Evolution of Herbivory in a Carnivorous Clade of Minnows (Teleostei: Cyprinidae): Effects on Gut Size and Digestive Physiology

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ABSTRACT

We constructed a phylogeny for 10 minnow species (family Cyprinidae) previously revealed to be members of sister genera with different dietary affinities and used the phylogeny to examine whether the evolution of digestive tract size and physiology is correlated with the evolution of diet in these fishes. We studied a total of 11 taxa: four herbivorous species in the genus *Campostoma* and six largely carnivorous species in the genus *Nocomis*, including two populations of *Nocomis leptocephalus*, the carnivorous Chattahoochee River drainage population and the herbivorous Altamaha River drainage population. Thus, we were able to compare digestive tract size and physiology among sister genera (*Campostoma* and *Nocomis*) and among sister taxa (*N. leptocephalus* Chattahoochee and *N. leptocephalus* Altamaha) in dietary and phylogenetic contexts. The herbivorous taxa had longer digestive tracts and higher activity of the carbohydrases amylase and laminarinase in their guts, whereas the carnivorous species had higher chitinase activity. Phylogenetic independent-contrast analysis suggested that the evolution of amylase and chitinase activities was correlated with the evolution of diet in these species, whereas trypsin and lipase activities showed no pattern associated with diet or phylogenetic history. Concentrations of short-chain fatty acids were low in all taxa, indicating that these fishes rely largely on endogenous digestive mechanisms to subsist on their respective diets. Subtle differences in tooth shape were observed between species in the two genera. Overall, our results suggest that dietary specialization can be observed on the level of anatomy and physiology of the digestive tracts of fishes but that such differences are most appropriately viewed in comparisons of closely related species with different diets.

Introduction

An animal's digestive tract and its associated organs can account for up to 40% of the animal's metabolic rate (Cant et al. 2006), and because of these costs, the digestive tract must be tightly regulated in relation to food intake and quality (Karasov and Martínez del Rio 2007). Thus, an animal's gut capacity, in terms of size and physiology, should maximize digestion from its natural diet; that is, dietary specialization should exist not only on the level of the community but also on the levels of gut size and digestive physiology (Whelan et al. 2000). Accordingly, many studies of digestion in natural fish populations have focused on comparing gut size and digestive enzyme activities among herbivores and carnivores in an attempt to identify specializations of the digestive tract for different diets. A few interesting patterns have emerged from these investigations. First, herbivorous fishes tend to have longer digestive tracts than carnivorous fishes (Al-Hussaini 1947; Zihler 1982; Ribble and Smith 1983; Kramer and Bryant 1995; Elliott and Bellwood 2003; German and Horn 2006; Horn et al. 2006). Herbivores presumably have longer guts to allow for higher intake of a low-quality food and to maintain some minimum retention time of food in the gut (Ribble and Smith 1983; Sibly and Calow 1986; Horn 1989; Starck 2005), thereby increasing digestive efficiency (Clements and Raubenheimer 2006). Second, herbivores consume more carbohydrates than carnivores, and hence, carbohydrate-degrading enzymes (e.g., amylase, α-glucanase) tend to be higher in activity in the guts of herbivorous fishes than in those of carnivores (Fish 1960; Cockson and Bourne 1972; Reimer 1982; Sabapathy and Teo 1993; Hidalgo et al. 1999; Fernandez et al. 2001; Horn et al. 2006).

In the context of nutrient acquisition matching dietary nu-

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trient content, also known as the "adaptive-modulation hypothesis" (summarized in Karasov and Martínez del Rio 2007), it is logical that animals would efficiently digest the nutrients most available to them in their food. However, most of the comparisons of gut size and digestive enzyme activities in fishes (and vertebrates in general) have emphasized completely unrelated taxa and were likely confounded by phylogenetic distance among the species studied. Thus, better phylogenetic control in comparative studies of digestion has been called for on several occasions (Karasov and Hume 1997; Horn et al. 2006; Karasov and Martínez del Rio 2007).

Perhaps not surprisingly, recent investigations have shown that the phylogenetic history of an animal can have meaningful effects on its gut size and digestive enzyme activities and can result in comparative data that conflict with the adaptive-modulation hypothesis. Elliott and Bellwood (2003) illustrated that gut size corrected for body size correlates with diet (i.e., gut lengths followed the pattern carnivores < omnivores < herbivores) in fishes, but only within particular fish families. Comparisons of gut length among species in different families did not necessarily show a correlation with diet. Similarly, German and Horn (2006) showed that the carnivorous prickleback fish *Xiphister atropurpureus* had body-size-corrected gut lengths that were more similar to those of its sister taxon, the herbivorous *Xiphister mucosus*, than to those of another carnivore, *Anoplarchus purpurescens*. The authors argued that *X. atropurpureus* was constrained by its phylogenetic history to have a longer gut despite having a carnivorous diet and presumably lower intake than its congener. In further support of this phylogenetic constraint, Chan et al. (2004) and German et al. (2004) found that *X. atropurpureus*, similar to *X. mucosus*, had higher activity levels of amylase (a carbohydrase) in their digestive tracts than did another herbivorous prickleback, *Cebidichthys violaceus*. These findings suggest that phylogenetic relationships among taxa must be taken into account in studies of gut size and digestive physiology in fishes, especially if dietary specialization is being examined. Although some recent studies have investigated gut length and digestive enzyme activities in closely related fish species with different diets (Chakrabarti et al. 1995; Hidalgo et al. 1999; Horn et al. 2006), to our knowledge, no studies have explicitly tested whether the evolution of a digestive physiological trait (e.g., digestive enzyme activity) is directly correlated with the evolution of diet in fishes. Such a study has been performed with phyllostomid bats, and dietary effects on digestive enzyme activities were observed in these species when phylogeny was controlled for (Schondube et al. 2001).

In addition, some marine herbivorous fishes possess microbial communities in their digestive tracts that ferment algal and plant polysaccharides, making the energy in these compounds available to the host fish in the form of short-chain fatty acids (SCFAs; Clements 1997; Burr 1998; Mountfort et al. 2002). Those fish species with high levels and rates of microbial fermentation in their guts (e.g., *Kyphosus sydneyanus*; Mountfort et al. 2002; Moran et al. 2005; Clements and Raubenheimer 2006) may meet a considerable amount of their daily energy budget from the SCFAs produced by microbes in their digestive tracts. However, few investigations have examined the concentration of fermentation end products (SCFAs) in freshwater fish species (Smith et al. 1996) to ascertain whether they also use microbial endosymbionts to digest their food. Furthermore, we know of none that have examined levels of fermentation in a phylogenetic context, comparing SCFA concentrations among closely related herbivores and carnivores to observe whether there is dietary specialization in this facet of digestion.

North American minnows offer an array of feeding modes in which to study digestion in relation to dietary specialization and evolutionary history. The deeper North American minnow phylogeny (Simons et al. 2003) comprises primarily carnivorous species, but herbivory is observed in some derived taxa, providing ample opportunity to investigate the effects of evolution on gut size and digestive physiology. In this study, we examined gut size, digestive enzyme activities, and gastrointestinal fermentation in sister clades (Simons et al. 2003) of minnows that share a common carnivorous ancestor but have evolved different dietary specializations (Fig. 1): members of the genus *Campostoma* are herbivorous, feeding on algae, diatoms, and detritus (Kraatz 1923; Burr 1976; Evans-White et al. 2001; Boschung and Mayden 2004), whereas members of the genus *Nocomis* are generally carnivorous, feeding largely on insects (Lachner 1950; Gatz 1981; McNeely 1987; Cloe et al. 1995).

We chose to construct a phylogeny and measure gut size and digestive physiology in 10 species representing the two clades (Fig. 1; Table 1): four recognized species of *Campostoma* and six species of *Nocomis*, including two populations of *Nocomis leptocephalus* that have different diets, depending on the river drainage in which they are found—populations of *N. leptocephalus* from the Chattahoochee River drainage are carnivorous, whereas populations from the Altamaha River drainage are herbivorous (D. P. German, personal observation). Thus, we were able to examine characters of gut structure and function in sister clades of fishes with different diets (*Campostoma* and *Nocomis*) and in sister taxa with divergent diets (*N. leptocephalus* Chattahoochee and *N. leptocephalus* Altamaha).

Our study had six components relating to food processing and digestion in the minnow taxa. First, we analyzed the diets of the 11 taxa to confirm that during the period of study, the fish had the dietary affinities reported for them in the literature. Second, we compared gut length and gut content mass (GCM) as a function of body size among the fishes to determine whether a correlation exists between diet, gut size, and intake when phylogenetic history is controlled for. Third, we made qualitative comparisons of the shape of the pharyngeal teeth among the two genera. Minnows lack jaw teeth altogether but have pharyngeal teeth with which they can crush and grind ingested food items (Evans and Deubler 1955; Cloe et al. 1995), making them more digestible (Xie 1999, 2001). Because pharyngeal teeth are important in the digestive processes of carnivorous (Evans and Deubler 1955) and herbivorous cyprinids (Xie 1999, 2001), a comparison of tooth shape may provide insight into specializations of teeth for different diets in these fishes. Fourth, we compared the activity of five digestive enzymes (amylase, laminarinase, chitinase, trypsin, and lipase) among the 11 taxa, with

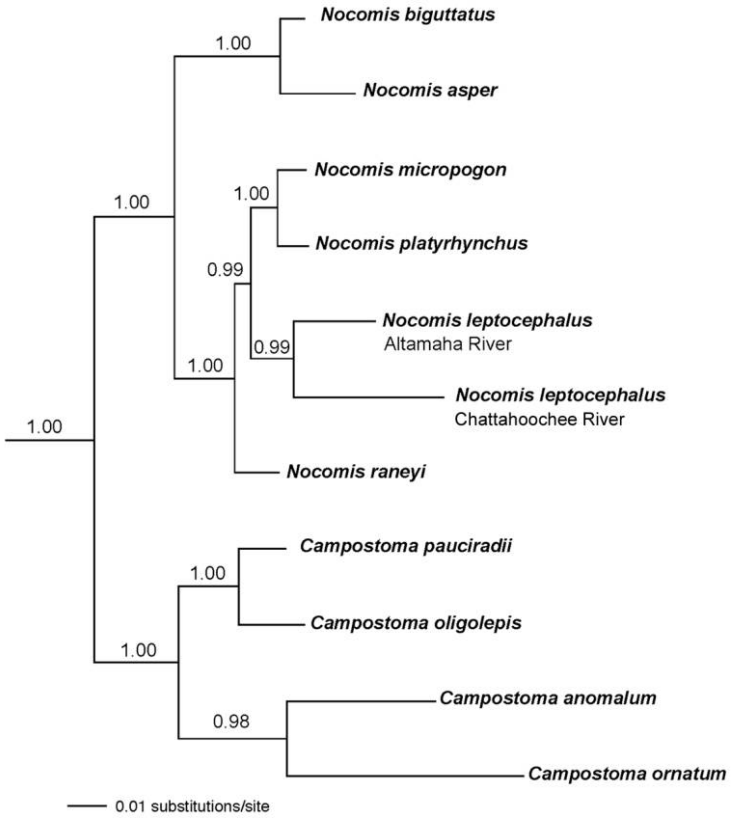

Figure 1. Phylogenetic hypothesis for the fish taxa used in this study of gut structure and function. The phylogeny was generated with gene sequences for rhodopsin, interphotoreceptor retinol binding protein, and cytochrome *b* by use of a mixed-model Bayesian approach and the Akaike Information Criterion. Numbers above branches are Bayesian posterior probabilities. The *Campostoma* species are herbivorous, and the *Nocomis* taxa are largely carnivorous, with the exception of *Nocomis leptocephalus*, of which the Chattahoochee River population is carnivorous and the Altamaha River population is herbivorous.

the expectation that enzyme activity would correlate with dietary nutrient composition (Table 2; Karasov and Martínez del Rio 2007). Fifth, we observed whether the evolution of carbohydrase activity (amylase, laminarinase, and chitinase) was correlated with the evolution of diet by using independent-contrast analyses (Felsenstein 1985). Because of the relatively consistent support in the literature for the adaptive-modulation hypothesis relating to carbohydrase activities (Schondube et al. 2001; Karasov and Martínez del Rio 2007), we expected to find such a correlation. Finally, we compared the SCFA concentrations in the gut fluid of the two genera, predicting a confirmation of the null hypothesis based on the few data sets available for comparison (Clements and Choat 1995; Smith et al. 1996). Overall, our investigation was designed to determine whether digestive tract size and physiology actually correlate with diet in these cyprinid fishes in an evolutionary context and/or whether phylogenetic history can affect digestive tract function, thereby obscuring characteristics of gut size and digestive physiology relating to dietary specialization.

Material and Methods

Fish Capture, Tissue Preparation, and pH Measurements

Fish were captured by seine and backpack electroshocker during the summer months (June–August) between 2004 and 2006 from streams throughout the eastern United States and northern Mexico (Table 1); *Nocomis biguttatus* is the only exception and was collected in October 2004. Because we used two populations of *Nocomis leptocephalus* in this investigation, for clarity we would like to differentiate these two taxa by their feeding habits: the carnivorous Chattahoochee drainage population of this species will be denoted *N. leptocephalus*-C and the herbivorous Altamaha drainage population *N. leptocephalus*-H. Upon capture, fish were placed in coolers of aerated stream water and held until killed (less than 2 h). Fish were killed in buffered stream water containing MS-222 (1 g L^{-1}), measured (standard length [SL] $\pm$ 1 mm), and weighed (body mass [BM] $\pm$ 0.01 g). Individual fish were dissected on a chilled (~4°C) cutting board, and guts were removed by cutting at the esophagus and the anus. The guts were then uncoiled, without stretching, and measured (gut length [GL] $\pm$ 1 mm). Because species of *Campostoma* and *Nocomis* have slightly different gut morphology and guts that differ in length (see Fig. A1 in the online edition of *Physiological and Biochemical Zoology*), 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 (PI)—including the liver, the gall bladder, and pancreatic tissue—the midintestine

Table 1: Collection locations for 11 minnow taxa of the genera *Campostoma* and *Nocomis*

Taxon (*N*)	Collection Location	Drainage	Latitude	Longitude
C. anomalum (10)	Flint Creek, Arkansas	Arkansas River	36°46.142′N	94°41.601′W
C. ornatum (10)	Río Santa Isabel, Chihuahua, Mexico	Río Conchos	28°32.463′N	106°30.218′W
C. oligolepis (10)	Wedowee Creek, Alabama	Tallapoosa River	33°18.496′N	85°26.068′W
C. pauciradii (7)[a]	Hillabahatchee Creek, Georgia	Chattahoochee River	33°18.632′N	85°11.288′W
C. pauciradii (4)[a]	Candler Creek, Georgia	Altamaha River	34°18.697′N	83°39.441′W
N. asper (7)	Flint Creek, Arkansas	Arkansas River	36°46.142′N	94°41.601′W
N. biguttatus (4)	Grindstone River, Minnesota	St. Croix River	46°07.450′N	93°00.420′W
N. micropogon (6)	Shoal Creek, Alabama	Tennessee River	34°57.140′N	87°35.410′W
N. platyrhynchus (4)	South Fork New River, North Carolina	Ohio River	36°13.150′N	81°38.250′W
N. raneyi (6)	Tar River, North Carolina	Tar River	36°10.300′N	78°29.470′W
N. leptocephalus-H (10)	Candler Creek, Georgia	Altamaha River	34°18.697′N	83°39.441′W
N. leptocephalus-C (6)[b]	Hillabahatchee Creek, Georgia	Chattahoochee River	33°18.632′N	85°11.288′W
N. leptocephalus-C (3)[b]	Pink Creek, Georgia	Chattahoochee River	33°23.154′N	85°03.468′W
N. leptocephalus-C (3)[b]	Cavender Creek, Georgia	Chattahoochee River	33°26.971′N	85°01.290′W

[a] Because of dietary and morphological similarities, individuals from the two populations of *Campostoma pauciradii* were pooled for all analyses.
[b] Similarly, individuals from the three Chattahoochee drainage populations of *Nocomis leptocephalus* were pooled for all analyses.

(MI), and the distal intestine (DI). For the *Nocomis* species, the gut was divided into two sections, the PI representing the proximal third of the intestine—including the liver, the gall bladder, and pancreatic tissue—up to a "bend" that is present in all the studied *Nocomis* taxa (Fig. A1), and the DI the remainder. Guts were divided differently among the two genera because most *Nocomis* species did not have enough gut tissue to further divide the intestine and allow for tissue homogenates to be made at a reasonable ($< \times 100$) dilution. Once the guts were measured and divided, the contents of each section were squeezed from the gut with the blunt side of a razor blade into sterile centrifuge vials. The vials containing these gut contents were then capped and immediately frozen, and the remaining gut tissues were individually placed in sterile centrifuge vials and frozen. The gut contents and tissues were then stored at −80°C until analyzed.

Gut tissues were defrosted, weighed (regional gut mass ± 0.001 g), and homogenized individually in ice-cold 0.05 M Tris-HCl buffer, pH 7.4, with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) using a 7-mm generator at 2,200 rpm for 3 × 30 s (German et al. 2004). Dilution varied between 3 and 100 volumes (v/w), depending on the mass of the gut region being homogenized. The homogenates were then centrifuged at 9,400 *g* for 2 min at 4°C, and the supernatants were collected and stored in small aliquots (100–200 μL) at −80°C until just before use in spectrophotometric assays of activities of the five digestive enzymes.

In June 2005, four individuals each of *Campostoma pauciradii* and *N. leptocephalus*-H from Candler Creek, Georgia, were used to estimate the pH of the gut in the two genera. Upon collection, guts from these fish were dissected, removed, and uncoiled as described above, and incisions were made in the gut wall of the PI, the MI, and the DI (PI and DI for *N. leptocephalus*). The intestinal tissue of each region was gently squeezed with forceps to force contents through the incision. We then dipped pH paper (ColorpHast indicator sticks; MCB Reagents, Gibbstown, NJ) into the gut contents and estimated pH to the nearest 0.5 units. Handling of fish from capture to kill was conducted under approved protocol E006 of the Institutional Animal Care and Use Committee at the University of Florida.

Phylogenetic Analysis

A phylogenetic hypothesis of the relationships among species used in this study was determined through a larger phylogenetic analysis of *Nocomis* and *Campostoma* taxa (B. C. Nagle, unpublished data). The phylogenetic study employed sequences of the single-copy, protein-coding nuclear markers rhodopsin and interphotoreceptor retinol binding protein and the mitochondrially encoded cytochrome *b* gene from 117 individuals

Table 2: Digestive enzymes assayed in North American minnows

Digestive Enzyme	Substrate	Substrate Source	Enzyme Hypothesis[a]
Amylase (α-glucanase)	Starch, $\alpha(1\rightarrow4)$-glucans	Green algae, microbes	Elevated in herbivores
Laminarinase	Laminarin	Diatoms	Elevated in herbivores
Chitinase	Chitin	Insect exoskeletons, fungi	Elevated in carnivores
Trypsin	Protein	Animal, plant, microbial material	Elevated in carnivores
Lipase	Lipid	Animal, plant, microbial material	Elevated in carnivores

[a] Hypotheses based on the adaptive-modulation hypothesis that enzyme activities will be elevated in the digestive tracts of animals consuming higher concentrations of substrates for particular enzymes (Karasov and Martínez del Rio 2007).

of *Nocomis* and *Campostoma* collected from across their geographic ranges. We also included multiple cyprinid outgroup taxa in the larger analysis. DNA extraction and PCR amplification protocols followed Berendzen et al. (2003). This larger hypothesis of relationships was generated with a mixed-model, partitioned Bayesian method as implemented in the software package MRBAYES, version 3.1.2 (Huelsenbeck and Ronquist 2001). The best-fitting models of DNA sequence evolution were determined with the Akaike Information Criterion (AIC) in the program MrModeltest (Nylander 2004). Ten million generations of Markov chain Monte Carlo were performed with a random starting topology and trees sampled every 100 generations. A plot of log-likelihood scores of sampled trees against generation time was produced, and all samples taken before a stationary likelihood value were discarded as burn-in. The retained trees were used to construct a 50% majority-rule consensus tree. The percentage of times that a particular node was recovered in the analysis is interpreted as the posterior probability of the occurrence of that node (Huelsenbeck and Ronquist 2001). All interspecific nodes in the expanded phylogeny were strongly supported (posterior probabilities of 95% or greater). The consensus tree from this expanded analysis was pruned to include only individuals taken from localities that were sampled in this study of gut structure and function. Branch lengths among terminals in the pruned tree were estimated by using sequence data from these individuals only.

Gut Dimension Analyses

Two indexes of gut length relative to body size were used to make comparisons of gut size among the 11 taxa: relative gut length (RGL = gut length [mm]/SL [mm]) and Zihler's index (ZI = GL [mm]/10 × BM $[g]^{1/3}$; Zihler 1982). These two digestive-somatic indexes have been used successfully in past comparisons of gut size in different fish taxa (Kramer and Bryant 1995; Elliott and Bellwood 2003; German and Horn 2006). The ratio of total GCM to BM (GCM/BM) was also compared among the species to examine the quantity of food in the gut in the herbivores and carnivores.

Assays of Digestive Enzyme Activity

All assays were carried out at 25°C in triplicate with the BioRad Benchmark Plus microplate spectrophotomer and Falcon flat-bottom 96-well microplates (Fisher Scientific, Pittsburgh). All pH values listed for buffers were measured at room temperature (22°C), and all reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO). All reactions were run at saturating substrate concentrations as determined for each enzyme with gut tissues from the 11 taxa. Each enzyme activity was measured in each gut region of each individual fish. These activities were then totaled for the whole gut and are expressed as total gut enzyme activity (Horn et al. 2006).

The ability of the fish to degrade starch (total amylolytic activity, which may encompass amylase and $\alpha[1\rightarrow4]$-glucanase activity) was measured according to the Somogyi-Nelson method (Nelson 1944; Somogyi 1952) as described by German et al. (2004). 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 μL of the starch solution was combined with 50 μL of a mixture of sodium citrate buffer and gut tissue homogenate. Homogenate volumes ranged from 5 to 25 μL, depending on α-amylase concentration in the homogenates. The incubation was stopped after 15–25 min by adding 20 μL of 1 M NaOH and 200 μL 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 water and centrifuged at 6,000 *g* for 5 min. The glucose content of the solution was then determined spectrophotometrically at 650 nm. The amylolytic activity was determined from a glucose standard curve and expressed in U (1 μmol glucose liberated min^{-1}) per gram wet weight of gut tissue. Blanks consisting of substrate only and homogenate only (in buffer) were conducted simultaneously to account for any background levels of reducing sugar in the substrate or homogenate and to account for "endogenous" substrate present in the homogenates.

Because laminarin is made up of $\beta(1\rightarrow3)$-glucan (Painter 1983), the same Somogyi-Nelson reducing-sugar analysis was used to assay for laminarinase (E.C. 3.2.1.6) activity. The substrate was prepared by dissolving 0.5% laminarin in 0.8 M sodium citrate buffer (pH 7.0) heated to ~95°C; the solution was allowed to cool before use in assays. In a microcentrifuge vial, 50 μL of the laminarin solution was combined with 20 μL of sodium citrate buffer and 30 μL of homogenate. The assay was stopped after 2 h, and laminarinase activity was determined as described above for α-amylase. Reaction times for α-amylase and laminarinase assays were determined by stopping a subset ($n = 4$ individuals per gut region per species) of the reactions at 1, 2, 5, 10, 15, 25, 30, 60, 120, 180, and 240 min. For α-amylase, the reactions were linear for up to 1 h, and laminarinase reactions were linear for up to 4 h.

Chitinase (E.C. 3.2.1.14) activity was determined by the release of N-acetyl-glucosamine (NAG), following Jeuniaux (1966), as described by Gutowska et al. (2004). Chitin substrate was prepared as a suspension (5 mg mL^{-1}) of native chitin (Sigma C9752) in 0.15 M citric acid and 0.3 M Na_2HPO_4 buffer, pH 7.0. The solution was constantly stirred on a magnetic stir plate during pipetting to maintain a uniform colloidal mixture. In a microcentrifuge vial, 125 μL of this suspension was combined with 75 μL of deionized water (two replicates) or, for another replicate, 75 μL of β-glucosidase (Sigma G4511, 6 U mL^{-1} in deionized water). The reaction was started with the addition of 50 μL of homogenate, and the assay mixture was incubated for 2 h under constant shaking. Afterward, the entire assay mixture (in the microcentrifuge vial) was placed in a boiling-water bath for 10 min to stop the reaction. After the vials cooled, the assay mixture was centrifuged at 13,000 *g* for 15 min. The amount of NAG released was then quantified with the method of Reissig et al. (1955). In a microcentrifuge vial, 125 μL of the supernatant was combined with 25 μL of 0.8 M potassium tetraborate, pH 9.3, and boiled for 3 min. After

cooling, this reaction mixture was combined with 750 μL of *p*-dimethylaminobenzaldehyde (DMAB) solution and incubated in a water bath at 37°C for 45 min. DMAB solution was prepared directly before use by adding 1.5 g of DMAB to 100 mL of glacial acetic acid containing 1.25% (v/v) of 12 M HCl. After the 45-min incubation, 100 μL of the assay mixture was pipetted into a microplate, and the NAG concentration of the mixture was determined spectrophotometrically at 585 nm. The chitinase activity was determined from a NAG standard curve and expressed in U (1 μmol NAG liberated h^{-1}) per gram wet weight of gut tissue. As in the case of α-amylase and laminarinase, blanks of substrate only and homogenate only (in buffer) were conducted to account for any background levels of NAG in the substrate or homogenate.

Trypsin (E.C. 3.4.21.4) activity was assayed with a modified version of the method designed by Erlanger et al. (1961), as described by Gawlicka et al. (2000). The substrate, 2 mM Nα-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 μL of BAPNA was combined with 5 μL 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 μmol *p*-nitroaniline liberated min^{-1}) per gram wet weight of gut tissue.

Lipase (nonspecific bile-salt-activated E.C. 3.1.1.-) activity was assayed with a modified version of the method designed by Iijima et al. (1998). In a microplate, 86 μL of 5.2-mM sodium cholate dissolved in 250 mM tris-HCl (pH 9.0) was combined with 6 μL of homogenate and 2.5 μL 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 μL 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 μmol *p*-nitrophenol liberated min^{-1}) per gram wet weight of gut tissue.

Michaelis-Menten constants (K_m) were determined for trypsin, amylase, and lipase in the PI region in the 11 taxa to examine possible differences among the species. Differences in the K_m values for an enzyme can indicate whether the species are expressing different isoforms of the enzyme of interest (Vonk and Western 1984; Voet and Voet 1995). The K_m for amylase was determined with starch concentrations ranging from 0.06% to 3%, for trypsin with BAPNA concentrations ranging from 0.13 to 2.0 mM, and for lipase with *p*-nitrophenol myristate concentrations ranging from 0.05 to 5 mM. We determined K_m values with the Hill equation,

$$[E] = \frac{V_{max}[S]^n}{(K_m)^n + [S]^n}, \quad (1)$$

where S is substrate and n is the Hill coefficient set to 1, using least squares nonlinear regression in Kaleidograph (Synergy, Reading, PA; Brandt and Vickery 1997).

Gastrointestinal Fermentation and Gut Content Analyses

Measurements of symbiotic fermentation activity were based on the methods of Pryor and Bjorndal (2005) and Pryor et al. (2006). Fermentation activity was indicated by relative concentrations of SCFAs in the fluid contents of the guts of the fish at the time of death. To prepare them for SCFA analysis, gut content samples from each region of the intestine (PI, MI, and DI for the *Campostoma* taxa and PI and DI for the species of *Nocomis*) were weighed, thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4°C) at 16,000 *g* for 10 min. The supernatants from each intestinal region were then pipetted into sterile centrifuge vials equipped with a 0.22-μm cellulose acetate filter (Costar Spin-X gamma sterilized centrifuge tube filters, Corning, NY) and centrifuged under refrigeration at 13,000 *g* for 15 min to remove particles from the fluid (including bacterial cells). The filtrates were collected for each intestinal region and frozen until they were analyzed for SCFA concentrations. The pelleted gut contents were refrozen at −80°C until used for dietary analysis. The masses of the gut contents for each intestinal region were added together to calculate the total GCM (±0.001 g) for each individual fish.

Concentrations of SCFAs in the gut fluid samples were measured by gas chromatography. Samples were hand-injected into a Shimadzu GC-9AM gas chromatograph equipped with a flame ionization detector (Shimadzu Scientific Instruments, Columbia, MD) and a Perkin Elmer LC-100 integrator (Perkin Elmer, Shelton, CT). Two microliters of each sample were injected onto a 2-m-long glass column (3.2 mm i.d.) packed with 10% SP-1000 and 1% H_3PO_4 on 100/120 Chromosorb W AW (Supelco, Bellefonte, PA). Carrier gas was N_2 at a flow rate of 40 mL min^{-1}. Temperatures of the inlet, column, and detector were 180°, 155°, and 200°C, respectively. An external standard containing 100 mg L^{-1} each of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate was used for calibration. The SCFA concentrations were expressed as millimoles per liter of gut fluid. Overall, we were able to recover enough gut fluid to measure SCFA concentrations in the guts of all four *Campostoma* species, the two *N. leptocephalus* populations, *N. biguttatus*, and *Nocomis micropogon*. SCFA concentrations were measured in the fluids of the PI, the MI, and the DI (PI and DI of *Nocomis* species) of the eight taxa. The concentrations of each intestinal region were then added together to calculate the total SCFA concentrations in the fish's intestines. Because of their small size, it was impossible to recover regional intestinal contents from *Campostoma ornatum*, and hence, fluid was extracted from the contents of the entire digestive tract for this species.

Gut contents were analyzed to confirm that the studied taxa were consuming the diets reported for them in the literature, but gut content analyses were carried out in different ways, depending on the diet of the fish. Gut contents from the PI of the four *Campostoma* species and herbivorous *N. leptocephalus* were suspended in deionized water, vortexed, and pipetted onto clean microscope slides. The contents were viewed at ×100, and the percentage area of each food type relative to all other

food types present was quantified for five random fields of view (Evans-White et al. 2001) with ImageJ analytical software (Abramoff et al. 2004). Five slides were analyzed in this manner for each individual fish (i.e., 25 images per fish). Carnivorous *Nocomis* PI gut contents were suspended in deionized water, poured into a petri dish, and analyzed under a dissecting microscope (equipped with a net reticle, 13 mm × 13 mm) with a point-contact method described by German and Horn (2006). It was necessary to analyze the gut contents of the herbivorous and carnivorous species by different methods because of differences in the particle sizes in the guts of the herbivores and carnivores. *Campostoma* have small algal and detrital particles in their guts, whereas *Nocomis* have discernible invertebrates in theirs. Percent area or point contacts were totaled for each food item in each individual fish and then totaled and expressed as a mean percent for each species. The results were expressed as proportions of animal, algal, diatom, and detrital material to provide a broad intraspecific and interspecific comparison of diet composition in the 11 taxa. Animal material was classified at the order level, algal material represents filamentous green algae and cyanobacteria, and unidentifiable organic material was classified as detritus.

Scanning Electron Microscopy of Pharyngeal Teeth

The fifth ceratobranchials (pharyngeal arches), which contain the pharyngeal teeth in minnows (Eastman and Underhill 1973), were removed with forceps from two specimens each of *Campostoma anomalum*, *Campostoma oligolepis*, *Nocomis asper*, *N. leptocephalus*-H, and *N. leptocephalus*-C. The left and right pharyngeal arches from each fish were then submerged in 1 M NaOH for 72 h to digest any adhering tissue. After the digestion period, the arches (with teeth attached) were rinsed in deionized water for 2 × 1 h and dried in a drying chamber at room temperature for at least 24 h. Once dry, the teeth were mounted on aluminum stubs and sputter-coated with gold palladium. Teeth were examined with a Hitachi S-4000 FE SEM (Hitachi Instruments, San Jose, CA), and qualitative comparisons of tooth shape were made between the two genera and between the two populations of *N. leptocephalus*.

Statistical Analyses

Before all significance tests, a Levene's test for equal variance was performed to ensure the appropriateness of the data for the analyses. If the data were not normal, they were log transformed, and normality was confirmed before analysis. All tests were run with Minitab statistical software (ver. 13; Minitab, State College, PA) unless specified otherwise. Interspecific comparisons of digestive enzyme activity, total gut SCFA concentrations, K_m, and GCM/BM were made with ANOVA followed by a Tukey's HSD multiple-comparisons test with a family error rate of $P = 0.05$. Interspecific comparisons of the gut dimensions (RGL and ZI) were made with ANCOVA, with BM as a covariate, followed by Tukey's HSD test with a family error rate set at $P = 0.05$.

Independent-Contrast Analyses

Associations were observed between carbohydrase (α-amylase, laminarinase, and chitinase) activity and percent animal material in the diets of the 11 minnow taxa. The extent to which these digestive carbohydrase activities reflect evolutionary specialization to diet was examined in the 11 minnow taxa by analyzing the data within a phylogenetic context with independent-contrast analyses (Felsenstein 1985). Phylogenetically independent contrasts of the activities of each carbohydrase with percent animal material in the diet were generated using COMPARE 4.6b software (Martins 2004). Branch lengths were taken directly from the phylogeny generated for this study (Fig. 1). To confirm that the branch lengths from the phylogeny were appropriate, we examined the correlations of the absolute values of the standardized independent contrasts with their standard deviations (Garland et al. 1992; Garland and Díaz-Uriarte 1999). The contrasts and standard deviations of amylase ($r = 0.24$, $P = 0.51$), laminarinase ($r = -0.09$, $P = 0.80$), and chitinase ($r = -0.33$, $P = 0.35$) were not correlated. Thus, the phylogenetically independent contrasts were used in linear-regression analyses, where the regression was forced through the origin, as is required for analyses of independent contrasts (Felsenstein 1985).

Results

Phylogeny

The phylogenetic hypothesis for the studied taxa is presented in Figure 1. Statistical support for the sister relationship between *Nocomis* and *Campostoma*, the monophyly of the two genera, and relationships among species within them was strong (Bayesian posteriors of 0.98 or greater) in the pruned analyses.

Gut Dimensions, pH, and Diets

The general gut shape and size of the two genera are illustrated in Figure A1. As guts were dissected from the animals, they were examined for ceca or elaborations of any kind. The only difference in gut shape between the two genera appears to be the length (see below) and the number of coils, with the *Campostoma* gut coiling at least 12 times around the swim bladder and the *Nocomis* gut folding two or fewer times in the peritoneal cavity before reaching the vent. Significant differences in mean RGL and ZI were detected among the 11 minnow taxa (Table 3). Each of the *Campostoma* species had guts that were longer relative to body size than any of the *Nocomis* taxa. *Campostoma pauciradii* and *Campostoma anomalum* had the longest guts, followed by *Campostoma oligolepis* and *Campostoma ornatum*, in that order. Among the *Nocomis* taxa, *Nocomis leptocephalus*-H had gut lengths that were significantly longer than those of the other *Nocomis* taxa, which did not differ from one another (Table 3). Significant differences in GCM/BM were also detected among the 11 taxa. *Campostoma oligolepis* and *C. pauciradii* had more food in their guts than any of the other species, and *Nocomis raneyi* displayed the lowest GCM. Notably, *N. lepto-*

Table 3: Interspecific comparisons of body mass (BM), relative gut length (RGL), Zihler's index (ZI), and gut content mass as a function of body mass (GCM/BM) in 11 minnow taxa

Species	BM (g)	RGL	ZI	GCM/BM
Campostoma anomalum	12.76 ± .82^{C}	5.16 ± .20^{E}	2.76 ± .78^{E}	.034 ± .006^{C}
Campostoma ornatum	4.48 ± .43^{AB}	3.24 ± .14^{C}	12.36 ± .54^{C}	.043 ± .008^{C}
Campostoma oligolepis	20.88 ± 1.15^{E}	5.41 ± .35^{E}	20.95 ± 1.31^{E}	.088 ± .007^{E}
Campostoma pauciradii	11.13 ± 1.45^{BC}	4.14 ± .16^{D}	16.04 ± .69^{D}	.061 ± .008^{D}
Nocomis biguttatus	13.52 ± 3.39^{CDE}	1.33 ± .14^{B}	4.95 ± .55^{B}	.021 ± .002^{BC}
Nocomis asper	8.16 ± 2.21^{BC}	.88 ± .09^{A}	3.40 ± .36^{A}	.017 ± .003^{BC}
Nocomis micropogon	13.21 ± 3.97^{CDE}	.87 ± .07^{A}	3.38 ± .28^{A}	.021 ± .005^{BC}
Nocomis platyrhynchus	4.60 ± .47^{ABCD}	.89 ± .10^{A}	3.37 ± .37^{A}	.023 ± .003^{BC}
Nocomis leptocephalus-C	14.13 ± 2.25^{DE}	.84 ± .04^{A}	3.23 ± .13^{A}	.011 ± .002^{B}
N. leptocephalus-H	9.61 ± 2.55^{CD}	1.66 ± .05^{B}	6.43 ± .18^{B}	.034 ± .007^{C}
Nocomis raneyi	.72 ± .05^{A}	1.05 ± .06^{A}	4.14 ± .22^{A}	.001 ± .000^{A}
Species:				
F (df)	7.83 (10, 89)	185.99 (10, 89)	170.56 (10, 89)	12.29 (10, 89)
P	<.001	<.001	<.001	<.001
BM:				
F (df)	…	.02 (1, 78)	.02 (1, 78)	…
P	…	.890	.891	…

Note. Values are means ± SEM (see table 1 for sample sizes). Interspecific comparisons of body mass (BM) and GCM/BM were analyzed with one-way ANOVA and Tukey's HSD with a family error rate of $P = 0.05$. Interspecific comparisons of gut dimension parameters were analyzed with ANCOVA (with BM as a covariate) and Tukey's HSD with a family error rate of $P = 0.05$. Values that share a superscript letter within a parameter are not significantly different.

cephalus-H had significantly more GCM than did *N. leptocephalus*-C (Table 3).

The digestive tracts of *C. pauciradii* and *N. leptocephalus*-H were nearly neutral in pH in all regions of the gut (Table 4). The lack of acidic conditions and the lack of a pyloric sphincter (Clements and Raubenheimer 2006) in the PI of either species is consistent with the general finding that cyprinids lack a gastric stomach (Kraatz 1924; Hofer et al. 1982; Chakrabarti et al. 1995).

The proportions of detrital, diatomaceous, algal, and animal material in the gut contents of all 11 taxa are shown in Figure 2. All four species of *Campostoma* represent true periphyton feeders, ingesting almost exclusively detritus, diatoms, and algae. *Campostoma pauciradii* consumed the highest proportion of detritus (58.7%), *C. anomalum* the highest proportion of diatoms (38.8%), and *C. oligolepis* and *C. ornatum* had similarly high proportions of algae in their diets, at 26.0% and 26.6%, respectively. *Campostoma ornatum* also consumed some animal material (5.3%), which consisted of chironomid larvae, unidentifiable insect parts, and fish scales. The diet of *Nocomis biguttatus* was almost entirely animal material (99.5%) and was dominated by clams, damsel fly larvae, and fragments of unidentifiable adult insects. Filamentous green algae composed the remaining 0.5% of the diet of *N. biguttatus*. *Nocomis asper* consumed only animal material (100%), primarily unidentifiable adult insect parts but also clams, damsel fly and chironomid larvae, and ostracods. The diet of *Nocomis micropogon* was more diverse, with animal material (72.9%) consisting of unidentifiable adult insect parts, caddis flies, and caddis fly larvae making up the largest proportion and algae and detritus making up the remainder, at 19.4% and 7.7%, respectively. The gut contents of *Nocomis platyrhynchus* were dominated by animal material (93%), mostly unidentifiable adult insect parts and caddis fly larvae, and some filamentous green algae (7%). Individuals of *N. leptocephalus*-C were mostly carnivorous (91.2% animal material), consuming snails, clams, adult and larval caddis flies, adult and larval midge flies, and chironomid larvae as well as some detritus (8.8%). Individuals of *N. leptocephalus*-H, on the other hand, had a diet similar to those of the *Campostoma* species, dominated by detritus (37.5%), diatoms (28.4%), and filamentous green algae (33.8%). Only 0.3% of their diet was animal material, and this was composed of

Table 4: Intestinal pH of *Campostoma pauciradii* and *Nocomis leptocephalus* captured from Candler Creek, Georgia

Species	PI	MI	DI	Whole Gut
C. pauciradii	6.88 ± .13	6.88 ± .13	7.38 ± .13	7.04 ± .04
N. leptocephalus	6.63 ± .13	NA	6.75 ± .13	6.69 ± .06

Note. Gut regions: proximal intestine (PI), medial intestine (MI), and distal intestine (DI). Values are means ± SEM ($N = 4$). NA = not available.

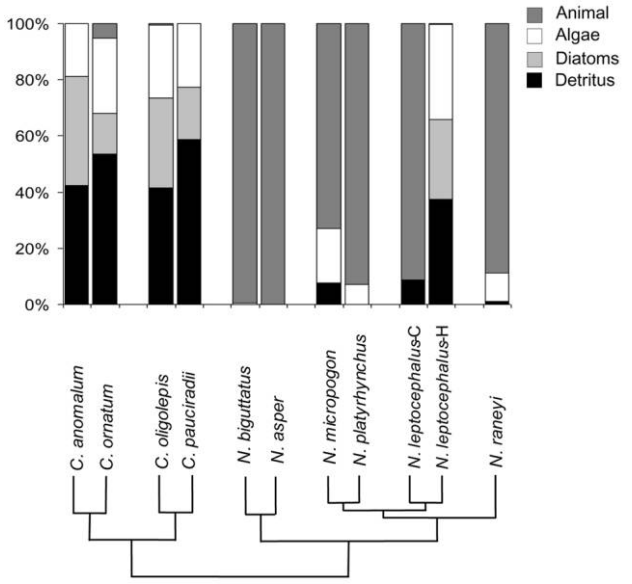

Figure 2. Proportions of animal, algal, diatomaceous, and detrital material in the gut contents of the 11 minnow taxa used in this study. Phylogenetic relationships at base are from Figure 1 and are used as a guide for the relationships among the studied species. *Nocomis leptocephalus*-C is the carnivorous population of this species from the Chattahoochee River drainage, whereas *N. leptocephalus*-H is the herbivorous population from the Altamaha River drainage.

unidentifiable insect parts. *Nocomis raneyi* consumed mostly animal material (88.8%), consisting of unidentifiable adult insect parts and clams, and filamentous green algae and detritus, at 10.2% and 1%, respectively.

Digestive Enzyme Activities

Significant differences in carbohydrase (amylolytic, laminarinase, and chitinase) activities were observed among the 11 minnow taxa (Fig. 3). The herbivorous species (all four *Campostoma* species and *N. leptocephalus*-H) generally had higher amylolytic activity (mean U g gut tissue^{-1} ± SEM) than the carnivorous *Nocomis* taxa. *Campostoma pauciradii* (837.04 ± 90.04), *C. oligolepis* (791.50 ± 87.80), and *C. ornatum* (641.81 ± 71.60) each displayed significantly higher amylolytic activity than any of the *Nocomis* taxa. *Campostoma anomalum* (539.75 ± 55.70) displayed significantly higher amylolytic activity than all of the *Nocomis* taxa except *N. biguttatus* (327.39 ± 107.35) and *N. leptocephalus*-H (418.72 ± 22.94), which in turn were not significantly different from one another. *Nocomis leptocephalus*-H had significantly higher amylase activity than the remaining *Nocomis* taxa, and *N. biguttatus* had higher amylolytic activity than *N. micropogon* (69.94 ± 20.21), *N. platyrhynchus* (125.29 ± 23.83), and *N. raneyi* (97.70 ± 22.88). Each of these three species had significantly lower activity than *N. asper* (266.37 ± 41.27) and *N. leptocephalus*-C (184.86 ± 25.19), which were not different from one another.

A similar pattern of higher activity in the herbivores was observed for the enzyme laminarinase as well. *Campostoma anomalum* (0.132 ± 0.017) and *C. ornatum* (0.119 ± 0.026) displayed laminarinase activities that were significantly higher than those of all the studied taxa except *N. leptocephalus*-H (0.091 ± 0.016), which in turn did not exhibit activity significantly higher than that of *C. pauciradii* (0.055 ± 0.017). *Campostoma oligolepis* (0.020 ± 0.005) displayed laminarinase activity that was not different from that of *N. biguttatus* (0.005 ± 0.002), *N. micropogon* (0.018 ± 0.011), or *N. platyrhynchus* (0.022 ± 0.013). *Nocomis asper*, *N. raneyi*, and *N. leptocephalus*-C had no detectable laminarinase activity (Fig. 3).

The opposite pattern was apparent for chitinase activities, with the carnivores displaying higher activities of this enzyme (Fig. 3). *Nocomis raneyi* had the highest chitinase activity (15.33 ± 3.48), but this activity was not significantly higher than those of *N. biguttatus* (8.64 ± 3.90) or *N. platyrhynchus* (8.68 ± 2.28). These two species, in turn, had activity similar to that of *N. asper* (6.49 ± 1.56), *N. micropogon* (3.21 ± 0.61), and *N. leptocephalus*-C (6.55 ± 1.84). *Nocomis leptocephalus*-H (2.30 ± 0.37) had the lowest chitinase activity of the *Nocomis* taxa and was statistically similar in activity to the herbivorous *C. anomalum* (1.22 ± 0.22), *C. ornatum* (1.37 ± 0.20), *C. oligolepis* (1.01 ± 0.12), and *C. pauciradii* (1.21 ± 0.29).

The patterns for trypsin and lipase activities were much more complicated and did not appear to correlate with diet as much as the carbohydrase activities appeared to (Fig. 4). Thus, we mention only notable activity levels and significant differences. *Nocomis platyrhynchus* (68.75 ± 15.21) displayed significantly higher trypsin activity than any of the other 10 taxa, and *N. raneyi* (5.49 ± 1.96) had the lowest. Individuals of *N. leptocephalus*-C (17.56 ± 2.22) displayed significantly higher trypsin activity than individuals of *Nocomis leptocephalus*-H (7.95 ± 1.59). *Campostoma pauciradii* (0.13 ± 0.01) had the highest lipase activities, and *N. leptocephalus*-H (0.06 ± 0.01) had the lowest (Fig. 4). *Nocomis leptocephalus*-C (0.11 ± 0.01) had significantly higher lipase activity than *N. leptocephalus*-H.

Significant differences were observed in the K_m values of amylase, trypsin, and lipase among the 11 minnow taxa, but these differences did not match dietary or phylogenetic patterns (see Table A1 in the online edition of *Physiological and Biochemical Zoology*).

Independent Contrasts

There was a negative correlation between the phylogenetically independent contrast of amylolytic activity and the contrast for percent animal material in the diet (Fig. 5), but no significant correlation was detected for the contrasts of laminarinase activity and diet. There was a strong positive correlation between the phylogenetically independent contrast of chitinase activity and the contrast for percent animal material in the diet (Fig. 5). Thus, the evolution of diet and enzyme activity appears to be correlated for amylolytic capacity and chitinase.

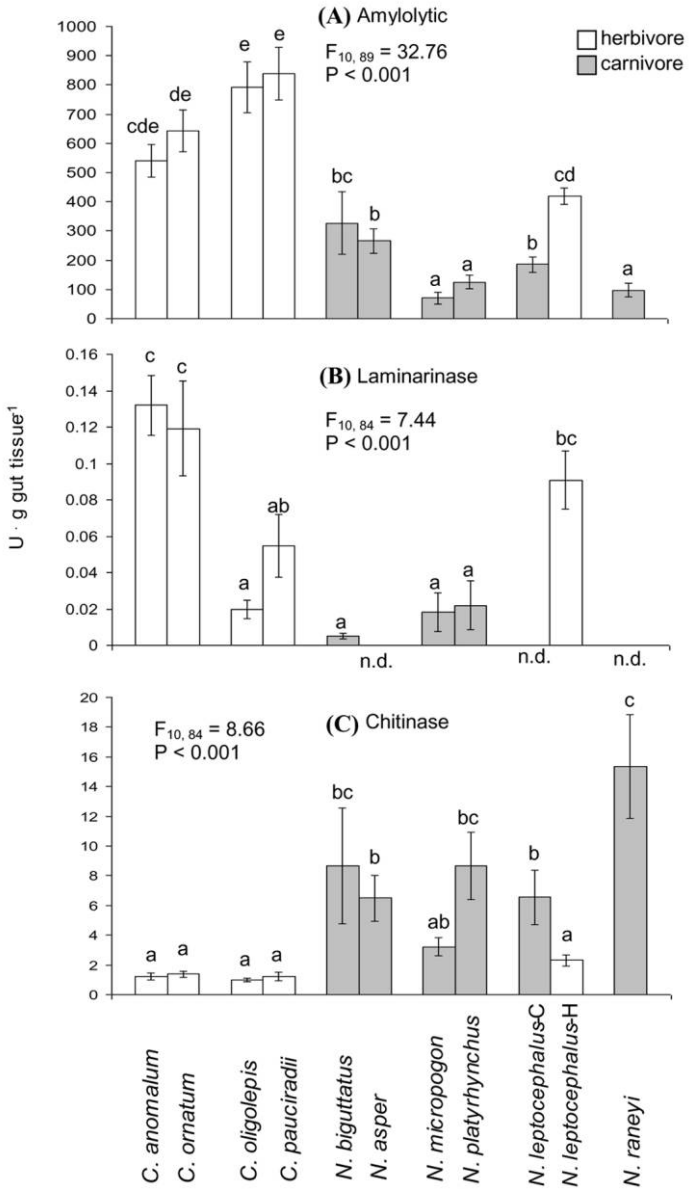

Figure 3. Amylolytic (*A*), laminarinase (*B*), and chitinase (*C*) activities in the 11 minnow taxa investigated in this study. Values are means ± SEM. Interspecific comparisons were made among the taxa for each enzyme with ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$. Values for a specific enzyme that share a superscript letter are not statistically different. n.d. = not detectable. Phylogenetic relationships among taxa are as in Figure 1.

Gastrointestinal Fermentation

Significant differences in intestinal SCFA concentrations were detected among the eight taxa in which we were able to measure these concentrations (Table 5). *Campostoma oligolepis* displayed the highest SCFA concentrations, and *C. pauciradii* had the lowest. All eight taxa showed at least some capability for microbial fermentation in their digestive tracts, and this was not limited to the more herbivorous species. *Nocomis biguttatus* and *N. micropogon*, both carnivores, showed the second- and third-highest SCFA concentrations, respectively, and these concentrations were not significantly different from the highest concentrations observed in *C. oligolepis* (Table 5).

Pharyngeal Teeth

Notable differences are apparent in the shape of the pharyngeal teeth between the species in the two genera (Fig. 6). Although these genera have the same number of pharyngeal teeth (4) on each cerratobranchial, their teeth do differ in shape and orientation: *Nocomis* species tend to have sharper, more curved teeth, whereas the *Campostoma* species have flatter, less curved

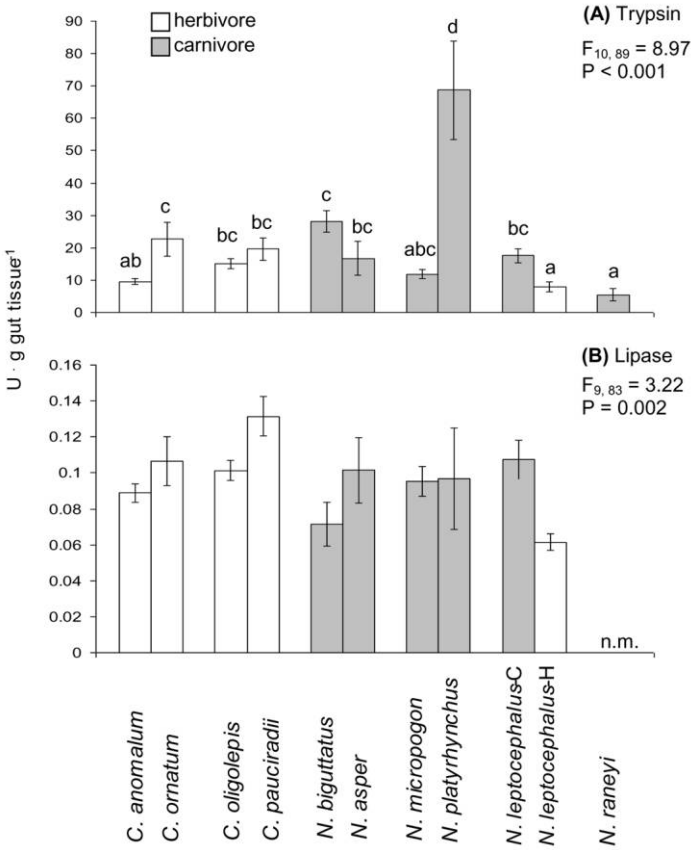

Figure 4. Trypsin (A) and lipase (B) activities in the 11 minnow taxa investigated in this study. Values are means ± SEM. Interspecific comparisons were made among the taxa for each enzyme with ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$. Bars for trypsin that share a superscript letter are not statistically different. The interspecific differences for lipase activity were too complicated to indicate with superscript letters, and thus are not shown. n.m. = not measured. Phylogenetic relationships among taxa are as in s Figure 1.

teeth. Despite their dietary differences, *N. leptocephalus*-C and *N. leptocephalus*-H had qualitatively very similar teeth that were much different from the teeth of the *Campostoma* species (Fig. 6).

Discussion

Overall, the results of this study provide evidence of both dietary and phylogenetic effects on gut structure and function in the 11 minnow taxa, and most of our hypotheses were supported. The four *Campostoma* species and *Nocomis leptocephalus*-H displayed gut lengths and digestive enzyme activities that largely matched their dietary preferences, but the gut size of *N. leptocephalus*-H appears to be somewhat constrained by its phylogenetic history. Despite the dietary similarity between this population of *N. leptocephalus* and the four species of *Campostoma*, the former had guts that were much shorter and more similar to the Chattahoochee population of *N. leptocephalus* than to the *Campostoma* species. Our hypothesis of no difference in tooth shape among the genera was rejected because the teeth of *Campostoma* species are flatter than those of the *Nocomis* taxa. Also, SCFA concentrations in the minnow intestines did not differ between the two genera, nor were they associated with a specific diet.

The paradigm for fish nutritional ecology has long been that digestive enzyme activities correlate with diet, and many studies have found support for this, especially for carbohydrate-degrading enzymes (Kapoor et al. 1975; Hidalgo et al. 1999; Fernandez et al. 2001; Moran and Clements 2002; Drewe et al. 2004; German et al. 2004; Horn et al. 2006), although there are exceptions (e.g., Skea et al. 2005). In the scope of the adaptive-modulation hypothesis and given the cost of regulating the digestive tract (Caviedes-Vidal et al. 2000; Karasov and Martínez del Rio 2007), it is efficient to have high digestive enzyme activities to degrade those compounds most available in the diet. In this study, we found clear associations between the ability to digest starch and dietary affinity—the more animal material consumed, the lower the amylolytic activity, even in a phylogenetic context—suggesting that the evolution of herbivory can lead to an increase in amylolytic activity. This may be adaptive, because starch and α-glucans probably constitute an important energy source for the *Campostoma* and herbivorous *N. leptocephalus*. The material consumed by these taxa is best described as periphyton, or the epilithic algal complex

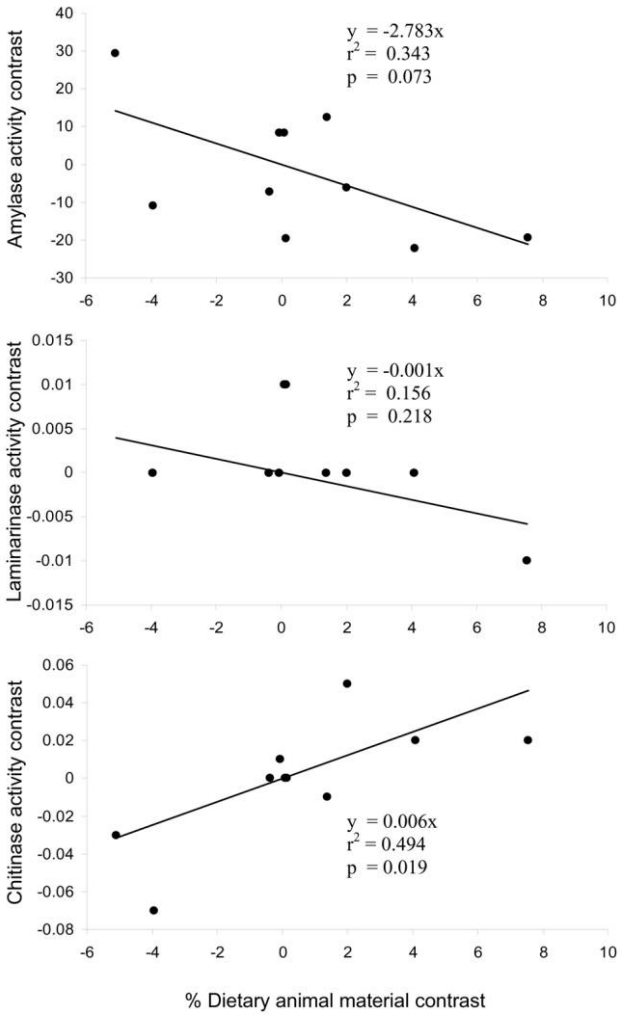

Figure 5. Relationships between digestive carbohydrase activity and percent animal material in the diet for 11 minnow taxa, plotted as phylogenetically independent contrasts. Regression coefficients are on the graphs. Relationships are considered significant at $P = 0.10$.

(EAC), which is a loose assemblage of bacteria, cyanobacteria, filamentous green algae, diatoms, and detritus that grows on hard substrates (Hoagland et al. 1982; van Dam et al. 2002; Klock et al. 2007). Starch is the storage polysaccharide of green algae (Painter 1983), and bacterial and algal mats contain a large proportion of soluble polysaccharides in exopolymeric substances (Leppard 1995; Wotton 2004; Klock et al. 2007). Thus, the elevated amylolytic activity in the minnows may be a combination of amylase and a general $\alpha(1\rightarrow4)$-glucanase (Skea et al. 2005), allowing the fishes to hydrolyze a variety of microbial and algal polysaccharides. Indeed, the general reducing-sugar assay we used to determine amylolytic activity probably measures amylase and $\alpha(1\rightarrow4)$-glucanase, not amylase alone.

Laminarinase activity was also clearly more important in the herbivorous taxa, although this was difficult to observe statistically because three *Nocomis* taxa (*Nocomis asper*, *Nocomis raneyi*, and *N. leptocephalus*-C) lacked laminarinase activity altogether. Laminarin is the storage polysaccharide of diatoms and brown algae (Painter 1983), and fishes that consume more laminarin have higher laminarinase activities (Sturmbauer et al. 1992; Moran and Clements 2002; Skea et al. 2005). Sturmbauer et al. (1992) showed that herbivorous and omnivorous cichlids consuming less of the EAC, and hence fewer diatoms, had no detectable laminarinase activity in their guts whereas EAC-consuming fishes had higher laminarinase activity. However, in accordance with previous studies (Moran and Clements 2002; Skea et al. 2005; German and Bittong 2009), laminarinase activities in the minnows are several orders of magnitude lower than the amylolytic activity, suggesting a lower importance of laminarin to the overall nutrition of the fish.

Campostoma anomalum and *Campostoma oligolepis* consumed the most diatoms in this study, yet *C. oligolepis* had the lowest laminarinase activity among the *Campostoma*. Individuals of *C. anomalum* have been shown to selectively feed on diatoms in some habitats, even when diatoms are not the most abundant food item (Napolitano et al. 1996). Furthermore, when sympatric, *C. anomalum* and *C. oligolepis* have been observed to exhibit niche partitioning and feed on different resources (Fowler and Taber 1985). Thus, it is possible that species in the clade to which *C. anomalum* and *Campostoma ornatum* belong are specialists on diatoms and hence have higher laminarinase activity than species in the *C. oligolepis–Campostoma pauciradii* clade, but this is purely speculative and is not necessarily supported by our gut content analyses. The activity of the brush-border disaccharidase maltase also follows the pattern of different activity between the two clades (German 2009*a*) and may reflect some deeper evolutionary and ecological

Table 5: Total short-chain fatty acid (SCFA) concentration (mM) in the digestive tracts of eight minnow species with different diets

Species (Diet)	*N*	SCFA Concentration[a]
Campostoma anomalum (herbivore)	6	12.96 ± 1.67[A]
Campostoma ornatum (herbivore)	8	9.60 ± 1.55[A]
Campostoma oligolepis (herbivore)	10	22.54 ± 2.39[B]
Campostoma pauciradii (herbivore)	7	7.45 ± .98[A]
Nocomis biguttatus (carnivore)	3	16.24 ± 3.98[AB]
Nocomis micropogon (carnivore)	5	14.42 ± 2.19[AB]
Nocomis leptocephalus (carnivore)	3	9.60 ± 2.36[A]
N. leptocephalus (herbivore)	10	8.06 ± 2.32[A]

Note. Values are means ± SEM. Interspecific comparisons of SCFA concentration were analyzed with one-way ANOVA and Tukey's HSD with a family error rate of $P = 0.05$. Values that share a superscript letter are not significantly different.

[a] $F_{7,47} = 6.62$, $P < 0.01$.

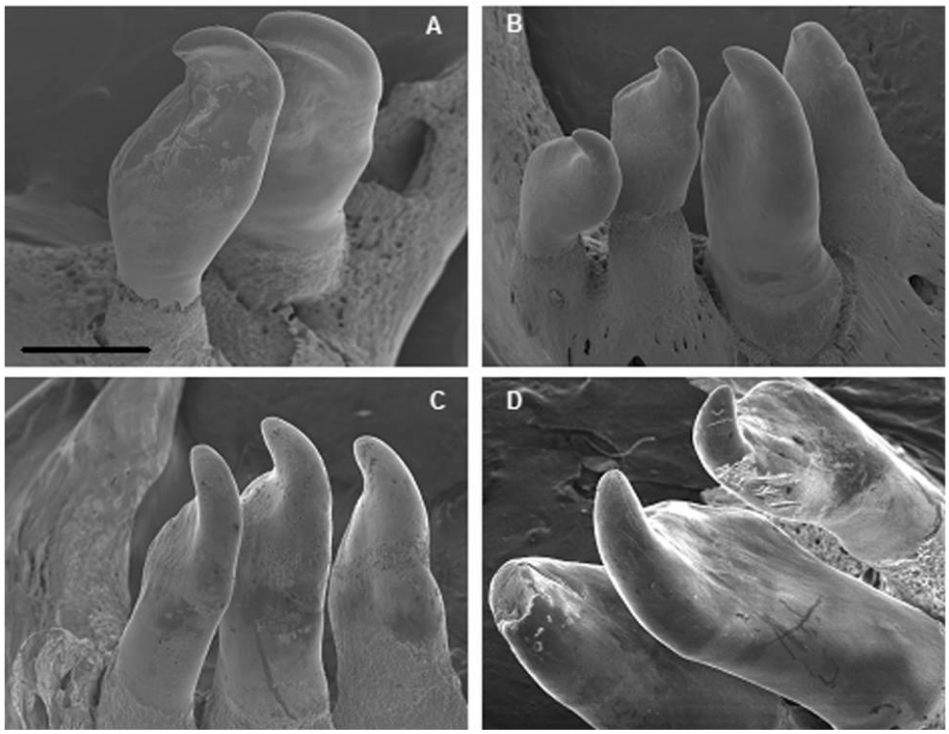

Figure 6. SEM micrographs of the pharyngeal teeth of herbivorous *Campostoma oligolepis* (*A*), carnivorous *Nocomis asper* (*B*), herbivorous *Nocomis leptocephalus* (*C*), and carnivorous *N. leptocephalus* (*D*). Scale bar = 1 mm.

differences between the two parts of the *Campostoma* phylogeny.

To our knowledge, there have been no comparisons of chitinase activity among herbivorous and carnivorous fishes in a phylogenetic context. A dietary effect on chitinase activity in fish digestive tracts is well known, however. Goodrich and Morita (1977*a*) found a relationship between diet and chitinase activity in *Enophrys bison* and *Platyichthys stellatus* (more chitin in the diet, higher chitinase activity). Danulat (1986) showed that *Gadus morhua* consuming crab and shrimp had higher chitinase activities in their guts than individuals of this same species consuming a fish diet. However, Gutowska et al. (2004) found that chitinase activity in 13 species of marine carnivorous fishes was associated more with intestinal length than with diet: fishes with shorter intestines had higher chitinase activity. The authors suggested that this elevated chitinase activity was more likely for the disruption of prey exoskeletons, preventing intestinal blockage, than for the digestion and absorption of NAG from chitin per se. Lindsay (1984) found no relationship between diet and chitinase activity in 29 species of marine and freshwater fishes but suggested that fishes with the ability to mechanically disrupt prey exoskeletons (e.g., with pharyngeal teeth) had lower chitinase activity than species that do not mechanically manipulate prey. The findings of Gutowska et al. (2004) and Lindsay (1984) beg for a comparison of chitinase activity among closely related fishes with similar gut morphology and ability to mechanically disrupt prey to discern whether a dietary effect on chitinase activity exists. Among the minnows—which all possess anatomically unspecialized intestines, albeit of different lengths, and have pharyngeal teeth—chitinase activity is clearly higher in fishes consuming more insects and thus more chitin. The higher chitinase activity in the *Nocomis* may be adaptive (as indicated in the independent-contrast analyses), although feeding trials would be necessary to test this assertion. Because the *Nocomis* taxa have pharyngeal teeth with which to disrupt prey exoskeletons and because chitin is composed of a usable energy source for vertebrates (i.e., NAG; Gutowska et al. 2004), chitin may represent an important additional source of nitrogen and energy to the *Nocomis* species. Moreover, these fishes may play a role in chitin degradation in North American freshwater ecosystems (Goodrich and Morita 1977*b*).

No pattern relating to diet has been established for protease activities. In fact, proteolytic enzyme activity in herbivorous fishes has often been found to be equal to or higher than that in carnivorous fishes (Sabapathy and Teo 1993; Hidalgo et al. 1999), even in closely related species (Chan et al. 2004). Hofer and Schiemer (1981) argued that because many herbivorous fishes have higher intake and more rapid gut throughput than their carnivorous relatives, they likely have higher daily production and activity of proteases than carnivores, regardless of the "snapshot" protease activities measured at the time of death. Given the observation in this study that the herbivorous species likely have higher intake (i.e., greater GCM) and thus would be expected to have more rapid gut transit rates (Sibly and Calow 1986), it is likely that they produce more proteolytic

enzymes (and all enzymes, for that matter) than the carnivores to ensure acquisition of protein from their low-protein diet (Hofer and Schiemer 1981; Smoot and Findlay 2000; Chan et al. 2004; German and Bittong 2009). In further support of this contention, *N. leptocephalus*-C had higher trypsin activity than *N. leptocephalus*-H, but *N. leptocephalus*-C also had lower GCM than *N. leptocephalus*-H. Lower intake can equate to longer retention of food in the gut (Sibly and Calow 1986), especially in predators that may experience extended times between meals. When they actually encounter food, carnivorous *Nocomis* may rapidly increase digestive enzyme secretion, which can result in higher digestive enzyme activity after consumption of a meal (Cox and Secor 2008), especially in comparison to herbivorous taxa that constantly have food in their guts.

Another way to examine patterns of protease activity is in the context of nutrient targets and daily protein requirements (Raubenheimer and Simpson 1998). Many fishes are known to feed to meet protein requirements, even if excess carbohydrates are consumed in the process (Raubenheimer et al. 2005; Clements and Raubenheimer 2006). Thus, if herbivores consuming a low-protein diet are found to exhibit relatively high proteolytic enzyme activities (e.g., loricariid catfishes; German and Bittong 2009), this may be due to the fishes scavenging any protein they can to meet their daily protein targets, whereas carnivores have a simpler task of meeting their daily protein needs with their higher-protein diet.

Like protease activity, lipase activities have often been found to be equal among herbivores and carnivores (Horn et al. 2006) or actually higher in herbivores (Nayak et al. 2003; German et al. 2004). Similar to what Hofer and Schiemer (1981) suggested for protease activity, German et al. (2004) argued that because an herbivorous diet is lower in lipid than a carnivorous one, herbivores attempt to maximize lipid digestion and assimilation (especially of essential fatty acids) by having higher lipase activities. This counterobservation to the adaptive-modulation hypothesis has been seen in vitamin and mineral transport activity along the intestinal epithelium (Karasov and Hume 1997) and may certainly apply to limiting nutrients (and their acquisition) across the board.

Cyprinid fishes are known to lack a gastric stomach (Kraatz 1924; Hofer et al. 1982; Chakrabarti et al. 1995), and our results support this notion because we measured alkaline conditions throughout the guts of *C. pauciradii* and *N. leptocephalus*-H. We also found no valves or ceca of any kind, further suggesting that minnows have anatomically unspecialized digestive tracts that are little more than an intestine from beginning to end. The length of the digestive tract, however, is definitely affected by diet and evolutionary history. The RGL and ZI of the *Campostoma* species were two to seven times those of the *Nocomis* species, consistent with many previous observations that herbivorous fishes have longer guts than carnivorous ones (Zihler 1982; Ribble and Smith 1983; Kramer and Bryant 1995; German and Horn 2006). *Campostoma anomalum* and *C. oligolepis* are known to feed on the EAC throughout the diel cycle and to pass food through their intestines in less than 8 h (Fowler and Taber 1985). With high intake and an anatomically unspecialized digestive tract, it makes sense to have an elongated intestine and most likely an expanded absorptive surface area (German 2009*b*), because such animals pass food through their guts in a rapid fashion (Sibly and Calow 1986; Horn et al. 2006; Karasov and Martínez del Rio 2007; German 2009*b*). Rapid transit of ingesta through the gut means that food passes through each region of the intestine only once and is not retained anywhere along the gut for any length of time. Thus, it is important to expose ingesta to as much absorptive surface as possible (Horn et al. 2006; German 2009*b*). Similarly, *N. leptocephalus*-H feeds on the EAC and has a longer gut than *N. leptocephalus*-C, but it is a gut that is one-half to one-third the length of the *Campostoma* intestines. This is the clearest example of a phylogenetic constraint in our data set, and it further illustrates why phylogenetic history must be taken into account in comparisons of gut size among fishes with different diets (German and Horn 2006).

We hypothesized that there would be no differences in the intestinal SCFA concentrations among species of *Campostoma* and *Nocomis*, and this appears to be true. Species of both genera have some level of gastrointestinal fermentation in their guts regardless of diet. Although some marine herbivorous fishes may meet a large proportion of their daily energy needs via SCFAs produced by endosymbiotic microorganisms (Mountfort et al. 2002), this is not necessarily true for all nominally herbivorous fishes, especially for those that consume more detritus (Crossman et al. 2005). The highest SCFA concentrations observed in this study—22.54 mM SCFA for the whole gut in *C. oligolepis*—still qualify as "low fermentation potential" among herbivorous fishes (Choat and Clements 1998), and the second- and third-highest SCFA concentrations in this study were observed in the carnivorous *Nocomis biguttatus* and *Nocomis micropogon*. This is consistent with a previous investigation in freshwater fishes that revealed higher SCFA concentrations in the hindgut of carnivorous *Micropterus salmoides* than in omnivorous *Cyprinus carpio* or detritivorous *Dorosoma cepedianum* (Smith et al. 1996). Furthermore, the SCFA concentrations in the minnow guts are not higher in one region of the intestine than another (i.e., foregut or hindgut; German 2009*a*), as is commonly the case in animals that rely on gastrointestinal fermentation (Stevens and Hume 1998). Thus, although species of *Campostoma* and *Nocomis* may meet some proportion of their daily energetic needs via gastrointestinal fermentation, they likely rely more on endogenous digestive mechanisms.

There are clear differences in the shape of the pharyngeal teeth between *Campostoma* and *Nocomis* that may be related to their phylogenetic history and evolution of dietary specialization. The *Nocomis* taxa clearly have curved, villiform teeth, whereas the *Campostoma* have flatter, less curved teeth. It is fitting that the *Nocomis* would have sharper teeth for piercing and shredding insect exoskeletons, whereas *Campostoma* have flatter teeth that act as a grinding surface to break apart diatom tests and algal cell walls. Differences in pharyngeal tooth shape in relation to diet have been observed in cichlids (Hulsey et al. 2005, 2006). In addition, *N. raneyi* apparently undergoes an

ontogenetic shift in diet in some locales from one dominated by insects to one dominated by molluscs, which results in a strengthening of the pharyngeal teeth in this species; the teeth become more rigid and blunt in larger fish than in smaller fish (Cloe et al. 1995). The lack of difference in pharyngeal tooth shape between the two *N. leptocephalus* populations, despite their dietary differences, suggests that tooth shape is not necessarily plastic in all *Nocomis* species and that a specific tooth shape is not required for a specific diet in these species.

In conclusion, this study is one of very few to investigate fish gut structure and function in a phylogenetic context, and the results are not necessarily surprising: there is a clear association between diet, gut size, and digestive enzyme activities in minnows of the genera *Campostoma* and *Nocomis*. However, this is the first study to actually evaluate whether the evolution of diet and gut structure and function can be correlated in fishes; they apparently can be in these minnows. We do caution that our results do not definitively answer whether the digestive physiology of the minnows is fixed in a manner appropriate for digesting a specific diet. Thus, in many ways our results are a first step, setting the stage for more complete analyses of the digestive physiology of *Campostoma* and *Nocomis* in a laboratory setting with diet-switching and common-diet experiments. The sister relationship of *Campostoma* and *Nocomis* and the dietary variability among populations of *Nocomis leptocephalus* provide ample opportunity to study digestive physiology and gut structure of fishes in a tight phylogenetic context. More complete sampling of the *Campostoma* taxa from across their biogeographic range (Blum et al. 2008) may clarify the differences among species in the different parts of the *Campostoma* clade. Nevertheless, even though many fishes have anatomically unspecialized digestive tracts in comparison with terrestrial vertebrates, there are subtle differences in the biochemistry of digestion and gut anatomy that allow for dietary specialization.

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