

Isolation of cellulolytic microbes from the intestinal tract of the pinfish, *Lagodon rhomboides*: size-related changes in diet and microbial abundance

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Abstract. Pinfish, *Lagodon rhomboides* (Linnaeus), undergo a gradual ontogenetic dietary shift during their first year of life, marked by an increase in the consumption of plant material. To determine if this shift in diet was associated with a change in the microbial flora of the intestinal tract that may assist in degradation of plant material, stomach contents were analyzed and microbes in the intestinal tract were isolated from fish ranging from 20 to 139 mm standard length. These fish were collected from Core Sound, North Carolina, USA between March and September 1991. Plant material increased from 16% of dry weight of stomach contents in pinfish under 40 mm standard length (SL) to 65% in pinfish above 120 mm SL, confirming previous observations of a diet-related ontogenetic change in *L. rhomboides*. Comparison of the total cultivatable facultative and anaerobic microbial flora isolated from the intestinal tract contents of pinfish ranging in size from 26 to 139 mm SL showed a 10-fold increase between fish <40 and fish >40 mm SL, with maximum population densities of approximately 2×10^7 colony forming units (CFU) g^{-1} of intestine including contents. The percentage of microbial isolates examined capable of hydrolyzing carboxymethylcellulose (CMC) increased from 12% in fish <40 mm SL to 13 to 50% in fish >40 mm SL, although there was no strict increase with increasing fish size classes. Although the percentage of CMC-hydrolytic microbial isolates varied with respect to fish SL, the percentage of skim-milk hydrolytic (proteolytic) isolates remained relatively constant (4% of total isolates) irrespective of fish SL and dietary composition. Results presented in this study document the first isolation of carboxymethylcellulase producing microbes from the intestinal tract of any fish and demonstrate that the ontogenetic dietary shift in *L. rhomboides* is paralleled by qualitative and quantitative changes in the intestinal microbial community. The use of strict anaerobic sampling methods in the preparation of intestinal contents from wild-captured fresh specimens was essential in obtaining these isolates.

Introduction

In seagrass meadows, herbivorous and omnivorous fishes and invertebrates are important trophic links between primary production in estuaries and higher trophic levels (Thayer et al. 1984, Horn 1989). Due to their great abundance in the southeastern United States and their propensity for consuming plant material, pinfish, *Lagodon rhomboides* (Family Sparidae), contribute significantly to the transfer of energy from estuarine habitats with high rates of primary productivity (seagrass meadows, marshes, mangroves, oyster reefs, etc.) to piscivorous fish species that support commercial and recreational fisheries [see Muncy (1984) and Darcy (1985) for a review of pinfish abundance rankings in these habitats and their trophic linkages]. Pinfish undergo a gradual ontogenetic change in diet, feeding largely on animals as larvae and juveniles, then changing to a predominantly plant diet as adults (Caldwell 1957, Hansen 1969, Carr and Adams 1973, Stoner 1980, Livingston 1982, Stoner and Livingston 1984, Huh and Kitting 1985, Luczkovich 1987). During this dietary transition, pinfish undergo significant changes in behavior, dentition, and gut anatomy corresponding to increased consumption of plant material in the diet (Caldwell 1957, Stoner and Livingston 1984). Similar ontogenetic dietary shifts have been documented in other members of the family Sparidae (i.e., *Diplodus holbrooki*, Carr and Adams 1973, Stoner and Livingston 1984; *Sarpa salpa*, Christensen 1977; *Archosargus rhomboidalis*, Vaughan 1978). Despite the fact that pinfish are major consumers of vegetation and can assimilate eelgrass consumed (Montgomery and Targett 1992), there is a paucity of information concerning the mechanisms responsible for the digestion and assimilation of dietary plant material in this species.

Although plant cell walls represent a formidable barrier to endogenous vertebrate digestive enzymes, recalcitrant components of the plant cell wall such as hemicellulose and cellulose are known to be hydrolyzed by the action of microbially produced cellulases in the digestive tracts of diverse taxa including termites, shipworms, sea

turtles, and ruminants (Hungate 1975, Bjorndal 1979, Breznak 1982, Waterbury et al. 1983). Although cellulase activity has been detected in the digestive tract contents of selected fish species (Stickney and Shumway 1974, Lindsay and Harris 1980, Moerland 1985), including pinfish (Weinstein et al. 1982), attempts to isolate cellulase-producing microbial symbionts from fishes have proven unsuccessful (Trust et al. 1979, Weinstein et al. 1982, Gerking 1984, Seiderer et al. 1987, Anderson 1991). While the existence of a microbial flora and fauna has been demonstrated in the gastrointestinal lumen of a number of herbivorous, carnivorous, and omnivorous fish species (Trust et al. 1979, Fishelson et al. 1985, Rimmer and Wiebe 1987, Seiderer et al. 1987, Sutton and Clements 1988, Anderson 1991, Clements 1991) including pinfish (Weinstein et al. 1982), the role of these microbes, particularly with regard to digestion of cellulose and other refractory plant polysaccharides, remains a matter of considerable controversy (see reviews by Horn 1989, 1992 and Choat 1991).

The present study was undertaken to determine if cellulolytic bacteria were present in the intestine of pinfish and whether changes in the dietary habits of the pinfish were accompanied by quantitative changes in the abundance as well as qualitative changes in the cellulolytic and proteolytic components of the intestinal tract microbial flora. In this study, we examined the stomach contents, quantified the proportion of plant material in the diet, isolated bacterial strains from the intestine, measured the abundance of cultivable anaerobic bacteria in the intestine, and tested isolated bacterial strains for the production of carboxymethylcellulase and protease activities.

Material and methods

Stomach content analysis

Pinfish, *Lagodon rhomboides* (Linnaeus), were collected from Core Sound, North Carolina, USA on the following dates: 30 March, 26 April, 31 May, 5 June, 12 June, 16 July, 14 August, and 16 September 1991. At least 15 fish were preserved on each date for examination of stomach contents using light microscopy. Whole fish were preserved in 10% formalin for examination of the stomach contents using a modification of the sieve-fractionation technique of Carr and Adams (1972). In this method, stomach contents from individual fish are passed through a series of sieves (2000, 850, 425, 250, 150, 75 μ) and food items are enumerated using low-power (7 to 60 \times) magnification. The counts are transformed to biomass (g dry weight) for each sieve fraction after drying for 48 h and determining the weight on a microbalance with a precision of 0.00001 g. Percentage dry weight for each prey category is calculated by determining the total number of particles in a sieve fraction then multiplying the proportion of the total comprised by a prey category by the dry weight for a sieve fraction. Dry weights for each category are summed across sieve fractions and divided by the total dry weight for each fish after subtracting the weight of sand grains and unidentifiable portions of the stomach contents. The average percentage dry weight per prey category (excluding sand grains and unidentifiable items) per fish is then calculated.

Microbiological sampling and analysis

Pinfish collected in April, May, June, July, and August 1991 were transported to the laboratory live in insulated ice chests containing

Table 1. *Lagodon rhomboides*. Proportion of various food categories in the stomach contents of four size classes of fish, separated by animals and plants and listed in decreasing proportion in the <39 mm standard length (SL) size class

Food category	Fish size class (mm SL)			
	20–39	40–79	80–119	120–139
Animals				
Polychaetes	0.37	0.25	0.25	0.04
Crustacean remains	0.16	0.10	0.04	0.07
Caprellid amphipods	0.14	0.00	0.00	0.00
Copepods	0.07	0.04	0.01	0.02
Hydroids	0.04	0.04	0.05	0.15
Polychaete fecal pellets	0.03	0.02	0.01	0.00
Fish scales	0.02	0.01	0.01	0.01
Invertebrate eggs	0.01	0.01	0.02	0.02
Gastropods	0.00	0.00	0.00	0.01
Gammarid amphipods	0.00	0.01	0.00	0.00
Shrimp	0.00	0.04	0.16	0.00
Fish remains	0.00	0.00	0.03	0.02
All animals	0.84	0.52	0.53	0.36
Plants				
Algae	0.14	0.38	0.30	0.26
Seagrasses	0.02	0.04	0.11	0.28
Diatoms	0.00	0.06	0.06	0.10
All plants*	0.16 ^a	0.48 ^{a,b}	0.47 ^{b,c}	0.64 ^c
n =	28	34	18	11

* The Bonferroni-adjusted multiple comparison procedure was used to compare the mean proportion of plant material consumed by each size class. Size classes with the same letter superscript do not differ significantly at an $\alpha=0.05$ for all contrasts

seawater from the collection site within 3 h of collection. Five fish of a similar size from each date were sacrificed immediately and their external surfaces sterilized using 70% ethanol. The entire intestinal tract was removed by aseptic surgery and the intestinal tract including contents were weighed, measured and placed intact into an anaerobic chamber (Forma Scientific, Model 1025) equilibrated with an atmosphere consisting of 85% N₂:10% H₂:5% CO₂. Intestinal tract samples used for microbiological analysis were prepared in one of two ways depending on fish size. Because of the small size of the intestinal tract of pinfish <40 mm standard length (SL) the entire intestinal tract, including contents, was homogenized in a glass tissue homogenizer containing 1.0 ml of anaerobic diluent. Samples from fish >40 mm SL were prepared by dissecting the intestinal tract, removing the contents, and homogenizing the contents in 1.0 ml of anaerobic diluent. Homogenized samples were diluted by ten-fold in a series, plated onto solid agar bacteriological media, and incubated at room temperature (20 to 22 °C) for 48 to 72 h under anaerobic conditions prior to enumeration or further manipulation.

The diluent used for homogenizing samples from the intestinal tract and plating media included per liter of distilled water: tryptone, 10 g; yeast extract, 10 g; casitone, 5 g; NaCl, 5 g; glucose, 1 g; MgSO₄ · 7 H₂O, 1 g; CaCl₂ · 2 H₂O, 0.5 g; MnCl₂ · 4 H₂O, 0.2 g; FeSO₄ · 7 H₂O, 0.2 g; resazurin, 0.001 g; hemin, 0.005 g; vitamin K, 0.01 g. Plating media differed from diluent by inclusion of 1.5% agar (Bacto-agar, Difco.). Sodium bicarbonate (0.5%) was added as a sterile solution after autoclaving other media components. Plating media used for enumeration of carboxymethylcellulase (CMCase-) and protease-producing microbial strains, in addition to the media components listed above, included 10 g l⁻¹ of carboxymethylcellulose [Low viscosity 50 to 200 centipoises (cps), Sigma Chemical Co.] or 5% sterile skimmed milk, respectively. Total viable cell counts

after anaerobic incubation were standardized based on the wet weight of the intestine including contents. CMCase- and protease-producing isolates were enumerated by selecting 50 colonies from serial dilutions of each fish and transferring each colony to plating medium containing either carboxymethylcellulose (CMC) or skimmed milk. CMCase-producing isolates were identified on CMC plates after flooding the plates with 5 ml of Congo red dye prepared in 0.7% agarose (Seakem HGT agarose) essentially according to the method of Teather and Wood (1982). In this assay, CMCase positive strains are identified by clear zones surrounding individual isolated colonies that have hydrolyzed CMC in the agar growth medium, because Congo red selectively binds with unhydrolyzed CMC. Protease-producing isolates were identified by the clearing of opaque milk proteins in the area surrounding isolates growing on the surface of skimmed milk plates as described by Durham et al. (1987).

In order to isolate any free-living bacteria in the environment, water and sediment core samples were taken from the collection sites on May, June, July, and August 1991. Water samples were taken by opening a sterile Whirl-Pak plastic bag in the water column at the site of fish collections. Core samples were taken with 16-cm long and 6-cm diameter plexiglass tubes that were pushed into the substrate, sealed top and bottom, and removed intact from the water. Core tubes were placed in ambient seawater in a cooler and transported along with the fish to the laboratory. Care was taken not to mix the sediment layers during transport in order to preserve the anaerobic conditions of the sediment. In the laboratory, serial dilutions of 1 ml of the water samples were made on anaerobic plating media (see above). In order to homogenize the sediment and water in each core sample immediately prior to microbial sampling, the cores were mixed thoroughly by inverting several times, allowed to settle for 2 min, and opened in the anaerobic chamber; 1 ml of water was then removed for serial dilutions on anaerobic plating media (as described above). This procedure was designed to minimize exposure of anaerobic sediments to oxygen in the water in the core.

Statistical analyses

Analysis of variance (ANOVA) was used to compare the plant and animal material in the diets of pinfish in four size classes (20 to 39, 40 to 79, 80 to 119, 120 to 139 mm SL), and multiple regression analysis was used to explain variation by month of collection and SL (Wilkinson 1990). A Bonferroni-adjusted multiple comparison procedure was used to generate pairwise significance tests of the mean difference in proportion of plant material consumed by fish in the four size classes (Wilkinson 1990). The size classes were chosen based on previous studies that have documented natural size groupings (i.e., trophic units) of pinfish with similar dietary composition, determined using cluster analysis (Stoner 1980, Livingston 1982, Luczkovich 1987). Because of the large amount of work involved in screening and quantifying bacterial populations, the number of fish surveyed on each date for microbial flora was small (five) and the underlying distributions of the populations unknown, so non-parametric statistical analyses were used. We used exact Wilcoxon Rank Sum and Kruskal-Wallis non-parametric statistical procedures to compare the bacterial abundances among the fish size classes and months of capture listed above (StatXact, Cytel Corp. 1989). The Wilcoxon test is analogous to a Student's *t*-test, and the Kruskal-Wallis test is analogous to a parametric ANOVA testing the hypothesis that at least one of the groups differs from the others in the median number of bacteria. The Jonckheere-Terpstra non-parametric procedure was used to test the hypothesis that there was an increase in the median number of bacteria with increased fish size. All non-parametric procedures are exact procedures, which means that the probabilities reported are obtained by randomizing the data and resampling them 2000 times to determine the exact probability of getting a statistic as large as the one calculated from the observed data (StatXact, Cytel Corp. 1989). For each fish size class or month of collection, box plots were created that show the medi-

an, the interquartile range or mid-range, which contains the middle 50% of the data, and a region which is equivalent to 1.5 × the interquartile range (Wilkinson 1990). Data values falling outside this latter region were considered outliers.

Results

Stomach content analysis

Stomach content analysis revealed that pinfish of all sizes consumed both plant and animal foods. The percentage of plant material in the diet increased from 16% in fish < 39 mm SL to 64% after fish reached 120 mm SL, with a decline in animal material from 84 to 36% (Table 1). The mean proportion of plant material among the four size classes (20 to 39, 40 to 79, 80 to 119, 120 to 139 mm SL) differed significantly (ANOVA, $F_{3,63} = 13.171$, $P = 0.0005$). Pinfish in the largest size class (120 to 139 mm SL) consumed significantly more plant material than did pinfish in the two smallest size classes (20 to 39, 40 to 79 mm SL), but did not consume more than the 80 to 119 mm SL fish; the 80 to 119 mm SL pinfish consumed significantly more plant material than the 20 to 39 mm SL pinfish; no other pairwise comparisons were significant (Table 1). There was a significant effect of collection date as well as fish size on the proportion of plant material in the diet (multiple regression analysis; month of collection: $P = 0.008$; SL: $P = 0.010$; $R^2 = 0.456$). Small pinfish (< 39 mm SL) consumed animal material in the spring and early summer, intermediate-sized pinfish (40 to 120 mm SL) consumed a mixture of plants and animals in the summer, and large pinfish (> 120 mm SL) consumed primarily seagrasses and macroalgae in the late summer and fall (Fig. 1 A, B). The major plant and animal components of the stomach contents of the smallest size class (< 39 mm SL) included polychaetes (*Axiiothella mucosa*, *Nereis* sp., 37% dry weight), crustacean remains (16%), caprellid amphipods (*Caprella penantis*) (14%), algae (filamentous red algae, 14%), and harpacticoid copepods (7%) (Table 1). The major plant and animal components of the stomach contents of the largest size class (> 120 mm SL) included algae (*Hypnea musciformis* and other unidentifiable foliose and filamentous algae, 26% dry weight), seagrasses (*Zostera marina* and *Halodule wrightii*, 28%), epiphytic diatoms (10%), crustacean remains (7%), and polychaetes (4%) (Table 1). Other minor food categories present in some fish of all size classes included isopods, gastropods, hydroids, fish scales, polychaete fecal pellets, gammarid amphipods, and invertebrate eggs. These results demonstrate that a change from a predominantly carnivorous diet to a predominantly herbivorous diet occurs when pinfish attain a size of ca. 120 mm SL.

Microbiological survey

Colony morphology and direct microscopic examination of individual microbial isolates demonstrated the presence of a diverse microbial flora even in samples obtained from a single fish. Results of microscopic observation

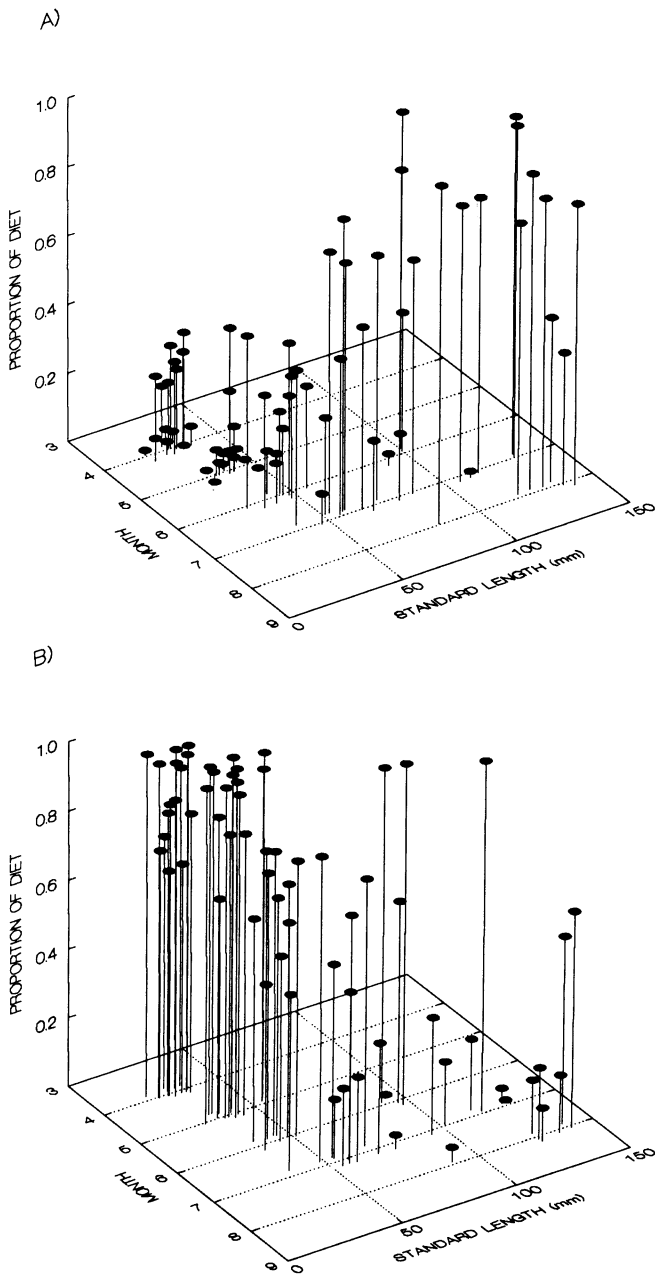


Fig. 1. *Lagodon rhomboides*. (A) The proportion of dry weight of the stomach contents comprised of plant material (seagrasses, algae, and diatoms) and (B) animal material (shrimp, amphipods, copepods, fish eggs, fish scales, invertebrate eggs, polychaetes, polychaete fecal pellets, and hydroids) vs month of capture and fish standard length in mm. Each spike represents the data from an individual fish

and Gram staining of individual isolates cultivated on bacteriological media revealed the presence of differentiated bacillary, coccobacillary, coccoid, and fusiform-shaped Gram-positive and Gram-negative bacteria.

Based on the results of growth on solid agar plating medium, the total number of viable facultative and anaerobic microorganisms measured as the median number of colony forming units (CFUs) present g^{-1} of intestinal tract including contents varied from a median of approximately 1.8×10^6 in pinfish < 39 mm SL, in-

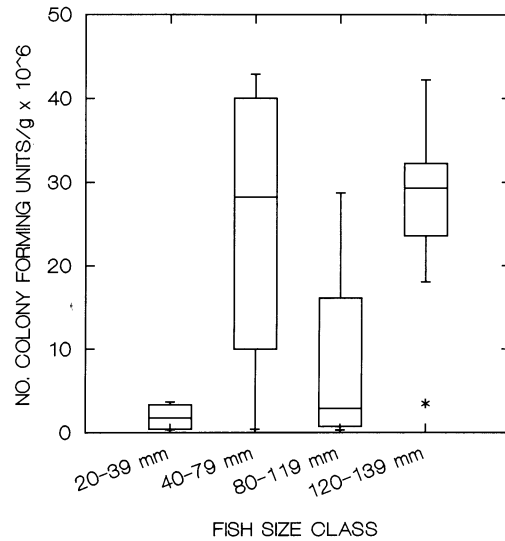


Fig. 2. *Lagodon rhomboides*. Median number of cultivable anaerobic bacteria g^{-1} of intestine plus contents weight in four fish standard length classes: 20 to 39 mm ($n=6$), 40 to 79 mm ($n=6$), 80 to 119 mm ($n=6$), 120 to 139 mm ($n=7$). Box plots indicate median number of colony forming units (horizontal line), the interquartile range (box), $1.5 \times$ interquartile range (bar), and data values that fall outside this range (asterisk)

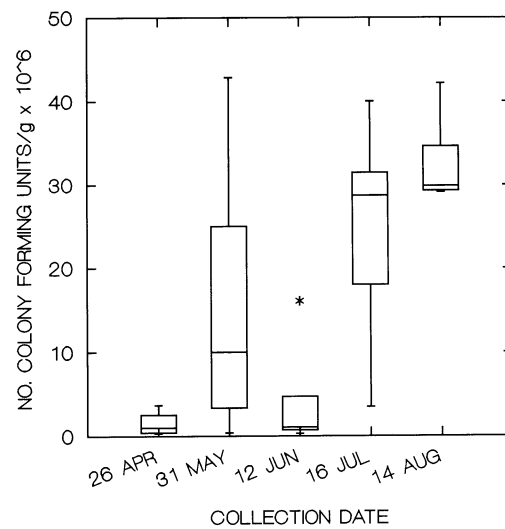


Fig. 3. *Lagodon rhomboides*. Median number of cultivable anaerobic bacteria g^{-1} of intestine plus contents weight from five fish collected each month during April to August 1991. Size range (standard length) for fish collected each month: April, 25 to 31 mm; May, 38 to 47 mm; June, 90 to 106 mm; July, 76 to 134 mm; August, 127 to 137 mm. Box plots and asterisk explained in Fig. 2

creased to 2.8×10^7 in fish 40 to 79 mm SL, dropped to a level of 2.9×10^6 in fish 80 to 119 mm SL, and increased to 2.9×10^7 in fish > 120 mm SL (Fig. 2). The number of CFUs g^{-1} differed significantly among size classes (Kruskal-Wallis test, $P=0.004$) and increased with increasing fish size (Jonckheere-Terpstra test, $P=0.019$). The median number of CFUs g^{-1} differed significantly among collection dates (Kruskal-Wallis test, $P=0.002$; Fig. 3) with the greatest number occurring in fish caught during July and August.

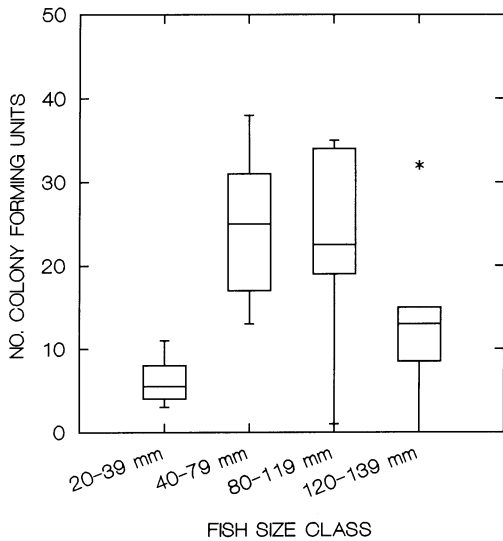


Fig. 4. *Lagodon rhomboides*. Median number of colonies out of 50 selected colonies that tested positive for the ability to degrade carboxymethylcellulose vs fish standard length classes. See Fig. 2 for sample sizes and explanation of asterisk and box plots

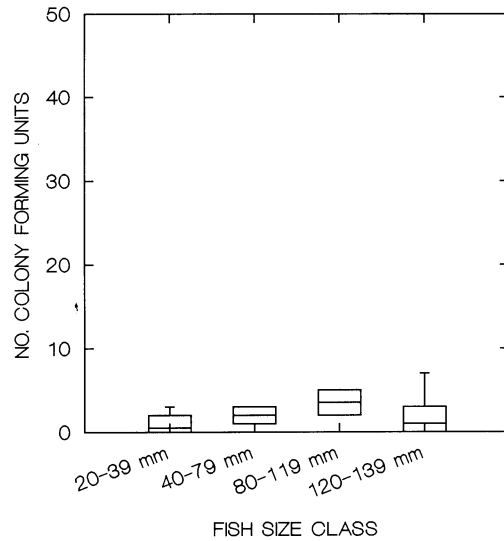


Fig. 6. *Lagodon rhomboides*. Median number of colonies out of 50 selected colonies that tested positive for the ability to degrade skim milk (proteolytic strains) vs fish standard length (mm) classes. See Fig. 2 for sample size and explanation of box plots

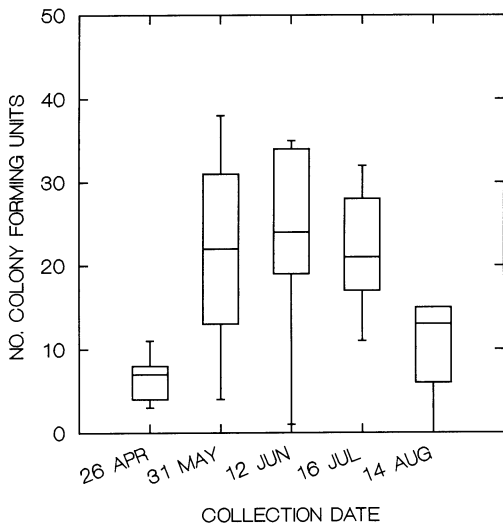


Fig. 5. *Lagodon rhomboides*. Median number of colonies out of 50 selected colonies that tested positive for the ability to degrade carboxymethylcellulose vs month of fish collection. Box plots explained in Fig. 2

Quantitative changes in the CMCase-producing, but not the protease-producing, intestinal microflora varied with fish size and month of collection. The median number of CMCase-producing isolates (CMCase isolates) increased from an average of 5.5 of 50 isolates screened (averaging 12.4% of isolates) from fish < 39 mm SL to 25 of 50 isolates (averaging 49.6% of isolates) screened from fish 40 to 79 mm SL, then declined to 13.0 of 50 isolates (averaging 26.2% of isolates) in fish 120 to 139 mm SL (Fig. 4). There was a significant difference between pinfish < 39 mm SL and fish > 40 mm SL (Wilcoxon Rank Sum, $P < 0.01$) and at least one significant difference among all size classes (Kruskal-Wallis test, $P < 0.007$), although there was not a direct increase in median num-

ber of CMCase isolates with increasing fish size class (Jonckheere-Terpstra test, $P > 0.244$). The median number of CMCase isolates varied significantly with month of collection (Kruskal-Wallis test, $P = 0.049$; Fig. 5) with the greatest number occurring in mid-summer (May, June, and July). The median number of protease-positive isolates was low in all sizes of fish surveyed, ranging between 0.5 and 3.5 out of 50 CFUs selected (averaging between 2 and 7% of isolates) from fish intestinal contents (Fig. 6). There was no significant difference in the median number of protease-positive CFUs among the four fish size classes (Kruskal-Wallis test, $P > 0.07$).

Although CMCase isolates were identified in 96% (24 of 25) of fish surveyed, no CMCase isolates were identified from four water and four sediment samples obtained from the same site and at the same time fish were collected.

Discussion

We have demonstrated that large numbers of carboxymethylcellulolytic bacteria were present in the intestinal tracts of 96% of pinfish with diverse omnivorous diets ranging in size from 25 to 137 mm SL that were captured in seagrass meadows in North Carolina from April through August. CMCase-producing bacteria were absent from water and sediment samples taken at the time of collection, suggesting that these bacteria may be endosymbiotic. Further time-course studies are required to confirm that these bacteria are resident in the intestine before endosymbiosis can be demonstrated. Our results indicate clearly that bacteria in the intestinal tracts of pinfish hydrolyze CMC. Whether the resultant products of hydrolysis contribute to the nutritional needs of this species has not been documented. The mode of intestinal colonization is unknown and may result from consump-

tion of bacterially colonized plant material, coprophagy, or ingestion of invertebrate herbivores that themselves contain cellulolytic bacteria. To our knowledge, the present study is the first demonstration of cellulolytic bacteria in fishes. This microbial flora is the most likely source of the cellulase activity in pinfish.

The present study confirmed the dietary transition of pinfish from a primarily carnivorous diet to a primarily herbivorous diet, which had been observed previously in Florida (Stoner 1980, Stoner and Livingston 1984, Luczkovich 1987). Although a general pattern of increasing herbivory was observed as fish grew, variation in the amount of plant material in the stomach contents of individual fish was noted for all sizes of fish, indicating that pinfish are capable of opportunistic switches in food selection due to changes in prey availability. Similar dietary shifts have been noted previously in pinfish populations in Texas, where fish <60 mm SL switched from animal prey (largely amphipods) to epiphytic algae, diatoms, and seagrass pieces as the number of amphipods declined in the seagrass meadow (Huh and Kitting 1985).

Comparison of results from stomach content analysis and microbial abundance showed that significant changes in the proportion of plant material consumed in the diet were reflected by changes in the proportion of CMCase-producing bacteria present in intestinal tract contents. While the proportion of plant material consumed in the diet increased with increasing fish SL reaching a value of 64% of total dietary intake in the largest size class of fish, the percentage of CMCase-producing bacteria attained a maximum value of 50% in 40 to 79 mm SL fish and declined to 13% in the largest size class of fish. The lack of correlation between the percentage of plant material consumed in the diet and the proportion of CMCase-producing bacteria present in the gut contents of pinfish indicates that the proportion of CMCase-producing bacteria is independent of the proportion of plant material in the diet. The changes in abundance of the CMCase-producing bacteria may be related to the presence of alternative carbon sources for growth, such as xylan and pectin, which also derive from plant material. The bacterial community comprising the CMCase-producing flora is presently being characterized and appears to be a heterogeneous assemblage of strains (Stellwag et al. in preparation). Competition between various strains for substrate and strain-specific patterns of carbon substrate utilization may be responsible for the fish size and seasonal variation in the proportion of CMCase-producing bacteria observed in this study.

Much of the controversy concerning the source of cellulase activity in the intestinal tract of fishes has arisen due to the inability to isolate cellulase-producing microorganisms from the intestinal contents and to document diet-related fluctuations in the level of cellulase activity. Several explanations have been proposed to account for the presence of cellulase in the digestive tracts of fish. The first suggests that intestinal tract-associated cellulase is produced by an endosymbiotic microbial flora resident in the intestinal tract. This hypothesis is supported by the fact that no vertebrate has been shown to produce endogenous cellulase (Yokoe and Yasumasu 1964,

Barnard 1973), by the presence of large numbers of bacteria in intestinal tracts of fishes (Trust et al. 1979, Moerland 1985, Seiderer et al. 1987, Sutton and Clements 1988, Clements 1991), some of which produce volatile fatty acids (Rimmer and Wiebe 1987), and by the inhibition of cellulase production after treatment with antibiotics (Stickney and Shumway 1974). In all of these studies, the evidence presented to support this hypothesis is indirect in that presumptive cellulase-producing microorganisms were neither identified nor isolated from gut contents. The results of the present study show that carboxymethylcellulolytic bacteria exist in the intestinal tract of pinfish and support the hypothesis that bacteria contribute to the production of cellulase in fishes.

The second major explanation for the presence of cellulase activity in the digestive tracts of fishes is that cellulase may be derived from ingestion of plant detritus. Prejs and Blaszczyk (1977) showed that in six cyprinid and salmonid species examined the activity of cellulase was correlated positively with the amount of dead plant material, presumably detritus, present in the digestive contents. The authors suggested that the detritus was colonized intensively by bacteria before ingestion and implied that the detritus-colonizing bacteria were responsible for the cellulase activity detected in the gut contents. Moerland (1985) also indicated that at least a proportion of cellulase activity in *Fundulus heteroclitus* gut contents may derive from ingested detritus, because cellulase activity was higher in field-collected fish than in laboratory-acclimated specimens fed a cellulose-free diet. Neither study measured the intrinsic cellulase activity of detritus upon which fish were feeding nor did either document the presence of a cellulolytic microbial flora associated with ingested detritus. Our findings do not address this hypothesis and as a consequence we cannot rule out plant and detritus-associated cellulase as a source of cellulase in the intestinal tracts of pinfish.

Thirdly, Niederholzer and Hofer (1979) and Lindsay and Harris (1980) suggest that digestive tract-associated cellulase activity is the result of cellulase or microbial flora present in the digestive tracts of invertebrates ingested as food. Niederholzer and Hofer (1979) demonstrated that the highest levels of cellulase activity were measured in roach, *Rutilus rutilus*, and rudd, *Scardinius erythrophthalmus*, feeding on zooplankton and arthropods. Lindsay and Harris (1980) concluded that, because most "invertivores" (carnivorous fish that feed almost exclusively on invertebrates) exhibited moderate or high cellulase activity in gut contents compared to omnivorous and piscivorous fish, cellulase activity in fishes is the direct result of the ingestion of invertebrates containing cellulase or a cellulolytic microflora. Neither study measured cellulase activity associated with invertebrates or other prey consumed in the diet, nor did these investigators demonstrate that the microbial flora of ingested invertebrates or other prey were capable of cellulase production prior to or after ingestion. Our results are consistent with this hypothesis, and the bacteria present in the invertebrates consumed by pinfish may serve as a source for the establishment and maintenance of the microbial flora.

Finally, Weinstein et al. (1982) have suggested that cellulase activity, at least in the pinfish, *Lagodon rhomboides*, is the result of endogenous synthesis. They reported cellulase activity as indicated by the release of reducing sugars in the presence of CMC and tissue homogenates from various regions of the gastrointestinal tract. Although a diverse and abundant anaerobic bacterial flora was present in the intestinal tracts of the eight fish surveyed, they concluded the source of the cellulase was endogenous synthesis based on the absence of any bacteria isolated that could produce CMCase. The bacterial populations isolated from the gastrointestinal tracts of fish by Weinstein et al. (1982) may have been reduced greatly due to the method of storage of fish transported to the laboratory and subsequent sample handling techniques. Because anaerobic bacteria comprised greater than 80% of the CMCase-producing isolates obtained in the present study (Stellwag et al. in preparation), aerobic exposure may have affected the viability and recovery of CMCase-producing bacteria from intestinal contents. While pinfish used in the present study were transported to the laboratory live and dissected within 3 h of capture, the eight pinfish surveyed by Weinstein et al. (1982) were shipped to the laboratory dead and on ice. In the absence of respiration and active metabolic activity by the pinfish, it is possible that gut contents were exposed to aerobic low temperature conditions for a prolonged period of time, adversely affecting or eliminating the anaerobic microbial flora. Moreover, although Weinstein et al. (1982) cultivated bacteriological samples under anaerobic conditions, gut contents were not collected or processed anaerobically, further reducing the CMCase-producing anaerobes. Therefore, it is possible that the CMCase activity detected by Weinstein et al. (1982) in tissue homogenates represented residual CMCase activity originally produced by bacteria that failed to survive transportation and sample handling procedures. This explanation is supported by the failure to document endogenous production of cellulase by any vertebrate or higher eukaryote (Yokoe and Yasumasu 1964, Barnard 1973).

Other studies have suggested that gut bacteria in fishes lack the ability to produce cellulase. Gerking (1984) examined the aerobic digestive tract microflora of two specimens of *Sarpa salpa* for cellulase activity. While six strains of bacteria were isolated and none produced cellulase, Gerking stressed the results were inconclusive inasmuch as anaerobic bacteria were not isolated or tested. Seiderer et al. (1987) cultured anaerobic bacteria in large numbers from the intestinal lumen of the anchovy, *Engraulis capensis*, but failed to find any cellulase activity in the intestine or associated with bacteria. Seiderer et al. (1987) concluded that their failure to isolate CMCase either in bacteria or in gut homogenates was associated with the absence of cellulose in the diet of this pelagic species.

Our study suggests that the microbial community may contribute to the breakdown of plant material by pinfish and may be the major source of cellulase. Although pinfish do not possess a specialized chamber for bacterial fermentation such as a hind-gut caecum, which is present in the Kyphosidae (Rimmer and Wiebe 1987, Horn 1989), their intestinal tract lengthens relative to body

length and becomes convoluted as they grow (Stoner and Livingston 1984), suggesting that the entire intestine may function as a fermentation chamber. Pinfish can utilize eelgrass, but may assimilate only 2 to 7% of the complex cell wall carbohydrates (Montgomery and Targett 1992). However, the cellulases produced by the bacteria may be important in liberating cell contents after the cell wall is hydrolyzed. The presence of cellulolytic bacteria alone does not provide evidence that the pinfish tested utilize plant material; however, the large amount of plants consumed by pinfish and the presence in their digestive tracts of abundant bacterial populations capable of cellulase production suggests that this bacterial association is an important but understudied trophic link in food webs of shallow seagrass ecosystems worldwide.

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