates were collected and frozen until they were analyzed for SCFA 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) and attached to a Hewlett-Packard HP3392A integrator (Hewlett-Packard Co., Palo Alto, CA). Two µL of each sample were injected onto a 2 m-long stainless steel column
(3.2 mm ID) packed with 10% SP-1000 and 1% H₃PO₄ on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, Pennsylvania). 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, a Levene’s test for equal variance was performed and residual versus 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 a Tukey’s HSD with a family error rate of \( P = 0.05 \). Interspecific comparisons of short chain fatty acid (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 a Tukey’s HSD 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. purpureascens* (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 fish with different diets.
3. Results

3.1. Diet, Relative Gut Length, and Gut content masses

*Cebidichthys violaceus* (84%) and *Xiphister 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). *Phytichthys 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 (ANOVA 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. *Cebidichthys 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 weren’t 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). $Xiphister mucosus$ and $X. atropurpureus$ showed no differences in relative regional gut content 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

$Cebidichthys violaceus$ 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 weren’t 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 TGSA, $X. atropurpureus$ possessed significantly higher amylase activity than all 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, wasn’t different from *C. violaceus* (Fig. 6). *Phytichthys chirus* and *A. purpurescens* had the lowest amylase activities that weren’t 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 weren’t 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. *Phytichthys 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). *Anoplarchus purpurescens* also showed increasing activity moving distally along the intestine, with each intestinal region significantly greater ($F_{2,17} = 25.43$, $P < 0.001$) than the one before. In terms of TGSA, *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 weren’t 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 TGSA activity for amylase and aminopeptidase generally follow those seen for relative total gut
mass and body size (see Table 2, and Supplementary Figure S1 in the online version of this
manuscript). 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 TGSA, 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 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 Supplemental Figures 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 (Supplemental Fig. S2). TGSA for these enzymes follow a similar pattern
to those for amylase, aminopeptidase, and RGM (see Supplemental Figure S3 in the online
version of this manuscript). 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 Supplemental Table S1 in the online
version of this manuscript). 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

*Cebidichthys violaceus* possessed significantly greater SCFA concentrations (*F*<sub>3,33</sub> = 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*<sub>3,33</sub> = 8.63, *P* < 0.001), and proportionately more propionate (*F*<sub>3,33</sub> = 18.61, *P* < 0.001) in their hindguts than the other species, which didn’t differ in this manner (Table 3). No significant differences in butyrate proportion were detected (*F*<sub>3,33</sub> = 2.01, *P* = 0.134). Consistent with its carnivorous diet, *A. purpurescens* had measureable isovalerate (0.25 ± 0.08 mM) in four out of six tested individuals, whereas none of the other species had measurable isovalerate in more than two individuals.

### 4. Discussion

The results of this study show that sympatric, closely-related animals that convergently evolve similar diets can specialize on 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 (Karasov and Martínez del Rio, 2007; Stevens and Hume, 1998). 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” (Horn and Messer, 1992; Ralston and Horn, 1986) 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 this 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 hours on an algal diet (Fris and Horn, 1993), but times exceeding 50 hours have been documented for this species (Urquhart, 1984). Transit times exceeding 20 hours are consistent with other fishes that utilize a Yield-maximizing strategy and hold digesta in their hindguts (Choat et al., 2004; Clements and Rees, 1998). Moreover, the distal intestine contents of *C. violaceus* contain more fluid and smaller particles than the other stichaeid species (D.P. 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 (Felicetti et al., 2000; Stevens and Hume, 1998; Vispo and Hume, 1995). 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*, but 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; Clements et al., 2014; German, 2009a).

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). Here, *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 (German and Bittong, 2009; German et al., 2010a; Skea et al., 2005, 2007), 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 Figure 1. Amylase and trypsin activities decreased moving distally along the intestine, which are common
observations for these two pancreatic enzymes (German, 2009a; German and Bittong, 2009; German et al., 2010b; Skea et al., 2005). 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 seen in comparisons of other herbivores and carnivores (Day et al., 2011; German, 2009a). 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 Martinez del Rio, 2007). Maltase and lipase showed varying activity patterns, which are not unknown for these enzymes (Day et al., 2011; German, 2009a; German and Bittong, 2009; German et al., 2010b), but make interpretations of activity difficult for this carbohydrase and lipolytic enzyme, respectively.

N-acetyl-β-D-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 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 (Danulat, 1986; German et al., 2010a; Goodrich and Morita, 1977; Gutowska et al., 2004), 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-D-glucosaminidase in fishes, but clear positive correlations between N-acetyl-β-D-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-D-glucosaminidase activities than all of them. Thus, given that *P. chirus* consumed more crustaceans than the other taxa (including *A. purpurescens*; Fig. 3, and see results section), *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; German et al., 2014; Chereshnev et al., 2013; 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 diversity of amylase isoforms, whereas the *Xiphister* species only have four copies of amylase genes with little variation among them (D.P. German and D.M. Foti, unpubl. 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; Crossman et al., 2005; German, 2009a, b; German and Bittong, 2009; German et al., 2010a; Lujan et al., 2015; Mountfort et al., 2002), 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 take 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 Martinez del Rio, 2007). Thus, understanding the post-ingestive
processes that occur within an animal are crucial to understanding the role that animal plays in its ecosystem (Choat and Clements, 1998; Clements et al., 2014; Clements et al., 2009).

5. Acknowledgements

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6. Appendices

Supplementary data related to this article can be found at http://XXX.

References


Saba, M., 2004. Trophic ontogeny in four closely related species of herbivorous and carnivorous prickleback fishes (Stichaeidae), Master's Thesis. Biological Sciences, California State University, Fullerton, CA, USA. 56 Pg.


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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Synthesis</th>
<th>Substrate</th>
<th>Dietary source</th>
<th>Expected pattern</th>
<th>&gt;Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>Pancreatic</td>
<td>Starch, α-glucans</td>
<td>Algae</td>
<td>Decrease</td>
<td>Tissue</td>
</tr>
<tr>
<td>Maltase</td>
<td>Brush border</td>
<td>Maltose</td>
<td>Algae</td>
<td>Middle spike</td>
<td>Tissue</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>Microbial</td>
<td>β-glucosides</td>
<td>Algae</td>
<td>Distal spike</td>
<td>Contents</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucos(^4)</td>
<td>Brush border</td>
<td>N-acetyl-β-D-glucosaminide</td>
<td>Crustaceans</td>
<td>Middle spike</td>
<td>Tissue</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Pancreatic</td>
<td>Protein</td>
<td>Algae, animals</td>
<td>Decrease</td>
<td>Tissue</td>
</tr>
<tr>
<td>Aminopeptidase</td>
<td>Brush border</td>
<td>Dipeptides</td>
<td>Algae, animals</td>
<td>Middle spike</td>
<td>Tissue</td>
</tr>
<tr>
<td>Lipase</td>
<td>Pancreatic</td>
<td>Lipid</td>
<td>Algae, animals</td>
<td>Decrease</td>
<td>Tissue</td>
</tr>
</tbody>
</table>

\(^1\) Indicates where the enzyme is synthesized, either from fish (pancreatic or brush border), or from microbial sources.

\(^2\) This column shows the expected patterns of activity along the GI 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).

\(^3\) Predictions of which assayed fractions will have higher activity of a particular enzyme. For example, “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 this microbially-produced enzyme should be greater in the gut contents than in the gut tissue.

\(^4\) Complete name of the enzyme is N-acetyl-β-D-glucosaminidase, and the substrate is N-acetyl-β-D-glucosaminides.
Table 2. Body sizes of prickleback fishes used for measurements of digestive enzyme activities.

<table>
<thead>
<tr>
<th>Species</th>
<th>Standard length (mm)</th>
<th>Body mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cebidichthys violaceus</em> (H)</td>
<td>152.82 ± 6.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.52 ± 3.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Phytichthys chirus</em> (O)</td>
<td>108.33 ± 6.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.67 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Xiphister mucosus</em> (H)</td>
<td>160.85 ± 11.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.52 ± 4.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Xiphister atropurpureus</em> (O)</td>
<td>183.48 ± 6.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.77 ± 1.99&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Anoplarchus purpurescens</em> (C)</td>
<td>80.42 ± 1.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.63 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

\[ F_{4,32} \quad 30.12 \quad 13.64 \]

\[ P \quad <0.001 \quad <0.001 \]

Note. Values are mean ± SEM. H = herbivore, O = omnivore, C = carnivore. 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*. 
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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total SCFA (mM)</th>
<th>Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cebidichthys violaceus</em> (H)</td>
<td>11.68 ± 0.51b</td>
<td>53:32:11</td>
</tr>
<tr>
<td><em>Xiphister mucosus</em> (H)</td>
<td>1.83 ± 0.39a</td>
<td>76:17:5</td>
</tr>
<tr>
<td><em>Xiphister atropurpureus</em> (O)</td>
<td>2.76 ± 0.34a</td>
<td>77:10:13</td>
</tr>
<tr>
<td><em>Anoplarchus purpurescens</em> (C)</td>
<td>2.72 ± 0.39a</td>
<td>78:6:12</td>
</tr>
</tbody>
</table>

\[ F_{3,33} = 127.92 \]  

\[ P < 0.001 \]

Note. Values are mean ± SEM. H = herbivore, O = omnivore, C = carnivore. 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*.

† If sum does not equal 100, the remainder is composed of isobutyrate, isovalertae, 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 didn’t differ from each other. There were no differences in butyrate proportions among species \( F_{3,33} = 2.01, P = 0.134 \).
Figure 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 ($t_1$). A portion of the nutrient consumed is lost in the feces (“Wastage”), but at $t_1$ 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 ($t_2$), however, this is done at the cost of 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 made 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 2009; German and Bittong 2009).
Figure 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 **bolded** and indicated with **.** H=herbivory, O=omnivory, C=carnivory. Evolution of herbivory (-----) and omnivory (…………) are shown. Numbers in parentheses show number of taxa evaluated at that branch. Boxes highlight 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 Cebrichthyidae, 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.
Figure 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. Herbivory, Omnivory, and Carnivory indicated by colors. The clade shows a pruned tree for the analyzed taxa. See Fig. 2 for full phylogeny.
Figure 4. A: Relative regional gut mass; and 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. Herbivory, Omnivory, and Carnivory indicated by color. 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.
Figure 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*. Herbivory, Omnivory, and Carnivory indicated by color. 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*. 
**Amylase**

- Canyon (Cv)
- P. cancrivora (Pc)
- X. maculata (Xm)
- X. ariakensis (Xa)
- A. pacifica (Ap)

**Total gut standardized enzymatic activity (µmol min⁻¹)**

- Cv: b
- Pc: a
- Xm: bc
- Xa: c
- Ap: a

**Aminopeptidase**

- Cv: b
- Pc: a
- Xm: b
- Xa: b
- Ap: a
Figure 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*. Herbivory, Omnivory, and Carnivory denoted by color. Interspecific comparisons of enzyme activity were made with ANOVA, where bars for a specific enzyme that share a letter are not significantly different.
Figure 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. PI = proximal intestine, MI = mid intestine, and DI = distal intestine. “Contents” indicate activities measured in the gut contents from the different gut regions, whereas the intestinal fractions are the fishes’ gut wall tissue.
### Supplemental Table S1. Digestive enzymes activities in intestinal contents of prickleback fishes.

<table>
<thead>
<tr>
<th>Intestinal Region</th>
<th>Amylase</th>
<th>Maltase</th>
<th>NAG</th>
<th>Trypsin</th>
<th>Aminopeptidase</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cebidichthys violaceus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>4.81 ± 1.22</td>
<td>0.18 ± 0.08</td>
<td>0.92 ± 0.14</td>
<td>0.78 ± 0.20</td>
<td>0.31 ± 0.10</td>
<td>0.94 ± 0.15b</td>
</tr>
<tr>
<td>Mid</td>
<td>3.68 ± 0.79</td>
<td>0.08 ± 0.02</td>
<td>0.54 ± 0.21</td>
<td>0.66 ± 0.07</td>
<td>0.27 ± 0.08</td>
<td>0.35 ± 0.09a</td>
</tr>
<tr>
<td>Distal</td>
<td>2.27 ± 0.38</td>
<td>0.04 ± 0.01</td>
<td>2.59 ± 1.51</td>
<td>0.51 ± 0.09</td>
<td>0.31 ± 0.04</td>
<td>0.68 ± 0.08ab</td>
</tr>
<tr>
<td>( F_{2,17} )</td>
<td>2.14</td>
<td>2.82</td>
<td>1.53</td>
<td>1.10</td>
<td>0.22</td>
<td>6.62</td>
</tr>
<tr>
<td>( P )</td>
<td>0.152</td>
<td>0.091</td>
<td>0.249</td>
<td>0.359</td>
<td>0.809</td>
<td>0.009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Herbivory, Omnivory, or Carnivory</th>
<th>Carnivory</th>
<th>Omnivory</th>
<th>Herbivory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytichthys chirus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t (df=16) )</td>
<td>1.58</td>
<td>0.96</td>
<td>0.84</td>
</tr>
<tr>
<td>( P )</td>
<td>0.134</td>
<td>0.351</td>
<td>0.414</td>
</tr>
</tbody>
</table>

| Xiphister mucosus                 |           |          |           |
| Proximal                          |           |          |           |
| Mid                               |           |          |           |
| Distal                            |           |          |           |
| \( F_{2,17} \)                   | 7.53      | 8.47     | 2.57      |
| \( P \)                           | 0.005     | 0.003    | 0.109     |

| Xiphister atropurpureus           |           |          |           |
| Proximal                          |           |          |           |
| Mid                               |           |          |           |
| Distal                            |           |          |           |
| \( F_{2,17} \)                   | 5.08      | 1.05     | 1.61      |
| \( P \)                           | 0.021     | 0.380    | 0.232     |

| Anoplarchus purpurescens          |           |          |           |
| Proximal                          |           |          |           |
| Mid                               |           |          |           |
| Distal                            |           |          |           |
| \( t (df=10) \)                   | 0.39      | 2.23     | 0.35      |
| \( P \)                           | 0.823     | 0.061    | 0.736     |

Note: Activities are \( \mu \text{mol product min}^{-1} \text{g}^{-1} \), except for N-acetyl-\( \beta \)-D-glucosaminidase (NAG), for which the values are nmol product min\(^{-1}\) g\(^{-1}\). Values are mean ± SEM. **Herbivory**, **Omnivory**, or **Carnivory** denoted by colors. Intraspecific comparisons of enzyme activities among intestinal regions were made with ANOVA followed by Tukey's HSD, except for **P. chirus** and **A. purpurescens**, where \( t \)-tests.
were used instead. Significant effects are **bolded**. Enzyme activities for gut regions within a single 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*. 
Supplemental Figure S1. Relative Total Gut Mass 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*. Herbivory, Omnivory, and Carnivory denoted by color. Interspecific comparisons of Relative Total Gut Mass were made with ANCOVA, with body mass as the covariate; bars that share a letter are not significantly different. ANCOVA: $F_{4,32} = 11.75$, $P < 0.001$; Body mass $F_{1,27} = 0.03$, $P = 0.857$. 
Enzyme activity (µmol g\(^{-1}\) min\(^{-1}\))
**Supplemental Figure S2.** Maltase, N-acetyl-β-D-glucosaminidase, trypsin, and lipase 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*. Herbivory, Omnivory, and Carnivory indicated by color. 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. Phylogenetic relationships among species as in Figures 2 and 3. Note that there is no mid intestine section for *Pc* and *Ap*. Statistical results are below.

**Maltase**

*C. violaceus*: $F_{3,23} = 15.45$, $P < 0.001$

*P. chirus*: $F_{2,26} = 0.30$, $P = 0.741$

*X. mucosus*: $F_{3,23} = 1.17$, $P = 0.348$

*X. atropurpureus*: $F_{3,19} = 3.91$, $P = 0.029$

*A. purpurescens*: $F_{2,17} = 0.60$, $P = 0.563$

**N-acetyl-β-D-glucosaminidase**

*C. violaceus*: $F_{3,23} = 4.86$, $P = 0.011$

*P. chirus*: $F_{2,26} = 6.02$, $P = 0.008$

*X. mucosus*: $F_{3,23} = 2.61$, $P = 0.079$

*X. atropurpureus*: $F_{3,23} = 3.57$, $P = 0.032$

*A. purpurescens*: $F_{2,17} = 1.31$, $P = 0.299$

**Trypsin**

*C. violaceus*: $F_{3,23} = 4.01$, $P = 0.022$

*P. chirus*: $F_{2,26} = 1.98$, $P = 0.161$

*X. mucosus*: $F_{3,23} = 14.94$, $P < 0.001$

*X. atropurpureus*: $F_{3,23} = 6.18$, $P = 0.004$

*A. purpurescens*: $F_{2,17} = 6.40$, $P = 0.010$

**Lipase**

*C. violaceus*: $F_{3,23} = 1.54$, $P = 0.235$

*P. chirus*: $F_{2,26} = 1.86$, $P = 0.177$

*X. mucosus*: $F_{3,23} = 2.46$, $P = 0.092$

*X. atropurpureus*: $F_{3,23} = 2.00$, $P = 0.147$

*A. purpurescens*: $F_{2,17} = 0.90$, $P = 0.429
Supplemental Figure S3. Total Standardized Gut Enzymatic Activity of maltase, N-acetyl-β-D-glucosaminidase (NAG), trypsin, and lipase 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*. *Herbivory, Omnivory*, and *Carnivory* denoted by color. Interspecific comparisons of enzyme activity were made with ANCOVA with standard length as the covariate. Bars for a specific enzyme that share a letter are not significantly different. Heterogeneity of slopes tested with the interaction term of species X body length (which was never significant). Maltase: Species $F_{4,32} = 18.02, P < 0.001$; Body length $F_{1,27} = 8.28, P = 0.008$. NAG: Species $F_{4,32} = 18.32, P < 0.001$; Body length $F_{1,27} = 19.86, P < 0.001$. Trypsin: Species $F_{4,32} = 5.83, P = 0.002$; Body length $F_{1,27} = 14.49, P = 0.001$. Lipase: Species $F_{4,32} = 8.93, P < 0.001$; Body length $F_{1,27} = 40.38, P < 0.001$. 