

# Do herbivorous minnows have “plug-flow reactor” guts? Evidence from digestive enzyme activities, gastrointestinal fermentation, and luminal nutrient concentrations

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**Abstract** Few investigations have empirically analyzed fish gut function in the context of chemical reactor models. In this study, digestive enzyme activities, levels of gastrointestinal fermentation products [short chain fatty acids (SCFA)], luminal nutrient concentrations, and the mass of gut contents were measured along the digestive tract in herbivorous and carnivorous minnows to ascertain whether their guts function as “plug-flow reactors” (PFRs). Four of the species, *Campostoma anomalum*, *C. ornatum*, *C. oligolepis*, and *C. pauciradii*, are members of a monophyletic herbivorous clade, whereas the fifth species, *Nocomis micropogon*, is a carnivore from an adjacent carnivorous clade. In the context of a PFR model, the activities of amylase, trypsin and lipase, and the concentrations of glucose, protein, and lipid were predicted to decrease moving from the proximal to the distal intestine. I found support for this as these enzyme activities and nutrient concentrations generally decreased moving distally along the intestine of the four *Campostoma* species. Furthermore, gut content mass and the low SCFA concentrations did not change (increase or decrease) along the gut of any species. Combined with a previous investigation suggesting that species of *Campostoma* have rapid gut throughput rates, the data presented

here generally support *Campostoma* as having guts that function as PFRs. The carnivorous *N. micropogon* showed some differences in the measured parameters, which were interpreted in the contexts of intake and retention time to suggest that PFR function breaks down in this carnivorous species.

**Keywords** Digestion · Digestive physiology · Nutritional ecology · Chemical reactor models · Microbial

## Introduction

The mechanisms of food ingestion and digestion influence nearly all aspects of an animal’s behavior and physiology (Karasov and Martínez del Río 2007). In turn, an animal’s foraging strategy—whether it is a predator consuming high-quality foods on an intermittent basis, or an herbivore continuously digesting low-quality food—will affect the structure and function of their digestive tract. Gut structure and function appear to match dietary intake and quality, theoretically (Sibly and Calow 1986; Jumars 2000; Whelan et al. 2000), and empirically in a range of vertebrate taxa (Stevens and Hume 1995; Karasov and Martínez del Río 2007). However, the diversity of gut morphologies and diets in fishes has made it difficult to apply theoretical considerations to fish gut function.

Horn and Messer (1992) modeled herbivorous fish guts as chemical reactors, ultimately arriving at the conclusion that gut morphology (especially length) and food passage rate can best describe how a fish gut functions in relation to diet quality. Under these models, herbivorous fishes are expected to have longer digestive tracts and longer retention of food in the gut than their carnivorous counterparts. However, several researchers have shown that simple gut

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morphology fails to adequately describe gut function in herbivorous fishes. For example, *Atherinops affinis* has a short, stomachless digestive tract and passes algae through its gut in 2 h (Logothetis et al. 2001). Conversely, *Odax pullus* and *O. cyanomelas* (family Labridae), which also have relatively short, anatomically unspecialized digestive tracts with no stomach, caeca, or valves of any kind, hold algae in their guts for nearly 20 h (Clements and Rees 1998) and harbor an endosymbiont community capable of fermenting indigestible algal compounds (e.g., mannitol; Seeto et al. 1996; Mountfort et al. 2002).

A more reasonable approach to understanding fish digestion can be followed by taking into account rates of reaction (e.g., digestive enzyme activities) and nutrient concentrations along the digestive tract (Crossman et al. 2005; Skea et al. 2005, 2007). Horn and Messer (1992) predicted that herbivorous fishes with “plug-flow reactor” (PFR) digestive tracts—those with unspecialized intestinal morphology (no kinks, valves, or caeca), and digesta that moves in a unidirectional trajectory down the intestine with no axial mixing (Penry and Jumars 1987; Jumars 2000)—should have high intake, and relatively short retention times of digesta in the alimentary canal. Thus, these fishes would rely almost entirely on endogenous digestive mechanisms and would have low levels of endosymbiont fermentation [i.e., low short chain fatty acid (SCFA) concentrations] in their guts. PFR guts, they predicted, should have steep gradients of nutrient concentration and digestive enzyme activities along the intestine, with both more concentrated in the foregut, and most of the absorption of nutrients taking place in the mid to distal intestine (Horn and Messer 1992; Jumars 2000).

Minnows in the genus *Campostoma* (family Cyprinidae, comprising minnows and carps) are small (<25 g), well-studied algal grazers in North American freshwater ecosystems. Although *Campostoma* have been investigated in terms of their effects on algal community dynamics (Power et al. 1988; Gelwick and Matthews 1997) and ecosystem health (Power et al. 1985; Gelwick and Matthews 1992; Evans-White et al. 2003), little is known about the structure and function of their alimentary tract. Generally, cyprinids are characterized as having long, thin-walled digestive tracts that lack a gastric stomach (Kraatz 1924; German et al. 2009), and they are known to possess pharyngeal teeth (German et al. 2009) with which they grind and crush (Evans and Deubler 1955; Cloe et al. 1995) foods into smaller, more digestible (Xie 1999, 2001) particles. With a pharyngeal grinding apparatus followed by what seems to be an anatomically unspecialized intestine, cyprinids appear to possess a PFR digestive tract as described by Horn and Messer (1992). Functional support for this comes from observations that *Campostoma* have rapid gut transit times (<8 h total gut clearance) and do not hold food in the gut overnight (Fowler and Taber 1985). However, what is

clearly missing is an evaluation of digestive enzyme activities, gastrointestinal fermentation, and luminal nutrient concentrations in digestive tracts of *Campostoma* in the context of chemical reactor models to address whether their guts function as PFRs.

In this study, I evaluated the function of the digestive tract in four species of *Campostoma* (*C. anomalum*, *C. ornatum*, *C. oligolepis*, and *C. pauciradii*), and in a closely related carnivore, *Nocomis micropogon* (German et al. 2009). I measured the activity levels of five digestive enzymes (amylase, maltase, trypsin, aminopeptidase, and lipase), luminal nutrient concentrations, the mass of gut contents, and levels of gastrointestinal fermentation along the digestive tract in the five minnow species to evaluate whether their gut functions as a PFR. If these minnow taxa possess a PFR gut, then nutrient concentrations and the activities of amylase, trypsin and lipase should decline distally in the digestive tract. Maltase and aminopeptidase activities might be expected to increase distally in the intestine (Fraisie et al. 1981; Harpaz and Uni 1999), as these enzymes hydrolyze maltose and dipeptides, respectively, just preceding absorption; a PFR model predicts that absorption will preferentially take place in the mid- to distal intestine (Horn and Messer 1992; Jumars 2000). Under the PFR model, digesta content mass (Parra 1978) and concentrations of fermentation end products (SCFAs) should not be localized to any one region of the alimentary canal, and SCFA concentrations should be low (Stevens and Hume 1998). Additionally, comparisons of the *Campostoma* taxa with *N. micropogon* may provide evidence of functional differences between herbivores and carnivores.

## Materials and methods

### Fish capture and tissue preparation

Fishes were captured by seine and a backpack electroshocker during the summer months (June–August) between 2004 and 2006 from streams throughout the eastern United States and northern Mexico (Table 1). Upon capture, fishes were placed in coolers of aerated stream water and held until euthanized (<2 h). Specimens were prepared following German et al. (2009). Briefly, fishes were euthanized in buffered water containing 1 g l<sup>-1</sup> tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA), measured [standard length (SL) ± 1 mm], weighed [body mass (BM) ± 0.01 g], and dissected on a chilled (~4°C) cutting board. Guts were removed by cutting at the esophagus and at the anus. The guts were then uncoiled, without stretching, and measured [gut length (GL) ± 1 mm]. Because species of *Campostoma* and *Nocomis* have slightly different gut morphology and guts

**Table 1** Collection locations for five minnow taxa used in this study of digestive characters of the genera *Campostoma* and *Nocomis*

Taxon ( <i>N</i> )	Diet <sup>b</sup>	Collection location	Drainage	Latitude	Longitude
<i>C. anomalum</i> (10)	H	Flint Creek, Arkansas, USA	Arkansas River	36°46.142"N	94° 41.601" W
<i>C. ornatum</i> (10)	H	Río Santa Isabel, Chihuahua, Mexico	Río Conchos	28°32.463"N	106° 30.218" W
<i>C. oligolepis</i> (10)	H	Wedowee Creek, Alabama, USA	Tallapoosa River	33°18.496"N	85° 26.068" W
<i>C. pauciradii</i> <sup>a</sup> (6)	H	Hillabahatchee Creek, Georgia, USA	Chattahoochee River	33°18.632"N	85° 11.288" W
<i>C. pauciradii</i> <sup>a</sup> (5)	H	Candler Creek, Georgia, USA	Altamaha River	34°18.697"N	83° 39.441" W
<i>N. micropogon</i> (6)	C	Shoal Creek, Alabama, USA	Tennessee River	34°57.140"N	87° 35.410" W

<sup>a</sup> Because of dietary and morphological similarities, individuals from the two populations of *C. pauciradii* were pooled for all analyses

<sup>b</sup> "H" denotes herbivore, whereas "C" denotes carnivore

that differ in length, digestive tracts from the two genera were processed in slightly different ways. For the *Campostoma* species, the gut was divided into three equal sections representing the proximal intestine, mid intestine, and distal intestine. For *N. micropogon*, the gut was divided into two sections, the proximal intestine representing the proximal third of the intestine up to a small "bend" that is present in *Nocomis* taxa (German et al. 2009), and the remaining distal intestine. Guts were divided differently among the two genera because *N. micropogon* did not have enough gut tissue to further divide the intestine and allow for tissue homogenates to be made at a reasonable dilution. Once the guts were measured and divided, the gut contents were pushed out of the intestinal tissues with forceps and the blunt side of a razorblade, and into sterile centrifuge vials (Horn et al. 2006; German 2008; German et al. 2009). The evacuated intestinal tissues were then individually placed into sterile centrifuge vials. Both, the gut contents and the intestinal tissues were then frozen (in their vials) on dry ice, transported back to the University of Florida, and placed at  $-80^{\circ}\text{C}$  until analyzed.

Gut tissues from each gut region from individual fish were weighed (regional gut mass  $\pm 0.001$  g) and homogenized in ice-cold 0.05 M Tris-HCl buffer pH 7.4 using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) with a 7-mm generator at a setting of 2,200 rpm for  $3 \times 30$  s (German et al. 2004). Dilution varied between three and 60 volumes (v/w) depending on the mass of the gut region being homogenized. The homogenates were then centrifuged at  $9,400 \times g$  for 2 min at  $4^{\circ}\text{C}$  and the supernatants collected and stored in small aliquots (100–200  $\mu\text{l}$ ) at  $-80^{\circ}\text{C}$  until just before use in spectrophotometric assays of activities of the five digestive enzymes. Following centrifugation, the protein content of the homogenates was measured using bicinchoninic acid (Smith et al. 1985).

#### Assays of digestive enzyme activity

All assays were carried out at  $25^{\circ}\text{C}$  in triplicate using the Bio-Rad Benchmark Plus microplate spectrophotometer and

Falcon flat-bottom 96-well microplates (Fisher Scientific). All pH values listed for buffers were measured at room temperature ( $22^{\circ}\text{C}$ ), 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. 2009). 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.

The  $\alpha$ -amylase (EC 3.2.1.1) activity was measured according to the Somogyi-Nelson method (Nelson 1944; Somogyi 1952), as described by German et al. (2004, 2009). Briefly, starch substrate was prepared by boiling 2% soluble starch in 0.8 M sodium citrate buffer (pH 7.0) for 5 min. In a microcentrifuge vial, 50  $\mu\text{l}$  of the starch solution was combined with 50  $\mu\text{l}$  of a mixture of sodium citrate buffer and gut tissue homogenate. Homogenate volumes ranged from 5 to 25  $\mu\text{l}$  depending on  $\alpha$ -amylase concentration in the homogenates. The incubation was stopped after 15–25 min by adding 20  $\mu\text{l}$  of 1 M NaOH and 200  $\mu\text{l}$  of Somogyi-Nelson reagent A. Somogyi-Nelson reagent B was added after the assay solution was boiled for 10 min (see German et al. 2004 for reagent recipes). The resulting solution was diluted in deionized water and centrifuged at  $6,000 \times g$  for 5 min. The glucose content of the solution was then determined spectrophotometrically at 650 nm. The  $\alpha$ -amylase activity was determined from a glucose standard curve and expressed in U (1  $\mu\text{mol}$  glucose liberated per minute) per gram wet weight of gut tissue.

Maltase (3.2.1.20) activity was measured following Dahlqvist (1968) as described by German et al. (2004). In a microcentrifuge tube, 10  $\mu\text{l}$  of 56 mM maltose dissolved in 100 mM maleate buffer, pH 7.0, was combined with 10  $\mu\text{l}$  of diluted regional gut homogenate. After 10 min, the reaction was stopped by the addition of 300  $\mu\text{l}$  of assay reagent (Sigma GAGO20) dissolved in 1 M Tris-HCl, pH 7.0. The reaction mixture was incubated for 30 min at  $37^{\circ}\text{C}$ , and was

stopped by the addition of 300  $\mu\text{l}$  of 12 N  $\text{H}_2\text{SO}_4$ . The amount of glucose in the solution was then determined spectrophotometrically at 540 nm. The maltase activity was determined from a glucose standard curve and expressed in U (1  $\mu\text{mol}$  glucose liberated per minute) per gram wet weight of gut tissue.

Trypsin (E.C. 3.4.21.4) activity was assayed using a modified version of the method designed by Erlanger et al. (1961) as described by Gawlicka et al. (2000). The substrate, 2 mM *N* $\alpha$ -benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA), was dissolved in 100 mM Tris-HCl buffer (pH 8.0) by heating to 95°C (Preiser et al. 1975; German et al. 2004). In a microplate, 95  $\mu\text{l}$  of BAPNA was combined with 5  $\mu\text{l}$  of homogenate, and the increase in absorbance was read continuously at 410 nm for 15 min. Trypsin activity was determined with a *p*-nitroaniline standard curve, and expressed in U (1  $\mu\text{mol}$  *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase (EC 3.4.11.2) activity was measured according to Roncari and Zuber (1969) as described by German et al. (2004). In a microplate, 90  $\mu\text{l}$  of 2.04 mM L-alanine-*p*-nitroanilide HCl dissolved in 200 mM sodium phosphate buffer (pH 7.0) was combined with 10  $\mu\text{l}$  of homogenate. The increase in absorbance was read continuously at 410 nm for 15 min and activity determined with a *p*-nitroaniline standard curve. Aminopeptidase activity was expressed in U (1  $\mu\text{mol}$  *p*-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Lipase (nonspecific bile-salt activated E.C. 3.1.1.-) activity was assayed using a modified version of the method designed by Iijima et al. (1998). In a microplate, 86  $\mu\text{l}$  of 5.2 mM sodium cholate dissolved in 250 mM Tris-HCl (pH 9.0) was combined with 6  $\mu\text{l}$  of homogenate and 2.5  $\mu\text{l}$  of 10 mM 2-methoxyethanol and incubated at room temperature for 15 min to allow for lipase activation by bile salts. The substrate *p*-nitrophenyl myristate (5.5  $\mu\text{l}$  of 20 mM *p*-nitrophenyl myristate dissolved in 100% ethanol) was then added and the increase in absorbance was read continuously at 405 nm for 15 min. Lipase activity was determined with a *p*-nitrophenol standard curve, and expressed in U (1  $\mu\text{mol}$  *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.

Gut fluid preparation, gastrointestinal fermentation, and luminal nutrient concentrations

Measurements of symbiotic fermentation activity were based on the methods of Pryor and Bjorndal (2005).

Fermentation activity was indicated by relative concentrations of SCFA in the fluid contents of the guts of the fishes at the time of death. Gut content samples were weighed [gut content mass (GCM  $\pm$  0.001 g)], thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4°C) at 16,000g for 10 min. The supernatant was then pipetted into a sterile centrifuge vial equipped with a 0.22  $\mu\text{m}$  cellulose acetate filter (Costar Spin-X gamma sterilized centrifuge tube filters, Coming, NY, USA) and centrifuged under refrigeration at 13,000 $\times$ g for 15 min to remove particles from the fluid (including bacterial cells). The filtrates were collected and frozen until they were analyzed for SCFA and nutrient concentrations.

Concentrations of SCFA in the gut fluid samples from each gut region were measured using gas chromatography. Samples were hand-injected into a Shimadzu GC-9AM gas chromatograph equipped with a flame ionization detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and a Perkin Elmer LC-100 integrator (Perkin Elmer, Inc., CT, USA). Two  $\mu\text{l}$  of each sample were injected onto a 2-m long glass column (3.2 mm ID) packed with 10% SP-1000 and 1%  $\text{H}_3\text{PO}_4$  on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA). Carrier gas was  $\text{N}_2$ , at a flow rate of 40 ml  $\text{min}^{-1}$ . Temperatures of the inlet, column, and detector were 180, 155, and 200°C, respectively. An external standard containing 100 mg  $\text{l}^{-1}$  each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate was used for calibration. The SCFA concentrations are expressed as mmol  $\text{l}^{-1}$  of gut fluid, and are also presented as ratios of the percent of acetate, propionate, and butyrate in the samples, as is commonly practiced in studies of fermentation in domestic (Bergman 1990), and wild animals (Bouchard and Bjorndal 2005; Pryor and Bjorndal 2005; Pryor et al. 2006). Because of their small size, the gut contents of *C. ornatum* were pooled into one sample for each fish, and therefore, regional estimates of fermentation (and luminal nutrient concentrations) along the digestive tract were not possible.

Nutrient concentrations in the gut fluid were carried out to examine how the concentrations of glucose, protein, and lipid varied along the gut. Glucose concentrations were analyzed in 1–5  $\mu\text{l}$  of gut fluid using the same glucose content assay described for the maltase assay above. The only departure being that there was no pre-incubation with maltose; the gut fluid was immediately combined with the assay reagent and incubated at 37°C for 30 min, the reaction stopped with 12 N  $\text{H}_2\text{SO}_4$ , and the resulting mixture read in a spectrophotometer at 540 nm against a glucose standard curve.

Protein was measured with bicinchoninic acid (Smith et al. 1985) following the protocol of the BCA protein assay kit (Pierce, Rockford, IL, USA). In a microplate,

10 µl of diluted gut fluid was combined with 200 µl of BCA reagent and incubated at 37°C for 30 min. The plate was then read in a spectrophotometer at 562 nm, and the protein content determined from a standard curve made with bovine serum albumin. We recognize that this method determines total protein concentration, including free aromatic amino acids, peptides, and complete proteins. Therefore, digestive enzymes of endogenous and exogenous origin likely contributed to the protein concentrations in the gut fluid. More precise examination of different amino acid groups can provide more detail in terms of hydrolytic end products of digestive enzyme activity (Mayer et al. 1997; Crossman et al. 2005). However, I was interested in the relative pattern of protein concentration along the gut (Hofer 1982) so this “coarse” measurement was suitable to answer the question at hand.

Lipid content was assayed following the charring method of Marsh and Weinstein (1966). In a microcentrifuge vial, 10 µl of intestinal fluid was combined with 50 µl of 2:1 (v:v) chloroform/methanol and vortexed at 30-s intervals over 10 min. Then, 12.5 µl of 1 M NaCl (Neighbors and Horn 1991) was added and the mixture vortexed again for 1 min. The mixture was then centrifuged at 6,200×g for 2 min, and 5–10 µl of the bottom phase was pipetted into a glass vial. To evaporate the solvent, the vial was then baked at 60°C for 30 min. To the glass vial, I then added 200 µl of concentrated (36 M) H<sub>2</sub>SO<sub>4</sub> and baked the vial and its contents at 200°C for 15 min. The vials were then allowed to cool in a water bath, after which 300 µl of deionized water was added and 100 µl of the resulting fluid was read in a spectrophotometer at 375 nm. Lipid content was determined from a standard curve constructed with stearic acid.

Relative gut content mass

In terrestrial animals that utilize gastrointestinal fermentation to meet at least part of their daily energetic needs, nearly half the gut content mass at a given time is concentrated in a portion of the gut where fermentation actively takes place (Parra 1978; Pryor and Bjorndal 2005). Thus, in concert with my estimates of SCFA concentrations in each region of the gut, I examined the relative gut content mass [RGCM = GCM (g)/BM (g)] of each of the gut regions in the five species to determine whether gut contents are more concentrated in one portion of the gut over the other.

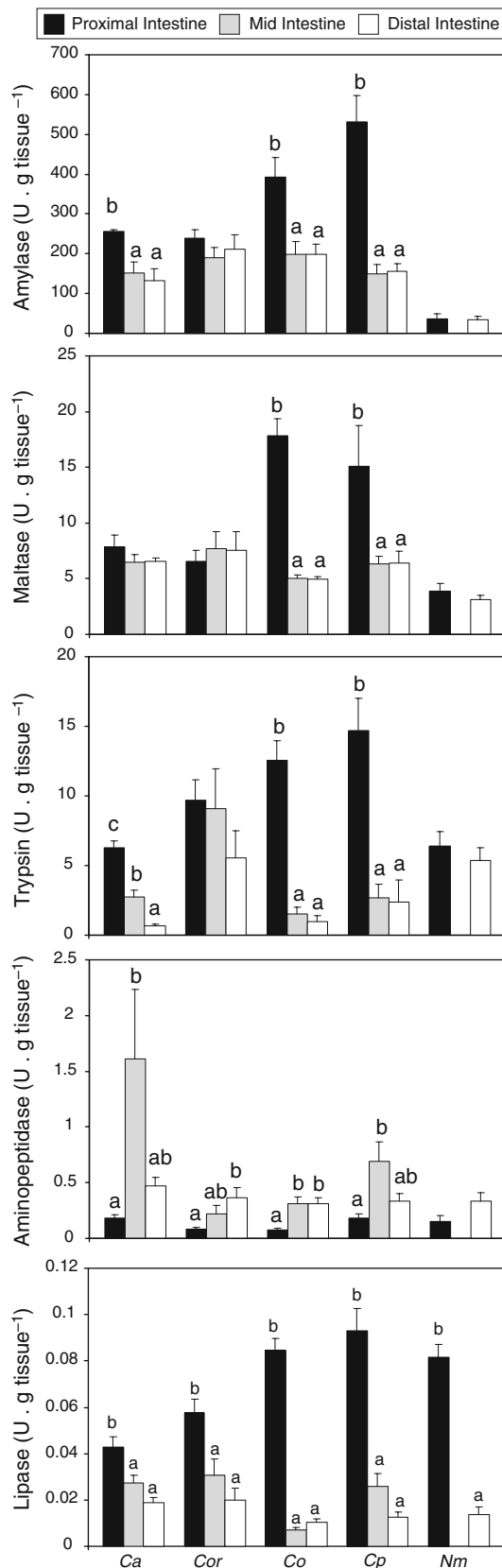
Statistical analyses

Prior to all significance tests, a Levene’s test for equal variance was performed to ensure the appropriateness of the data for parametric analyses. If the data were not normal, they were log transformed, and normality confirmed prior to analysis. All tests were run using Minitab statistical software (version 13, State College, PA, USA). I was interested in the patterns of enzymatic activities, gastrointestinal fermentation, luminal nutrient concentrations, and gut content mass along the alimentary canal of each of the fish species. Thus, in the three species of *Campostoma* I used ANOVA, followed by a Tukey’s HSD test with a family error rate of  $P = 0.05$ , to examine how the enzyme activities, total SCFA concentrations, nutrient concentrations, and gut content mass varied among the proximal, mid, and distal intestines. I used *t*-tests to compare the same variables among the proximal and distal intestine in *N. micropogon*. ANOVA and *t*-test statistics are shown in Table 2, separately from the data, which are graphed in Fig. 1. Because I was unable to measure regional SCFA, nutrient concentration,

**Table 2** Summary of ANOVA statistics for intraspecific comparisons of digestive enzyme activities among gut regions in five species of minnows

Enzyme	<i>C. anomalum</i>	<i>C. ornatum</i>	<i>C. oligolepis</i>	<i>C. pauciradii</i>	<i>N. micropogon</i>
Amylase	$F_{2,29} = 8.06$ $P < 0.01$	$F_{2,29} = 0.78$ $P = 0.47$	$F_{2,29} = 9.87$ $P < 0.01$	$F_{2,32} = 25.34$ $P < 0.01$	$t = 0.19$ $P = 0.85$
Maltase	$F_{2,23} = 0.51$ $P = 0.61$	$F_{2,17} = 0.19$ $P = 0.83$	$F_{2,29} = 94.42$ $P < 0.01$	$F_{2,29} = 4.17$ $P = 0.03$	$t = 1.04$ $P = 0.32$
Trypsin	$F_{2,29} = 53.98$ $P < 0.01$	$F_{2,23} = 1.09$ $P = 0.36$	$F_{2,29} = 48.10$ $P < 0.01$	$F_{2,32} = 16.83$ $P < 0.01$	$t = 0.77$ $P = 0.46$
Aminopeptidase	$F_{2,23} = 4.37$ $P = 0.03$	$F_{2,14} = 4.35$ $P = 0.04$	$F_{2,29} = 9.04$ $P < 0.01$	$F_{2,29} = 5.55$ $P = 0.01$	$t = 2.01$ $P = 0.07$
Lipase	$F_{2,29} = 12.68$ $P < 0.01$	$F_{2,29} = 9.92$ $P < 0.01$	$F_{2,29} = 187.79$ $P < 0.01$	$F_{2,32} = 42.51$ $P < 0.01$	$t = 10.61$ $P < 0.01$

Data are presented in Fig. 1. Sample sizes for each species and enzyme are as follows: *C. anomalum*, amylase  $n = 10$ ; maltase  $n = 8$ ; trypsin  $n = 10$ ; aminopeptidase  $n = 8$ ; lipase  $n = 10$ . *C. ornatum*, amylase  $n = 10$ ; maltase  $n = 6$ ; trypsin  $n = 8$ ; aminopeptidase  $n = 5$ ; lipase  $n = 10$ . *C. oligolepis*,  $n = 10$  for all enzymes. *C. pauciradii*, amylase  $n = 11$ ; maltase  $n = 10$ ; trypsin  $n = 11$ ; aminopeptidase  $n = 10$ ; lipase  $n = 11$ . *N. micropogon*,  $n = 6$  for all enzymes



and gut content mass in *C. ornatum*, only the enzyme activities were compared among regions as described above for the other species of *Campostoma*. Interspecific

comparisons of the various variables were not made in this study, mainly because the division of the digestive tract was different for *N. micropogon*, making direct comparisons impossible.

◀ **Fig. 1** Digestive enzyme activities in three regions of the intestine of five minnow species. Values are mean  $\pm$  SEM. Intraspecific comparisons among the gut regions for each enzyme were made with ANOVA followed by Tukey's HSD with a family error rate of  $P = 0.05$ . Bars for a species and enzyme that share a *superscript* letter are not significantly different. Interspecific comparisons were not performed. ANOVA statistics are summarized in Table 2. *Ca*, *Campostoma anomalum*; *Cor*, *C. ornatum*; *Co*, *C. oligolepis*; *Cp*, *C. pauciradii*; and *Nm*, *Nocomis micropogon*

## Results

### Digestive enzyme activities

With the exception of *C. ornatum*, the *Campostoma* species showed significant decreases in amylase activity toward the distal intestine (Fig. 1; Table 2). *N. micropogon* showed no change in its amylase activity between the proximal and distal intestine. The amylase activity of *C. anomalum*, *C. ornatum*, *C. oligolepis*, and *C. pauciradii* decreased by 48, 12, 50, and 70%, respectively, from proximal to distal portions of the intestine. *C. anomalum*, *C. ornatum*, and *N. micropogon* did not show significant changes in maltase activity along the gut, whereas *C. oligolepis* and *C. pauciradii* significantly decreased their maltase activity by 70 and 60%, respectively, in the mid- and distal intestine in comparison to the activities in the proximal intestine.

Similar to amylase, trypsin activity significantly decreased toward the distal intestine in *C. anomalum*, *C. oligolepis*, and *C. pauciradii*, but not in *C. ornatum* (Fig. 1; Table 2). The trypsin activity in the distal intestine of *C. anomalum*, *C. oligolepis*, and *C. pauciradii* was reduced by 90, 93, and 85%, respectively, of its activity in the proximal intestine. Although the change in trypsin activity was not statistically significant in *C. ornatum*, it decreased by 43% in the distal intestine in comparison to the proximal intestine. In *N. micropogon*, trypsin activity decreased by only 16% in the distal intestine when compared to the proximal intestine, which was not a significant difference. In contrast to the other enzymes, aminopeptidase activities increased distally in the alimentary tracts of all four *Campostoma* species, with *C. anomalum* and *C. pauciradii* showing peaks in the mid intestine. *C. anomalum* had mean aminopeptidase activities that were an order of magnitude higher in the mid than in the proximal intestine, and *C. pauciradii* increased its aminopeptidase activity nearly fourfold in the mid compared to the proximal intestine. The aminopeptidase activities of *C. anomalum*, *C. ornatum*, *C. oligolepis*, *C. pauciradii*, and *N. micropogon* increased by 2.5, 4.5,

**Table 3** Fluid nutrient concentrations and digesta content masses in different regions of the gut of four species of herbivorous (*Campostoma*) and carnivorous (*Nocomis*) minnows

Gut region	<i>C. anomalum</i>	<i>C. oligolepis</i>	<i>C. pauciradii</i>	<i>N. micropogon</i>
<b>Glucose (mM)</b>				
Proximal	4.19 ± 0.40	5.49 ± 0.94 <sup>b</sup>	6.07 ± 0.49	3.41 ± 1.58
Mid	3.97 ± 0.51	2.54 ± 0.39 <sup>ab</sup>	3.22 ± 0.36	–
Distal	5.69 ± 1.17	2.50 ± 0.42 <sup>a</sup>	3.22 ± 1.88	3.74 ± 1.82
	$F_{2,17} = 1.47$ $P = 0.26$	$F_{2,29} = 4.05$ $P = 0.03$	$F_{2,14} = 2.43$ $P = 0.13$	$t = 0.14$ $P = 0.89$
<b>Protein (mg/ml)</b>				
Proximal	14.69 ± 2.90	30.41 ± 4.86 <sup>b</sup>	26.26 ± 4.81 <sup>b</sup>	17.60 ± 5.39
Mid	11.12 ± 1.31	11.73 ± 1.93 <sup>a</sup>	13.53 ± 4.14 <sup>ab</sup>	–
Distal	11.37 ± 1.28	12.04 ± 2.21 <sup>a</sup>	9.51 ± 3.66 <sup>a</sup>	18.86 ± 5.32
	$F_{2,17} = 1.01$ $P = 0.39$	$F_{2,29} = 10.66$ $P < 0.01$	$F_{2,14} = 4.27$ $P = 0.04$	$t = 0.17$ $P = 0.87$
<b>Lipid (mg/ml)</b>				
Proximal	14.84 ± 5.00	40.72 ± 5.09 <sup>b</sup>	18.43 ± 6.04 <sup>b</sup>	12.71 ± 2.04
Mid	12.78 ± 2.81	10.96 ± 1.19 <sup>a</sup>	10.71 ± 2.54 <sup>b</sup>	–
Distal	5.21 ± 0.34	12.40 ± 3.86 <sup>a</sup>	3.40 ± 2.08 <sup>a</sup>	8.81 ± 2.53
	$F_{2,17} = 0.35$ $P = 0.71$	$F_{2,29} = 25.63$ $P < 0.01$	$F_{2,14} = 5.69$ $P = 0.02$	$t = 1.20$ $P = 0.27$
<b>Gut content mass (GCM/BM)</b>				
Proximal	0.013 ± 0.002	0.032 ± 0.004	0.019 ± 0.003	0.012 ± 0.002
Mid	0.015 ± 0.006	0.027 ± 0.002	0.022 ± 0.004	–
Distal	0.006 ± 0.001	0.029 ± 0.003	0.024 ± 0.007	0.013 ± 0.003
	$F_{2,26} = 1.22$ $P = 0.31$	$F_{2,29} = 0.86$ $P = 0.43$	$F_{2,29} = 0.29$ $P = 0.75$	$t = 0.37$ $P = 0.72$

Values are mean ± SEM. Comparisons were made among gut regions for each nutrient class and gut content mass for the *Campostoma* species with ANOVA followed by Tukey’s HSD with a family error rate of  $P = 0.05$ . Nutrient concentrations or gut masses for a particular species that share a *superscript letter* are not significantly different. Comparisons were made among the two gut regions in *N. micropogon* with *t*-test. Sample sizes (nutrient analyses; gut content mass): *C. anomalum* (6; 9), *C. oligolepis* (10; 10), *C. pauciradii* (5; 10), and *N. micropogon* (5; 5)

4.0, 2.0, and 2.0 fold, respectively, in the distal intestine in comparison to the proximal intestine, but this increase was only statistically significant in *C. ornatum* and *C. oligolepis*. Nonetheless, all five taxa showed increased ability to hydrolyze dipeptides in the mid- and distal intestine in comparison to the proximal intestine.

All five species significantly decreased their lipase activities proximal to distal along the gut (Fig. 1). Individuals of *C. anomalum*, *C. ornatum*, *C. oligolepis*, *C. pauciradii*, and *N. micropogon* decreased their lipase activities by 56, 65, 88, 84, and 83%, respectively, in the distal intestine compared to the proximal intestine, indicating that their ability to hydrolyze lipids is lower in the distal intestine.

**Luminal nutrient concentrations and gut content mass**

*Campostoma oligolepis* and *C. pauciradii* decreased the glucose concentrations in the fluid of their distal intestine compared to the proximal intestine by 55 and 47%, respectively, but this difference was only statistically significant

for *C. oligolepis*. *C. anomalum* and *N. micropogon* increased their glucose concentrations by 36 and 10%, respectively, in the distal intestine (Table 3). The protein concentration in the distal digestive fluids of *C. anomalum*, *C. oligolepis*, and *C. pauciradii* decreased by 23, 60, and 64%, respectively, in comparison to protein concentrations in the proximal gut fluid, but only significantly so for *C. oligolepis* and *C. pauciradii*; *N. micropogon* showed a slight, but non-significant increase (7%) in protein concentration in the distal intestine (Table 3). All four minnow taxa decreased the lipid concentrations in the gut fluid of the distal intestine in comparison to the proximal intestine, but only significantly so in *C. oligolepis* and *C. pauciradii*. No differences were observed in the RGCM in any gut region in any of the four species (Table 3).

**Gastrointestinal fermentation**

None of the minnow taxa showed significant changes in SCFA concentrations along the gut, and overall levels of

**Table 4** Total short chain fatty acid concentrations in each gut region of four minnow species

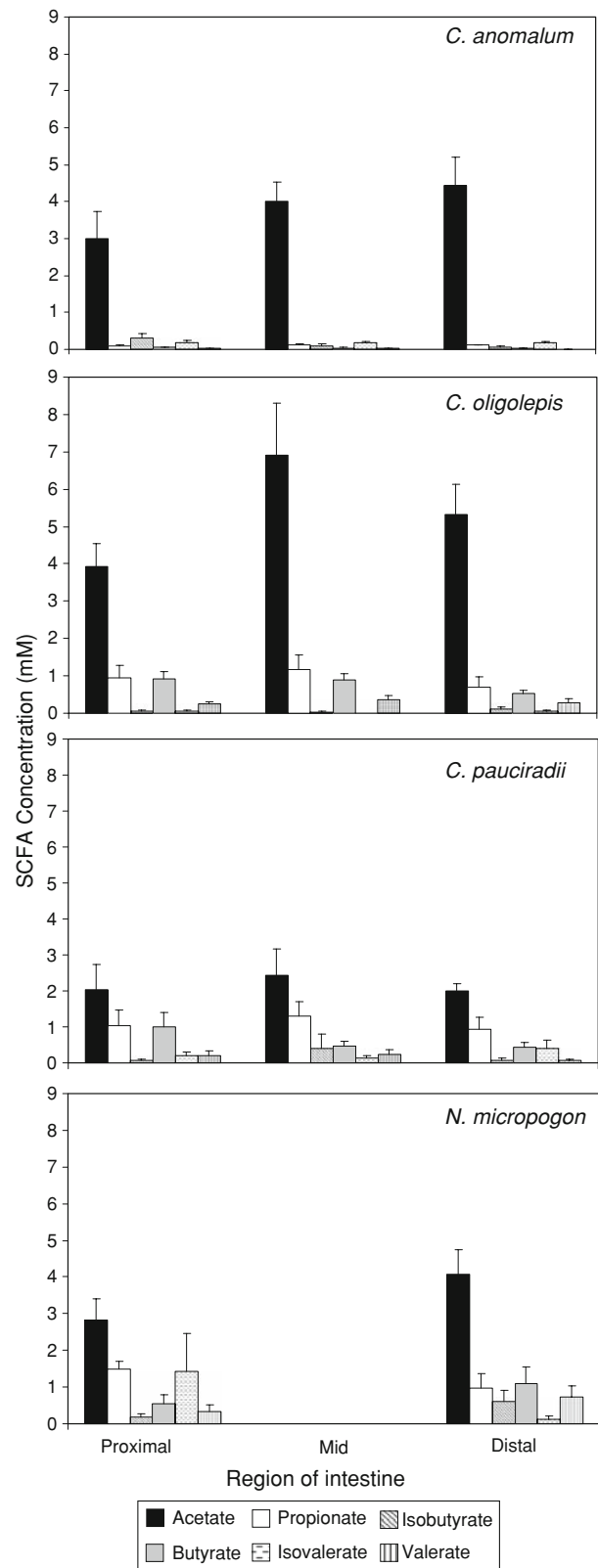
Gut region	<i>C. anomalum</i>	<i>C. oligolepis</i>	<i>C. pauciradii</i>	<i>N. micropogon</i>
Proximal	3.65 ± 0.84	6.15 ± 0.75	4.55 ± 0.76	6.83 ± 0.89
Mid	4.48 ± 0.51	9.39 ± 1.56	4.93 ± 1.44	–
Distal	4.83 ± 0.77	7.00 ± 0.82	3.91 ± 0.62	7.59 ± 1.72
	$F_{2,17} = 0.71$	$F_{2,29} = 2.31$	$F_{2,20} = 0.26$	$t = 0.39$
	$P = 0.51$	$P = 0.12$	$P = 0.77$	$P = 0.71$

Values are  $\text{mM l}^{-1}$  intestinal fluid, are presented as mean ± SEM. Comparisons of SCFA concentrations among gut regions for the *Campostoma* species were made with ANOVA, with differences considered significant at  $P = 0.05$ . SCFA concentrations in the PI and DI of *N. micropogon* were compared with *t*-test. Sample sizes: *C. anomalum* (6), *C. oligolepis* (10), *C. pauciradii* (7), and *N. micropogon* (5)

fermentation were not concentrated in one gut region (Table 4; Fig. 2). All four minnow species in which I measured SCFA concentrations showed a dominance of acetate over the other SCFA, and the proportion of acetate in comparison to the other SCFA increased distally along the gut. The ratios for acetate:propionate:butyrate in each gut region for the four minnow taxa were as follows: *C. anomalum*, PI—82:3:2, MI—89:3:1, DI—92:2:0.4; *C. oligolepis*, PI—64:15:15, MI—74:13:9, DI—76:10:8; *C. pauciradii*, PI—45:22:22, MI—49:26:9, DI—51:24:11; *N. micropogon*, PI—42:22:8, DI—54:13:14. Notably, *N. micropogon* had the highest proportions of isobutyrate and isovalerate (PI—30%, DI—20%) in its gut, and these two SCFA were low (<15%) in all gut regions of the *Campostoma* species.

## Discussion

The results of this study essentially support the hypothesis that small herbivorous minnows have intestines that function as PFRs. With few exceptions, the activities of amylase, trypsin, and lipase decreased in the distal region of digestive tract of the *Campostoma* species. Maltase activities tended to remain the same (*C. anomalum*, *C. ornatum*, and *N. micropogon*) or to decrease distally in the gut (*C. oligolepis* and *C. pauciradii*), concomitant with regional glucose concentrations along the intestines of these species. In contrast, aminopeptidase activities generally increased distally in the alimentary tract. Protein and lipid concentrations decreased in the distal intestine, whereas SCFA and gut content mass were not localized to one gut region, which, along with the unspecialized gut morphology of these minnows (German et al. 2009), suggests that their guts function as PFRs. Differences between the herbivorous species of *Campostoma* and the carnivorous *N. micropogon*



**Fig. 2** Concentrations (mM) of short-chain fatty acids (SCFA) acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate among gut regions of four minnow species



show that the guts of these herbivores and carnivore function in slightly different ways, most likely relating to dietary quality and intake.

Although digestive enzyme activities have been measured along the digestive tracts of numerous wild-caught (e.g., Fish 1960; Hofer 1982; Chakrabarti et al. 1995; Smoot and Findlay 2000; Logothetis et al. 2001; Gawlicka and Horn 2005; Skea et al. 2005, 2007), and cultured fish species (e.g., Chiu and Benitez 1981; Harpaz and Uni 1999; Tengjaroenkul et al. 2000; Hakim et al. 2006, 2007), few have considered these activities in the context of chemical reactor models. Amylase and trypsin activities are known to decrease distally in the intestine of several cyprinid taxa (Hofer and Schiemer 1981; Hofer 1982; Bitterlich 1985; Das and Tripathy 1991; Chakrabarti et al. 1995). Given that amylase and trypsin are pancreatic in origin, and are therefore secreted in the proximal intestine, it makes sense that the activities of these enzymes decrease in the hindgut as their substrates are diminished and the enzymes themselves are broken down or reabsorbed (Hofer 1982; Clements and Raubenheimer 2006). Lipase activity decreases distally in the guts of several cyprinids (Das and Tripathy 1991; Chakrabarti et al. 1995) and other detritivorous fish (e.g., gizzard shad; Smoot and Findlay 2000; Loricariid catfishes; German 2008), most likely for the same reason, as this enzyme is also secreted by the pancreas.

Maltase activities were expected to increase in the mid- and distal intestine of the minnows. However, two patterns of maltase activities along the digestive tract emerged in this study: one where maltase activities decreased moving distally in the intestine (*C. oligolepis* and *C. pauciradii*), and one where maltase activities were relatively similar throughout the gut (*C. anomalum*, *C. ornatum*, and *N. micropogon*). Among the *Campostoma*, these differences fall directly along phylogenetic lines, as the two groups of sister-taxa (*C. oligolepis* and *C. pauciradii* vs. *C. anomalum* and *C. ornatum*) show differing patterns. Sister-taxa similarity in digestive enzyme activities has been observed in prickleback fishes (German et al. 2004), and a similar split relating to laminarinase activity was observed in these same minnow species (German et al. 2009). Exactly why the patterns of maltase activity would differ among these two clades is unknown but may have to do with dietary selection.

The diet of the *Campostoma* species is best described as “periphyton” or the epilithic algal complex (EAC), which is composed of bacteria, cyanobacteria, filamentous green algae, diatoms, and detritus growing on hard substrates (Hoagland et al. 1982; van Dam et al. 2002; Klock et al. 2007). The EAC can be rich in soluble polysaccharides (Leppard 1995; Wotton 2004; Klock et al. 2007) as well as in lipids (Napolitano et al. 1996) and proteins (Bowen et al. 1995; Wilson et al. 2003; Crossman et al. 2005). Thus, the

EAC can be considerably more nutritious and easier to digest and subsist on than a purely algal diet, and likely would allow a fish to rely on endogenous digestive mechanisms (Crossman et al. 2005) and have a PFR gut.

Measurable glucose in the gut fluid of these fishes illustrates that they are consuming a highly digestible food that is rich in soluble polysaccharides. Other herbivorous fishes that consume macro- and microalgae, which are lower in soluble polysaccharides, have little or no detectable glucose in their digestive tracts (Skea et al. 2005, 2007; German 2008). Given that there is measurable glucose left in the distal intestine of these minnows suggests that some glucose may even escape the gut unabsorbed. Protein is known to be the limiting nutrient for fishes grazing on the EAC (Bowen et al. 1995) and algae (Raubenheimer et al. 2005). Thus, in the context of compartment models of digestion and nutrient targets (Raubenheimer and Simpson 1998), if protein is the limiting nutrient and carbohydrates are overly abundant to these minnows, then perhaps they have optimized intake and retention time to maximize protein digestion and assimilation, even at the cost of losing some glucose in their feces (i.e., “wastage”; Raubenheimer and Simpson 1998; Clements and Raubenheimer 2006). This may be especially true for *C. anomalum* and *C. ornatum*. The patterns of aminopeptidase activity along the gut support this idea, as the activity of this dipeptidase increased distally in the guts of all five minnow taxa. Aminopeptidase indicates the ability of the fish to hydrolyze dipeptides just preceding absorption of amino acids in the gut (Fraisse et al. 1981; Harpaz and Uni 1999). So it seems logical that increasing activities of this enzyme reflect an increased capacity to absorb proteins distally in the intestine. A similar pattern has been observed in tilapia (Harpaz and Uni 1999), which are known to be detritivorous in natural settings, and feed to meet protein requirements (Bowen 1980, 1981; Bowen et al. 1995).

Trypsin activity is generally not different in the guts of *Campostoma* and *Nocomis* (German et al. 2009), and in this study, it is apparent that the *Campostoma* have qualitatively higher aminopeptidase activities than *N. micropogon*. By incorporating intake and retention time, the *Campostoma* taxa may actually synthesize more of these proteases (German et al. 2009), and therefore, have higher trypsin and aminopeptidase activities on a daily basis than the carnivorous *Nocomis* (Hofer and Schiemer 1981), showing that these EAC feeders efficiently digest and assimilate protein from their diet.

Some interesting differences were observed in the digestive enzyme activity and luminal nutrient concentration patterns along the intestine between the *Campostoma* species and *N. micropogon*. Whereas amylase and trypsin activities generally decreased in the distal intestines of the herbivores, these enzyme activities remained constant in the

proximal and distal intestine of *N. micropogon*. Furthermore, amylase was noticeably lower in *N. micropogon*, and each of the *Campostoma* taxa had significantly higher amylase in their gut than *N. micropogon* (German et al. 2009). Amylase activity has been shown to be higher in herbivorous than in carnivorous fishes (Fernandez et al. 2001; German et al. 2004; Horn et al. 2006), so this finding is not surprising. However, the lack of any patterns of activity of amylase and trypsin, nor in the concentrations of glucose and protein along the *N. micropogon* gut, challenge the PFR model in this species. Their gut is considerably shorter ( $0.86\times$  their body length) than the guts of the *Campostoma* species ( $3.2\text{--}5.4\times$  their body length; German et al. 2009), and, because their insect diet is likely higher in protein than an EAC diet, they probably have lower intake than the *Campostoma*. This is, in fact, predicted by their shorter guts (Sibly and Calow 1986; Horn and Messer 1992), and in further support, *N. micropogon* had lower overall gut content masses than any *Campostoma* species (German et al. 2009). With lower intake, a higher protein diet, and a shorter gut, *N. micropogon* may have more axial mixing of digesta within their intestine, or at least lower gut motility than the *Campostoma*. This slowing, or breakdown in PFR function has been observed in the herbivorous prickleback fish *Cebidichthys violaceus* feeding on a high-protein diet (Fris and Horn 1993), in the omnivorous *Girella tricuspidata* feeding on a more protein-rich alga (Raubenheimer et al. 2005), and in the halfbeak, *Hyporhamphus melanochir*, feeding on invertebrates in comparison to when feeding on sea grass (Klump and Nichols 1983).

Over the last 20 years it has become increasingly clear that herbivorous fishes do not all function like terrestrial vertebrate herbivores with respect to gastrointestinal fermentation (Choat and Clements 1998; Clements et al. 2009). In fact, many fish taxa categorized as nominally herbivorous (e.g., parrotfishes) include species that consume primarily detritus, which is moderately rich in protein and low in structural polysaccharides, unlike algae and plants (Crossman et al. 2005). Crossman and colleagues illustrated that there is a basic continuum of feeding habits in tropical “herbivorous” fishes, ranging from detritivores with rapid throughput rates and low levels of fermentation in their guts (i.e., guts that function like PFRs), to herbivores with lower throughput rates and high levels of fermentation, primarily localized in the hindgut. Foregut fermentation, which is found in ruminants, kangaroos, and a bird, the hoatzin (Stevens and Hume 1995; Karasov and Hume 1997; Stevens and Hume 1998), is unknown in fishes. The *Campostoma* showed no localization of SCFAs in the digestive tract, and the overall concentrations of SCFAs were low. For example, the highest SCFA concentrations observed in the mid intestine of *C. oligolepis* (9.39 mM total SCFAs) qualify as low fermentation poten-

tial (i.e.,  $<20$  mM) among fishes (Choat and Clements 1998). The lack of any localized region of fermentation in the minnow guts suggests that, similar to the fellow cyprinids grass carp and common carp (Smith et al. 1996; Stevens and Hume 1998), *Campostoma* do not rely on gastrointestinal fermentation to meet their daily energy needs. Furthermore, the *Campostoma* likely consume microbes with their food, which may already be engaged in fermentative digestion of detrital material. Horn and Messer (1992) predicted that fermentation in fishes with PFR intestines would likely come from microbes ingested with their food, rather than endosymbionts per se.

The proportional amount of acetate produced in the minnow guts increases toward the distal intestine. Acetate is produced in higher proportions by microbes digesting more structural polysaccharides (e.g., cellulose), whereas propionate is often produced in higher proportions when soluble polysaccharides are fermented (Bergman 1990). Because the *Campostoma* taxa are rapidly digesting the soluble component of the EAC in the proximal intestine, as evidenced by higher amylase activities and glucose concentrations in the foregut, this leaves less soluble and more structural elements toward the distal intestine, possibly accounting for the higher proportion of acetate produced there.

Some fishes (Titus and Ahearn 1988; Mountfort et al. 2002), and cyprinids in particular (Smith et al. 1996), are known to be able to absorb and utilize SCFA as a potential energy source, but given the low concentrations of SCFA in *Campostoma* guts, these by-products of fermentation probably contribute little to their daily energy budgets. Even small (3 g) detritivorous bullfrog tadpoles, which meet about 20% of their daily energy needs via gastrointestinal fermentation, have approximately 35 mM SCFA in their guts, mostly concentrated in their hindgut (Pryor and Bjorndal 2005). Additionally, animals can produce “endogenous” acetate by degrading acetyl-CoA in their tissues, and circulating acetate in the blood (Annison and White 1962; Clements et al. 1994); up to 40% of acetate in sheep blood may be from endogenous production (Annison and White 1962). Thus, it is possible that the low levels of fermentation in the minnow guts, and the low concentrations of acetate in particular, are low enough to allow a diffusion gradient of acetate from the blood stream into the intestinal lumen, leading to low concentrations of acetate in the intestinal fluid. However, explicit investigation of this hypothesis is necessary to test this assertion.

*Nocomis micropogon* also had low levels of fermentative digestion in its gut. However, this carnivore showed evidence (i.e., higher concentrations of isobutyrate and isovalerate) that amino acids were fermented in its intestine. The diet of *N. micropogon* is dominated by adult and larval insects, and includes some ( $\sim 25\%$ ) algae and detritus

(Lachner 1950; German et al. 2009). Thus, it is possible that microbes ingested with the algae and detritus ferment amino acids that are not directly absorbed by the fish.

In conclusion, the data gathered on digestive enzyme activities, luminal nutrient concentrations, and gastrointestinal fermentation suggest that herbivorous minnows of the genus *Campostoma* indeed have guts that function as PFRs as described by Horn and Messer (1992). Because they consume large amounts of a food (EAC) that may be of higher quality than their truly “herbivorous” counterparts that eat macroalgae or plants, they can rely more on endogenous digestive mechanisms. This appears to be common among cyprinids, as none has been reported to possess high concentrations of SCFA in their digestive tracts. The differences in maltase activity among members of the two *Campostoma* clades warrant further investigation, especially in the context of dietary selectivity. Nonetheless, it is clear that these minnows survive on a diet of EAC organisms and do not use gastrointestinal symbionts to digest algal polysaccharides. Given the important role of *Campostoma* species in North American freshwater systems, this may have important implications on nutrient cycling and trophodynamics of these habitats, >45% of which are severely threatened by human activities (Waters 1995).

The circumstantial nature of this investigation leads to a call for better predictive modeling of fish gut function and more research into fish nutritional ecology; the latter pales in comparison to the nutritional ecology of terrestrial vertebrates (Clements et al. 2009). Better knowledge of food biochemical composition, food selection, and digestive physiology can provide more insight into the variables that offer the most predictive power for modeling fish digestion. For example, how do food biochemical composition, gut capacity, gut transit times, digestive enzyme activities, absorptive efficiency, and algal and plant secondary metabolites interact to affect the digestive process? What defines food quality (e.g., protein content, Neighbors and Horn 1991; food stoichiometry, Mitra and Flynn 2007), and how does this affect diet choice in nature? Knowledge of these processes will allow us to better define fish feeding, digestion, and the ecological roles of fishes in the years to come (Clements et al. 2009).

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