Symbiotic Fermentation, Digesta Passage, and Gastrointestinal Morphology in Bullfrog Tadpoles (*Rana catesbeiana*)

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ABSTRACT

Relative to other herbivorous vertebrates, the nutritional ecology and digestive physiology of anuran larvae remain poorly understood. Our objective was to compare gut structure and inhabitants, digesta passage, and microbial fermentation in bullfrog tadpoles (*Rana catesbeiana*) to those in other herbivores. Bullfrog tadpole gastrointestinal tracts were long and voluminous, with an enlarged colon that harbored a diverse symbiotic community. The transit time for particulate markers passing through bullfrog tadpoles was 6 h, the median retention time was 8–10 h, and gut clearance was 10–14 h postingestion. Relatively high levels of short-chain fatty acids in the hindgut of tadpoles indicated active microbial fermentation in this gut region. This report represents the first account of gastrointestinal fermentation in the class Amphibia. On the basis of in vitro fermentation assays, we estimated that microbial fermentation in the hindgut provides 20% of the total daily energy requirement of bullfrog tadpoles. These tadpoles also exhibited coprophagy, a practice that provides important nutritive gains in other herbivores. The physiological and behavioral characteristics of these tadpoles are remarkably similar to those of other small-bodied, hindgut-fermenting vertebrates, suggesting convergent digestive strategies among a broad range of herbivorous taxa.

Introduction

Recognition of the importance of fermentative digestion in herbivorous vertebrates has proceeded sequentially from mammals (Hungate 1966; reviewed in Stevens and Hume 1995) to birds (Annison et al. 1968; reviewed in Stevens and Hume 1995), reptiles (Bjorndal 1979; reviewed in Bjorndal 1997), and fish (Rimmer and Wiebe 1987; reviewed in Clements 1997). Among these diverse herbivores, there are remarkable similarities in their morphological specializations, gastrointestinal symbiotic communities, fermentation activity, digesta passage, and coprophagous habits.

Oral and gastrointestinal specializations that characterize these herbivores include shearing and grinding teeth, relatively long digestive tracts, and enlarged regions of the gut that harbor microbial symbionts (reviewed in Stevens and Hume 1995). The symbionts digest the refractory components of the host diet, such as complex structural carbohydrates, and release fermentation by-products (e.g., short-chain fatty acids [SCFAs]) that are absorbed and used as an energy source by their herbivorous hosts. In some herbivores, most or all of their daily energy requirements are satisfied via symbiotic fermentation (Bergman 1990; Stevens and Hume 1995; Bjorndal 1997).

Gastrointestinal symbionts also provide to these herbivorous hosts essential nutrients (e.g., B-complex vitamins and amino acids) and make other important physiological contributions, ranging from sodium conservation to stimulated immunity (reviewed in Stevens and Hume 1995). To provide the time required by their symbionts for fermentation and to maintain symbiont populations in the gut, most herbivores exhibit delayed rates of digesta flow through those gastrointestinal regions populated by symbionts. Small-bodied herbivores often selectively retain relatively small food particles that can be rapidly fermented. Small herbivores also generally exhibit autoenzymatic digestion in their anterior gut regions and fermentative digestion in their posterior gut regions. This hindgut fermentation strategy prevents the loss of easily digested dietary components to a less energy-efficient fermentative metabolic pathway (reviewed in Hume 1997).

In addition to these morphological and physiological specializations, many species of small, herbivorous mammals regularly consume their own feces. Coprophagy provides essential nutrients to these hosts (e.g., microbial protein, SCFAs, vitamins, and minerals) that would otherwise be lost via defecation. Upon reingestion of feces, the hosts digest in their anterior gut regions both the symbionts and their metabolic by-products (reviewed in Hornicke and Bjornhag 1980).

Whether herbivorous amphibians exhibit the suite of characteristics that typify other herbivorous vertebrates has not yet been evaluated (Stevens and Hume 1995; Bjorndal 1997). Such a lack of investigation is surprising because the larvae of most

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anuran species feed on herbivorous diets (McDiarmid and Altig 1999) that include plants, plant-based detritus, periphyton, and phytoplankton (Sanderson and Kupferberg 1999). In this study we investigated gastrointestinal tract modifications, digesta passage, coprophagy, and gut symbiont communities in herbivorous bullfrog tadpoles (*Rana catesbeiana*) and conducted in vitro fermentation assays to estimate the energetic contributions provided by their gastrointestinal symbionts.

Material and Methods

Experimental Animals

Bullfrog tadpoles and eggs were collected from Green Pond on the University of Florida campus in Gainesville. Late-stage tadpoles (Gosner stages 31–41; Gosner 1960) were collected for a preliminary investigation of gastrointestinal morphology and gut inhabitants. To control for genetic, dietary, and environmental variability, other tadpoles were raised from a single clutch of eggs under laboratory conditions for quantitative analyses of gut morphology, digesta passage, and fermentation activity. For these analyses, bullfrog eggs were collected on March 17, 2002. These eggs were separated from pond water and transferred to naturally dechlorinated tap water (pH = 7.5). Hatchlings (Gosner stages 20–25) were not offered food until the larvae had attained an active feeding stage of development (Gosner stage 26). For the duration of these experiments, groups of 25 tadpoles per 37-L aquarium were maintained in dechlorinated tap water under cool white fluorescent lighting (14L : 10D) at 29°C. Tadpoles were used in compliance with and under supervision of the Institutional Animal Care and Use Committee at the University of Florida (IACUC Z011).

For maintenance, tadpoles were fed ad lib. levels of powdered, alfalfa-based rabbit food (Classic Blend Rabbit Food, L/M Animal Farms, Pleasant Plain, OH) mixed with flaked fish food (Wardley Premium Goldfish Flakes, Hartz Mountain, Secaucus, NJ). The ratio of rabbit food to fish food was 80 : 20. Every other day, tadpoles were transferred to newly prepared aquaria. This regime controlled water quality without the use of filters and pumps, which disturb normal feeding behavior in bullfrog tadpoles (G. S. Pryor, personal observation). Once a week, a small aliquot (5 mL) of suspended benthic substrate from Green Pond was added to each aquarium to expose tadpoles to the symbionts and other matter that would normally be ingested from the environment.

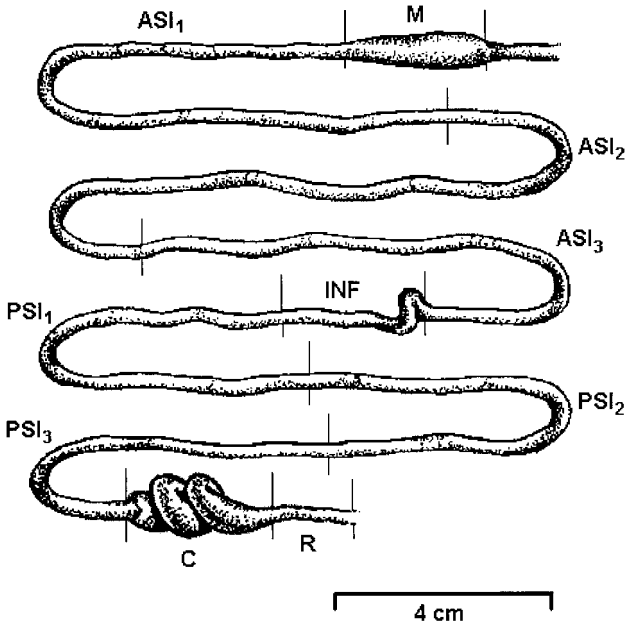

Figure 1. The bullfrog tadpole gastrointestinal tract. Gut regions: M = manicotto glandularae; ASI_1 = upper third of the anterior small intestine; ASI_2 = middle third of the anterior small intestine; ASI_3 = lower third of the anterior small intestine; INF = inflection region; PSI_1 = upper third of the posterior small intestine; PSI_2 = middle third of the posterior small intestine; PSI_3 = lower third of the posterior small intestine; C = colon; and R = rectum. Illustration by G. Pryor.

Experimental Protocol

Gut Structure and Inhabitants. To examine the gastrointestinal tract of wild-caught bullfrog tadpoles for general gut structure and inhabitants, 25 late-stage tadpoles (Gosner stages 31–41) collected from Green Pond were killed by overanesthetization in a water bath containing tricaine methanesulfonate (1 g L^{-1} of Tricaine-S; Western Chemical, Ferndale, WA) buffered with an equal amount of sodium bicarbonate. The gastrointestinal tracts were removed and preserved in formaldehyde (10% neutral-buffered formalin [NBF]) for gross examination or glutaraldehyde (2.5% neutral-buffered) for scanning electron microscopy (SEM). For SEM, samples were dehydrated with a graded ethanol series and hexamethyldisilazine, mounted on aluminum stubs, and sputter-coated with gold palladium. Specimens were examined using a Hitachi S-4000 FE SEM (Hitachi Instruments, San Jose, CA).

Gastrointestinal Tract Morphology. Gastrointestinal tract measurements were taken from 50 laboratory-reared tadpoles that were also used for studying symbiotic fermentation (see details below). For these tadpoles, total gut length, colon length, and colon width were measured to the nearest 0.1 mm using calipers. The full gastrointestinal tracts were measured after dissecting away the accessory organs and gently straightening the coiled tracts. Mass of the gut contents of these tadpoles, on both wet matter (WM) and dry matter (DM) bases, were measured from each of five distinct gut regions (Fig. 1): manicotto (M), anterior small intestine (ASI), posterior small intestine (PSI; including inflection region, INF), colon (C), and rectum (R).

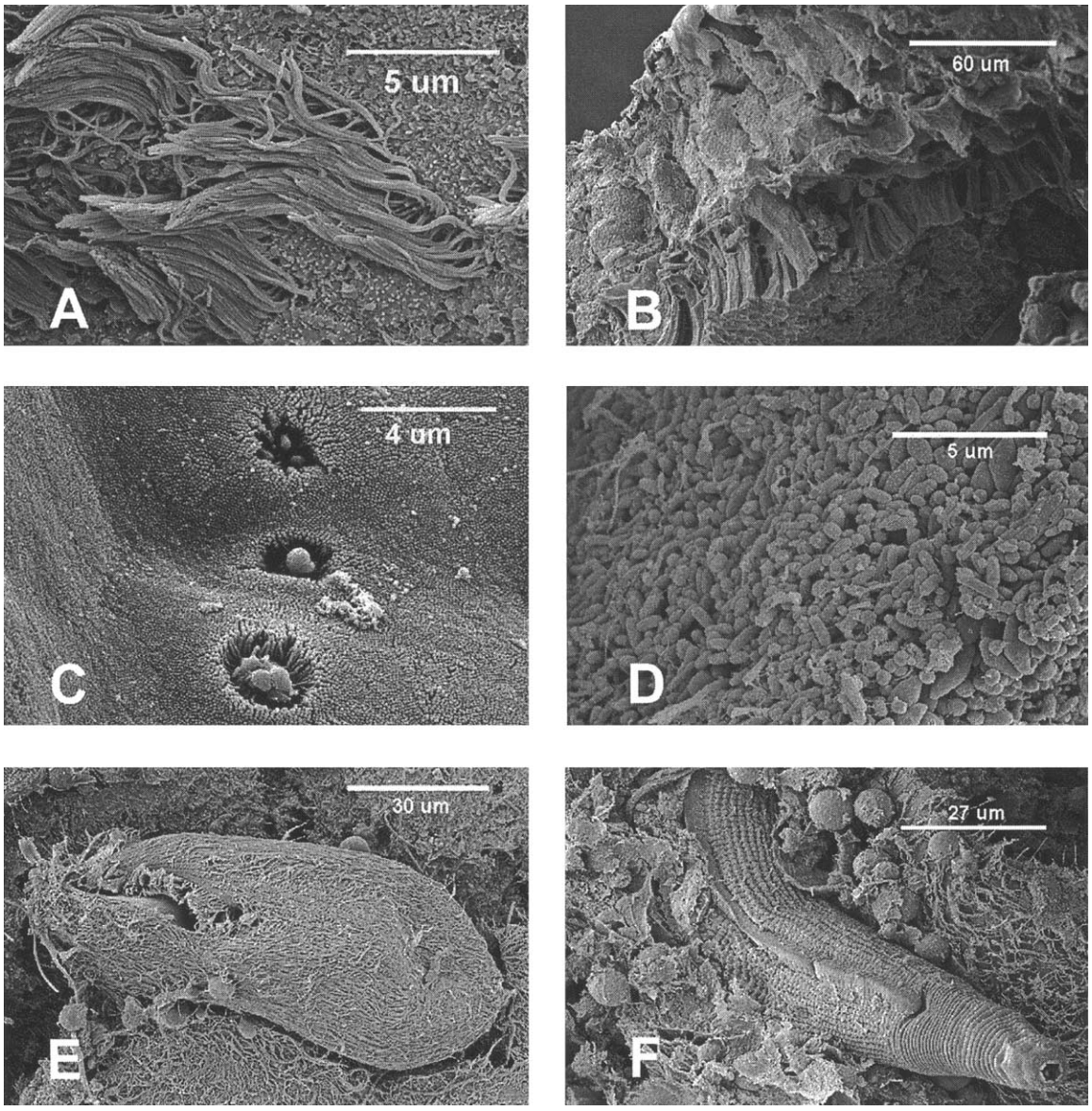

Figure 2. Scanning electron micrographs of the bullfrog tadpole gastrointestinal tract. Shown are cilia of the esophagus (*A*); mucous glands and epithelial cells of the manicotto glandularae (*B*); secretory glands and absorptive surface of the small intestine (*C*); and symbionts of the colon, including bacteria (*D*), the ciliate *Nyctotherus cordiformes* (*E*), and the nematode *Gyrinicola batrachiensis* (*F*).

Digesta Passage. To measure passage of digesta, two groups of tadpoles ($N = 16$ and $N = 18$) of similar sizes and developmental stages were placed in separate 37-L aquaria and served as experimental replicates. These tadpoles were allowed to acclimate for 7 d on a diet of flaked fish food.

Passage rates of two different sizes of indigestible markers were measured to determine whether selective retention of small particles occurs in these tadpoles. The markers were uniform-dyed, latex microspheres with similar physical properties (density = 0.95–1.05 g cm^{-3}) but different diameters and colors (pink microspheres = 112 μm [FSO8F]; yellow microspheres = 15 μm [DO150004PF]; Bangs Laboratories, Fishers, IN). The different sizes of microspheres approximated the sizes of unicellular algae (10–15 μm) and fragments of plant and filamentous algae (ca. 100 μm) consumed by the bullfrog tadpoles that were collected from the wild. Food marked with both sizes of microspheres was prepared by pipetting an aqueous solution of the markers onto a thin layer of flaked fish food, covering the wetted fish flakes with a layer of dry flakes, and drying at 60°C for 10–15 min.

Digesta passage trials were conducted by feeding each group of tadpoles the flaked fish food marked with both sizes of

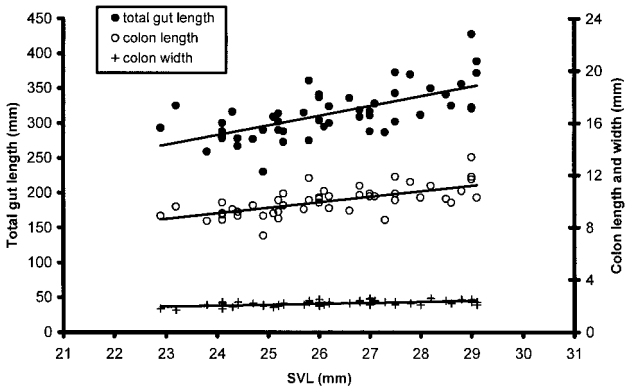

Figure 3. Morphological correlations of the bullfrog tadpole gastrointestinal tract. Plotted against snout-to-vent length (SVL) are total gut length ($y = 14.01x - 53.52$, $R^2 = 0.44$, $P < 0.001$, $N = 50$), colon length ($y = 0.43x - 1.04$, $R^2 = 0.44$, $P < 0.001$, $N = 50$), and colon width ($y = 0.07x + 0.30$, $R^2 = 0.37$, $P < 0.001$, $N = 50$). Colon length and width are plotted on the secondary *Y*-axis.

microspheres, collecting any uneaten food after 15 min, and continuing to feed tadpoles unmarked fish flakes for the remainder of the trials. To prevent reingestion of feces by tadpoles, nylon screening was installed 3 cm above the bottom of the aquarium. At 2-h intervals, two or three tadpoles were collected from each group, killed by overanesthetization, and preserved in NBF. Feeding trials lasted for 14 h after ingestion of marked food.

Digesta passage was quantified using compartmental analysis of dissected tadpoles. For each tadpole, the gut was divided into 10 regions (Fig. 1): manicotto glandularae (M), upper third of the anterior small intestine (ASI_1), middle third of the anterior small intestine (ASI_2), lower third of the anterior small intestine (ASI_3), inflection point of the small intestine plus a subsequent section equal to 10% of the total gut length (INF), upper third of the posterior small intestine (PSI_1), middle third of the posterior small intestine (PSI_2), lower third of the posterior small intestine (PSI_3), colon (C), and rectum (R). Contents of each gut region were added to a known volume of NBF (100–600 μL, depending on mass of gut contents) in a snap-top microcentrifuge vial and homogenized with a variable-speed vortex mixer. To determine the number of large (112 μm) microspheres per gut region, each sample was pipetted into a watch glass and examined under a dissecting microscope, and the total number of microspheres was counted. To determine the number of small (15 μm) microspheres per gut region, a single 10-μL aliquot of each homogenate was placed on a glass slide, the number of microspheres in each aliquot was counted using epifluorescent light microscopy, and the total number of microspheres was calculated based on the volume of NBF diluent added per gut region.

Transit time was estimated as the time of first appearance of marker in the rectum of dissected tadpoles. Because all markers that were ingested still remained in the gut (i.e., anterior to the rectum) 4 h after ingestion, the mean number of ingested markers was calculated from the number of markers in the gut at 2 and 4 h. Median retention time (M_{50}) was calculated as the time interval during which 50% of ingested markers remained in the gut. This measure is also known as the 50% excretion time and allows for comparison with the mean retention times (MRTs) reported by other authors (see discussions in Karasov et al. 1986; Stevens and Hume 1995). Total gut clearance was estimated as the first time interval at which 5% or fewer of ingested markers remained in the gut.

Gastrointestinal Fermentation. Measurements of symbiotic fermentation activity were based on the zero-time method of Carroll and Hungate (1954). Fermentation activity was calculated as the difference between concentrations of SCFAs at time zero (time of death) and after 1 h anaerobic incubation (in vitro).

The time-zero gut samples were collected from 25 tadpoles from the maintenance aquaria that were killed by pithing. The gastrointestinal tract was quickly removed, and the contents from each of five gut regions (M; ASI; PSI, including inflection region, INF; C; and R; Fig. 1) were emptied into a microcentrifuge vial, weighed, and frozen. These five gut regions were selected based on their distinct anatomical structure and physiological function (Hourdry et al. 1996; Viertel and Richter 1999). After all tadpoles were processed, the gut samples were thawed, homogenized with a vortex mixer, and centrifuged under refrigeration (4°C) at 16,000 *g* for 10 min. Supernatant was pipetted and combined to create five pooled samples (i.e., each pooled sample represented five tadpoles) for each of the five gut regions. Pooled batches of gut fluid were necessary because of the limited volume of gut fluid obtained from individual tadpoles. To remove bacteria and small particles from

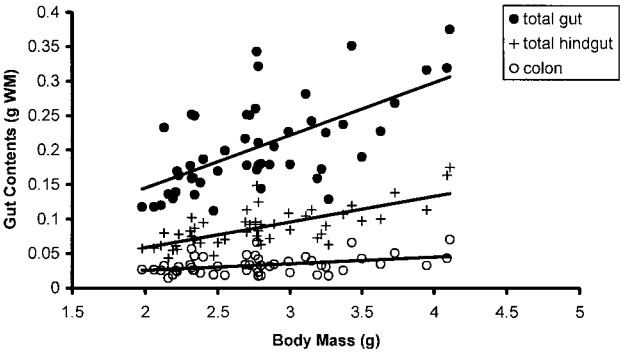

Figure 4. Gut content correlations of the bullfrog tadpole gastrointestinal tract. Plotted against total body mass are total gut contents ($y = 0.077x - 0.008$, $R^2 = 0.39$, $P < 0.001$, $N = 50$), total fermentation region (posterior small intestine, colon, and rectum) contents ($y = 0.037x - 0.016$, $R^2 = 0.51$, $P = 0.004$, $N = 50$), and colon contents ($y = 0.010x + 0.007$, $R^2 = 0.16$, $P < 0.001$, $N = 50$), on a wet matter (WM) basis.

the gut fluid, samples were purified using 0.22-μm cellulose acetate centrifuge tube filters (Costar Spin-X gamma sterilized centrifuge tube filters, Corning, NY). These samples were centrifuged under refrigeration at 13,000 *g* for 15 min and frozen until they were analyzed for SCFAs.

An additional 25 tadpoles were processed for the 1-h in vitro anaerobic incubations by pithing. Their gastrointestinal tracts were removed, and the contents from the five gut regions were placed in microcentrifuge vials. The vials were flushed with carbon dioxide gas for 30 s and then quickly capped. Samples were incubated for 1 h at ambient temperature (29°C) in the room where tadpoles were maintained, then frozen to stop fermentation activity, and processed using the same procedures as the time-zero samples. As with the time-zero samples, batches of gut fluid from five tadpoles were pooled together. Fermentation activity (1-h fermentation yield) was calculated as the difference between the SCFA concentrations after 1 h of incubation and the concentrations at time zero.

Concentrations of SCFAs in the gut samples were measured using gas chromatography. Samples were hand-injected into a Shimadzu GC-9AM gas chromatograph equipped with a flame ionization detector (Shimadzu Scientific Instruments, Columbia, MD) and a Perkin Elmer LC-100 integrator (Perkin Elmer). Two microliters of each sample were injected into a 2-m-long glass column (3.2 mm ID) 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 60 mL min^{-1}. Temperatures of the inlet, column, and detector were 180°, 155°, and 200°C, respectively. An external standard containing 90 mg L^{-1} each of acetate, propionate, butyrate, valerate, and isovalerate and 100 mg L^{-1} of isobutyrate was used for calibration.

To express SCFA concentrations relative to the DM of digesta, the DM of the gut contents of each gut region was determined by transferring the sediment remaining in each microcentrifuge vial to a ceramic crucible and drying at 60°C for 48 h. Energetic contributions of symbiotic fermentation were calculated from the daily energy requirements for bullfrog tadpoles (Crowder et al. 1998), the 1-h fermentation yields of individual SCFAs, and the energetic equivalents of SCFAs (see detailed calculations in "Results"). The pH of pooled gut fluid samples used in the fermentation analyses was determined to the nearest 0.5 unit of measure by pipetting 10 μL of the gut fluid onto pH paper (ColorpHast indicator sticks; MCB Reagents, Gibbstown, NJ).

Coprophagy. Focal animal sampling (Martin and Bateson 1986) was used to quantify the coprophagous habits of 18 bullfrog tadpoles. Individual tadpoles in two maintenance aquaria were observed constantly for 5 min, and the time they spent feeding on conspecific feces was recorded. To avoid repeated observations of the same individual tadpoles, tadpoles with distinctly different markings were chosen. Tadpoles that were actively feeding at the beginning of the observation period were selected for study. All observations were made sequentially on the same day to reduce confounding temporal effects. Food was present in the aquaria during these observation periods. Data were expressed as percent of time (mean ± SE) spent feeding on conspecific feces.

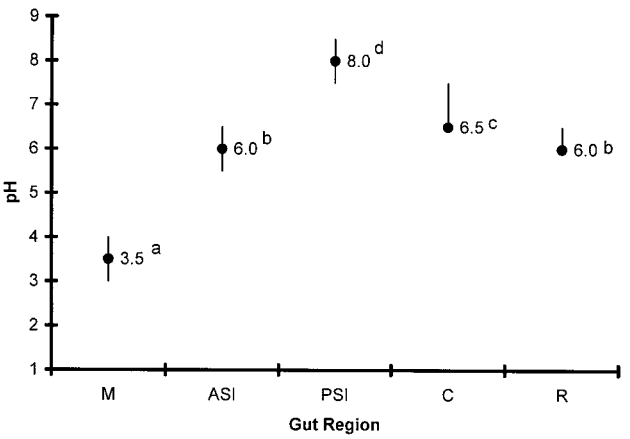

Figure 5. Medians and ranges of pH in distinct regions of the bullfrog tadpole gastrointestinal tract. Significant differences indicated by different superscripts (Kruskal-Wallis; $P < 0.001$). Sample $N = 5$ pooled samples of 5 tadpoles per gut region. Gut regions: *M* = manicotto; *ASI* = anterior small intestine; *PSI* = posterior small intestine; *C* = colon; and *R* = rectum.

Statistical Analyses

Linear regression was used to test for correlations between body size and gut size and between wet mass of gut contents and total body mass (i.e., body mass with gut contents included). For the digesta passage and fermentation experiments, mean body sizes and median stages of development (Gosner stages) of tadpoles were compared using appropriate parametric and nonparametric tests, which are specified for each contrast (see "Results"). For these analyses, means and standard errors (±SE) are presented. Levene's test and the Kolmogorov-Smirnov test (with Lilliefors significance correction) were used to test for homogeneity of variance and normality of data, respectively.

To contrast gut passage between the two different sizes of markers, data were first expressed as the mean percentage of marker in each gut region per time interval. Pairwise *t*-tests were then conducted for each gut region at each time interval using arcsine-transformed percentage data.

For the fermentation experiments, mean concentrations of SCFAs among gut regions were compared using one-way repeated-measures ANOVAs with Sidak's adjustments for multiple comparisons. Mean concentrations of SCFAs between time-zero and 1-h in vitro samples were compared with *t*-tests. Median pH values among gut regions were compared using Kruskal-Wallis one-way ANOVAs followed by Mann-Whitney

Table 1: Short-chain fatty acid (SCFA) concentrations and pH of the bullfrog tadpole gastrointestinal tract compared to the fermentative gut regions of other herbivorous vertebrates

Taxon, Species, and Gut Region	pH	SCFA Concentration		Source
		μmol g^{-1} DM	mmol L^{-1}	
Amphibian:				
Bullfrog tadpole (*Rana catesbeiana*)				This study
Manicotto	3.0–4.5	7–11	.9	
ASI	5.5–6.5	13–57	3.5	
PSI	7.5–8.5	30–147	7.6	
Colon	6.5–7.5	179–330	18.6	
Rectum	6.0–6.5	16–52	3.8	
Mammals:				
Cow (*Bos taurus*)				Bergman 1990; Van Soest 1994; Stevens and Hume 1995
Rumen	5.5–6.5	565–1,274	122–148	
Colon	7.4–7.6	300–500	70–120	
Rabbit (*Oryctolagus cuniculus*)				Parra 1978; Vernay et al. 1984; Vernay 1986; Garcia et al. 2002
Colon	7.1–8.1	226–291	42–46	
Cecum	5.7–6.3	317–368	75	
Horse (*Equus caballus*)				Parra 1978; Vernay et al. 1984; Drogoul et al. 2000
Colon	6.8–7.0	160–296	60–90	
Cecum	6.8–7.2	564–698	80	
Elephant (*Loxodonta africana*)				Clemens and Maloiy 1982
Colon	5.8–7.0	253–913	65–148	
Cecum	5.7	974	138	
Tree sloth (*Bradypus tridactylus*)				Foley et al. 1995
Foregut	5.2–6.7	…	51–78	
Birds:				
Ostrich (*Struthio camelus*)				Swart et al. 1993
Colon	8.2	527	171–195	
Cecum	6.9	1,260	140	
Emu (*Dromaius novaehollandiae*)				Herd and Dawson 1984
Ileum	8.2	101	14	
Rectum	7.2	86	17	
Hoatzin (*Opisthocomus hoazin*)				Grajal 1995
Crop	6.4	385	115	
Esophagus	6.6	431	170	
Cecum	7.5	402	95	
Reptiles:				
Florida red-bellied turtle (*Pseudemys nelsoni*)				Bjorndal and Bolten 1990
Small intestine	6.8	1,131	55	
Colon	6.8	1,091	61	
Cecum	6.8	1,282	60	
Green sea turtle (*Chelonia mydas*)				Bjorndal 1979
Colon	6.4	870	63	
Cecum	6.4	1,174	67	
Green iguana (*Iguana iguana*)				McBee and McBee 1982
Colon	7.5	…	16	
Cecum	7.5	…	51	
Egyptian spiny-tailed lizard (*Uromastyx aegyptius*)				Foley et al. 1992
Colon	7.0	324	60–76	
Cecum	6.9	569	120	

Table 1 (*Continued*)

Taxon, Species, and Gut Region	pH	SCFA Concentration		Source
		μmol g^{-1} DM	mmol L^{-1}	
Fish:				
Herring cale (*Odax cyanomelas*)				Clements et al. 1994
Hindgut	8.0–9.0	115–141	29–35	
Western buffalo bream (*Kyphosus cornelii*)				Rimmer and Wiebe 1987
Cecum	6.1	…	16–18	
Silver drummer (*Kyphosus sydneyanus*)				Rimmer and Wiebe 1987
Cecum	6.3–6.7	…	38	
Rectum	6.5–7.0	…	16	

Note. Species included are those for which SCFA concentrations and pH of gut contents have been reported; this is not an exhaustive list. Ellipses indicate that data are not available or cannot be calculated from the studies cited.

U-tests for multiple comparisons. Alpha values were set a priori at 0.05, and all statistical analyses were conducted using SPSS software (SPSS, Chicago).

Results

Gut Structure and Inhabitants

In bullfrog tadpoles, the short section of esophagus anterior to the manicotto was empty and ciliated (Fig. 2*A*). The manicotto and the small intestine were lined with tall, columnar, microvillous epithelial cells and punctuated with mucus-secreting goblet cells (Fig. 2*B*, 2*C*). No mucosal folds or villi projected into the lumen of the small intestine (Fig. 2*C*). The small intestine was tightly arranged into a double spiral in which the anterior small intestine coiled counterclockwise when viewed ventrally and the posterior small intestine coiled back clockwise from the point of inflection. The enlarged colon was also coiled, and the colonic epithelium was composed of cuboidal cells. There was a thin valve at the anterior aperture of the colon. The posterior colon narrowed gradually to merge with the rectum. The rectum and colon of pithed tadpoles exhibited peristalsis and antiperistalsis.

In both wild and laboratory-reared tadpoles, the colon was inhabited by a diversity of bacteria, protozoa, and nematodes (Fig. 2*D*–2*F*). These symbionts occupied a thick mucous matrix lining the colon wall. Colonic bacteria included bacilli, cocci, and spiral forms. Protozoan species living in the colon and posterior small intestine included *Opalina* spp. and *Nyctotherus cordiformes*. Gastrointestinal nematodes (*Gyrinicola batrachiensis*) were common in the colon and were occasionally found in the posterior small intestine and rectum. These nematodes were not found in metamorphosing, late-stage tadpoles (Gosner stages 42–46) or adult frogs. All bullfrog tadpoles collected in the wild ($N = 16$) were infected with nematodes.

Gastrointestinal Tract Morphology

Total gut length, colon length and width, and mass of gut contents were correlated with bullfrog tadpole body size (Figs. 3, 4). Gastrointestinal tracts of laboratory-reared tadpoles were 12 times longer than snout-to-vent length (SVL), and total gut contents, on a wet matter basis, represented approximately 7% of total tadpole body mass. In these tadpoles, the manicotto was acidic, the small intestine exhibited a variable pH along its length, and the colon and rectum were near neutral (Fig. 5; Table 1). These data are in agreement with those reported for wild-caught bullfrog tadpoles (Thrall 1972).

Digesta Passage

Tadpoles used in the first digesta passage trial did not differ in size (ANOVA; mass: $F_{6,9} = 0.389$, $P = 0.87$; SVL: $F_{6,9} = 0.510$, $P = 0.79$), total gut length ($F_{6,9} = 0.469$, $P = 0.82$), or developmental stage (Kruskal-Wallis; $\chi^2 = 4.08$, $P = 0.67$) among the 2-h collection intervals. For these tadpoles, mean mass was 2.37 ± 0.11 g; SVL was 23.0 ± 0.3 mm; and total gut length was 248.9 ± 7.4 mm. Median developmental stage was Gosner stage 36. Tadpoles used in the replicate trial also did not differ in size (mass: $F_{6,11} = 2.131$, $P = 0.13$; SVL: $F_{6,11} = 1.669$, $P = 0.22$), total gut length ($F_{6,11} = 0.618$, $P = 0.71$), or developmental stage ($\chi^2 = 10.09$, $P = 0.12$). Their mean mass was 2.12 ± 0.09 g; SVL was 22.3 ± 0.4 mm; and total gut length was 222.6 ± 6.9 mm. Median Gosner stage was 36. Between trials, tadpoles were similar in mass (*t*-test: $t_{32} = 1.80$, $P = 0.08$), SVL ($t_{32} = 1.54$, $P = 0.13$), and developmental stage (Mann-Whitney *U*-test: $\chi^2 = 103.50$, $P = 0.16$), but the mean gut length in tadpoles from the first trial was significantly greater than that of tadpoles from the second trial ($t_{32} = 2.60$, $P = 0.01$).

There was no evidence of differential passage between the two sizes of markers (Fig. 6; Table 2). Transit time for both sizes of markers was 6 h in each trial. M_{50} (50% loss) occurred between 8 and 10 h, and gut clearance (95% loss) occurred

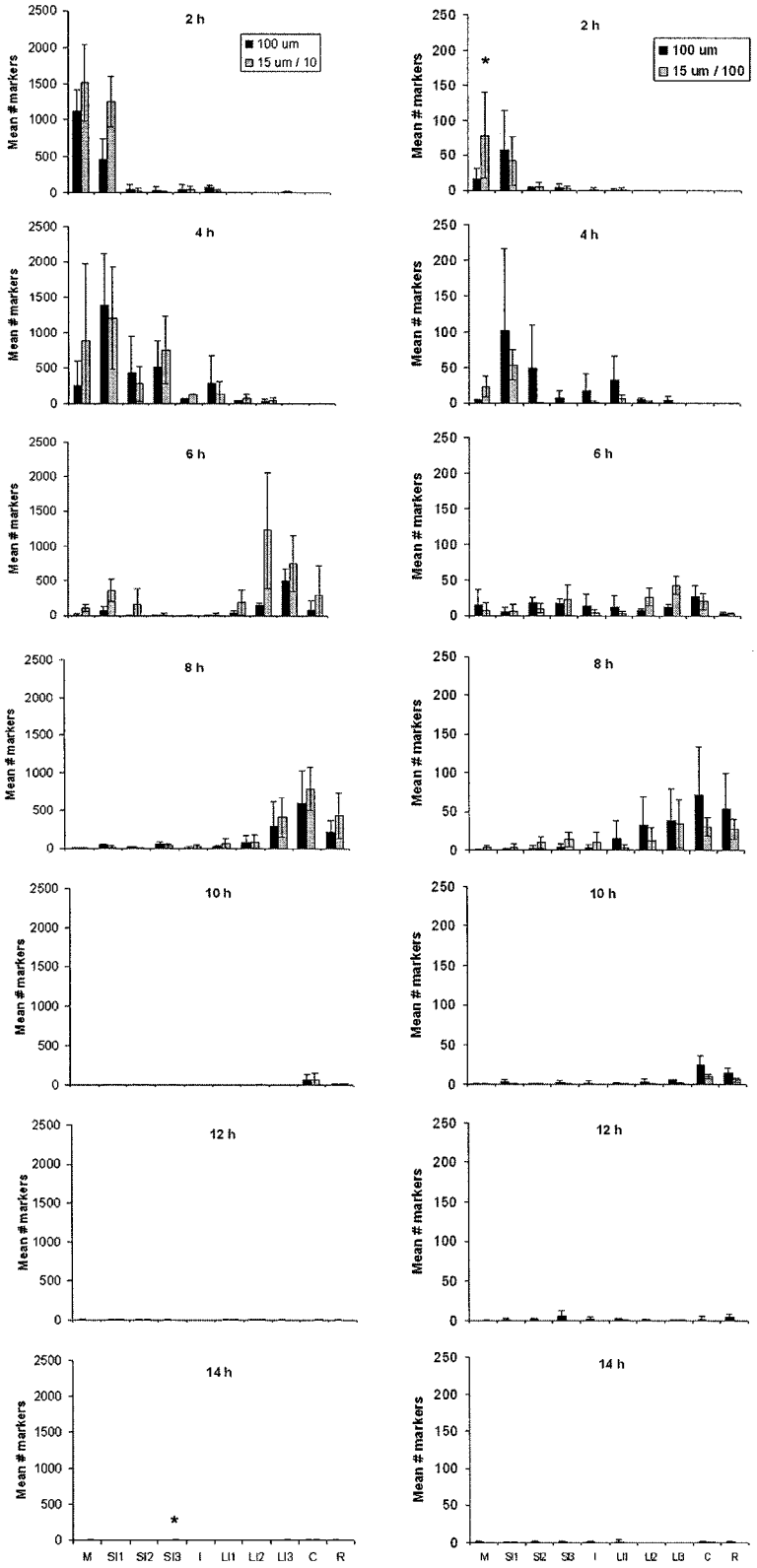

Figure 6. Digesta passage of two sizes of particulate markers in replicate groups of bullfrog tadpoles; $N = 18$ (*left*) and $N = 16$ (*right*). Postingestion time interval noted at the top of each plot. Dark bars represent the mean number of 100-μm markers and light bars represent the mean number of 15-μm markers in each gut region. To maintain the same scale (i.e., a shared Y-axis), the means for the 15-μm markers are divided by 100 and 10 for the initial and replicated trial, respectively. Error bars represent 1 SD. Significantly different pairwise contrasts (t-tests; $P < 0.05$) are indicated by asterisks.

between 10 and 14 h. There was a punctuated loss of microspheres between 8 and 10 h, during which period an average of 87% of markers from the previous time interval were defecated (Fig. 6). Mass-specific transit times, M_{50}, and gut clearance are presented in Table 3.

Gastrointestinal Fermentation

Active fermentation within bullfrog tadpoles was indicated by elevated concentrations of SCFAs in the colon relative to the other gut regions (Fig. 7; Table 1; repeated-measures ANOVA; $P < 0.001$ for all SCFAs). The molar ratio of the predominant SCFAs (acetate : propioniate : butyrate) in the colon was 56 : 31 : 13. In vitro fermentation yields for all SCFAs combined were highest in the colon; 1-h fermentation yields were 0.6, 3.3, 6.9, 7.8, and 5.8 mM of SCFAs h^{-1} for the manicotto glandularae, small intestine, posterior small intestine, colon, and rectum, respectively. These data indicate that fermentation is not restricted to the colon but also includes the posterior small intestine and rectum.

Estimated energetic contributions of symbiotic fermentation in the fermentation region (i.e., posterior small intestine, colon, and rectum combined) represent approximately 20% of the total daily energy requirement for a 3-g bullfrog tadpole (mean mass of the tadpoles used in fermentation analyses). Colonic fermentation alone would provide 0.0082 kJ d^{-1} or 8% of the total daily energy requirement. These estimates are based on an average daily energy requirement of 0.1056 kJ, as calculated from resting rates of oxygen consumption by bullfrog tadpoles at 23°C (Crowder et al. 1998; McNab 2002). Yields of SCFAs in the fermentation region over 24 h were calculated from the in vitro 1-h fermentation yields of individual SCFAs in the three gut regions comprising the fermentation region. Energetic equivalents of SCFAs were based on standard conversion factors (Weast 1985), and the resultant total energetic yield was 0.0210 kJ d^{-1}.

Coprophagy

The bullfrog tadpoles observed in this study were highly coprophagous. Grouped tadpoles spent 17.2% ± 2.5% of their time ($N = 18$) feeding on conspecific feces.

Discussion

The physiological traits we described in bullfrog tadpoles are consistent with those of herbivorous vertebrates that rely on hindgut fermentative digestion. These features include a lengthy, voluminous gut and an enlarged colon inhabited by dense populations of fermentative symbionts (reviewed in Stevens and Hume 1995). The relative gut length of laboratory-reared bullfrog tadpoles in this study was roughly 12 times SVL. This relative gut length is 1.5–8.4 times greater than those reported for 13 species of nonherbivorous anuran larvae (Altig and Kelly 1974). In another study, the average gut length of wild-caught bullfrog tadpoles was 15 times SVL (Pretty et al. 1995). Gut lengths of tadpoles are strongly correlated with food habits; herbivorous species have longer guts than omnivorous and carnivorous species (Altig and Kelly 1974), and within a species, individual tadpoles fed plant-based diets exhibit longer guts than conspecifics fed other diets (Alford 1999).

Table 2: Digesta passage of bullfrog tadpoles dosed with two sizes of inert, particulate markers

Marker Size	Mean (SE) Markers Ingested	Digesta Passage (h)		
		Transit Time[a]	M_{50}[b]	Gut Clearance[c]
100 μm:				
Trial 1	2,396 (485)	6	8–10	14
Trial 2	153 (66)	6	8–10	10
15 μm:				
Trial 1	31,833 (4,589)	6	8–10	10
Trial 2	10,855 (2,987)	6	8–10	12

Note. Spherical, solid latex microspheres (Bangs Laboratories, Fishers, IN); density = 0.95–1.05 g cm^{-3}.

[a] Time of first appearance of marker in the rectum of tadpoles.

[b] Median retention time: the time interval during which 50% of ingested markers remained in the gut.

[c] First time interval at which 5% or less of ingested markers remained in the gut.

Besides a lengthy gut, herbivores are also characterized by a relatively voluminous gut (Stevens and Hume 1995). Based on prediction equations presented by Parra (1978) for nonruminant herbivores, a 3-g hindgut fermenter is expected to have 0.19 g of total gut contents (6.4% of total body mass, on a wet matter basis), of which 0.11 g is within the fermentation region (3.7% of total body mass). The gut contents of bullfrog tadpoles conform closely to these predicted values. As calculated from prediction equations derived from tadpoles used in our study (Fig. 4), the gut contents of a 3-g bullfrog tadpole equal 0.23 g (7.7% of total body mass) and fermentative gut contents equal 0.10 g (3.3% of total body mass).

The colonic spiral in bullfrog tadpoles is anatomically similar to that of the herbivorous Scandinavian lemming (*Lemmus lemmus*; Sperber et al. 1983). In the lemming colon, digesta passage is slowed to allow sufficient time for symbiotic activity, and colonic bacterial populations are maintained by antiperistalsis and mucous entrapment mechanisms. In other hindgut fermenters, digesta passage is slowed and symbiotic populations are maintained by internal partitioning of the colon (e.g., in herbivorous iguanid lizards; Iverson 1982) or by diversion of digesta into a cecum (e.g., in many mammals and birds; Stevens and Hume 1995). In bullfrog tadpoles the colonic spiral, valve, contractions (antiperistalsis), and larger luminal diameter likely serve to delay the passage of digesta and main-

Table 3: Mass-specific digesta passage in bullfrog tadpoles compared to some representative herbivorous vertebrates

Taxon and species	Digesta Passage[a]	Mass-Specific Passage (h g^{-1})	Source
Amphibians:			
Bullfrog tadpole (*Rana catesbeiana*)	TT	2.53–2.83	This study
Bullfrog tadpole (*R. catesbeiana*)	M_{50}	3.38–4.72	This study
Bullfrog tadpole (*R. catesbeiana*)	GC	4.22–5.91	This study
Mammals:			
Horse (*Equus caballus*)	MRT	.0001	Stevens and Hume 1995
Cow (*Bos taurus*)	MRT	.0003	Stevens and Hume 1995
Goat (*Capra hircus*)	MRT	.001	Stevens and Hume 1995
Sheep (*Ovis aries*)	MRT	.001	Stevens and Hume 1995
Grey kangaroo (*Macropus giganteus*)	MRT	.001	Stevens and Hume 1995
Savanna baboon (*Papio cynocephalus*)	MRT	.004	Karasov et al. 1986
Blue monkey (*Cercopithecus mitis*)	MRT	.007	Karasov et al. 1986
Rabbit (*Oryctolagus cuniculus*)	MRT	.009	Stevens and Hume 1995
Vervet monkey (*Cercopithecus pygerythrus*)	MRT	.01	Karasov et al. 1986
Koala (*Phascolarctos cinereus*)	MRT	.02	Stevens and Hume 1995
Desert woodrat (*Neotoma lepida*)	MRT	.02–.04	Karasov et al. 1986
Sloth (*Bradypus tridactylus*)	MRT	.06	Foley et al. 1995
Hamster (*Mesocricetus auratus*)	MRT	.08	Stevens and Hume 1995
Rat (*Rattus norvegicus*)	MRT	.13	Stevens and Hume 1995
Snow vole (*Microtus nivalis*)	GC	.20	Karasov et al. 1986
Snow vole (*M. nivalis*)	TT	.02	Karasov et al. 1986
Townsend's vole (*Microtus townsendii*)	MRT	.22	Stevens and Hume 1995
House mouse (*Mus musculus*)	MRT	.23–.51	Karasov et al. 1986
Bank vole (*Clethrionomys glareolus*)	GC	.23	Karasov et al. 1986
Bank vole (*C. glareolus*)	TT	.005	Karasov et al. 1986
Birds:			
Emu (*Dromaius novaehollandiae*)	MRT	.0001	Stevens and Hume 1995
Ostrich (*Struthio camelus*)	MRT	.001	Stevens and Hume 1995
Ptarmigan (*Lagopus mutus*)	MRT	.004	Stevens and Hume 1995
Reptiles:[b]			
Yellowfoot tortoise (*Geochelone denticulata*)	TT	.02–.08	Bjorndal 1989
Galápagos land iguana (*Conolophus subcristatus*)	TT	.02–.04	Christian et al. 1984
Redfoot tortoise (*Geochelone carbonaria*)	TT	.03–.06	Bjorndal 1989
Gopher tortoise (*Gopherus polyphemus*)	TT	.04–.08	Bjorndal 1987
Green iguana (*Iguana iguana*)	TT	.09–.24	van Marken Lichtenbelt 1992
Loggerhead sea turtle (*Caretta caretta*)	GC	.10–.22	Birse and Davenport 1987
Green iguana (*I. iguana*)	MRT	.10–1.90	Karasov et al. 1986
Chuckwalla (*Sauromalus obesus*)	MRT	.42	Karasov et al. 1986
Green sea turtle (*Chelonia mydas*)	TT	.64–1.20	Davenport et al. 1989
Desert iguana (*Dipsosaurus dorsalis*)	MRT	1.19	Karasov et al. 1986
Desert tortoise (*Xerobates agassizii*)	TT	.12–2.24	Meienberger et al. 1993
Fish:[b]			
Gizzard shad (*Dorosoma cepedianum*)	TT	.74	Salvatore et al. 1987

Note. Not intended to be an exhaustive list.

[a] Digesta passage of particulate markers, expressed as transit time (TT), median retention time (M_{50}), gut clearance (GC), or mean retention time (MRT).

[b] For ectothermic herbivores, ambient temperature ranged from 20° to 36°C.

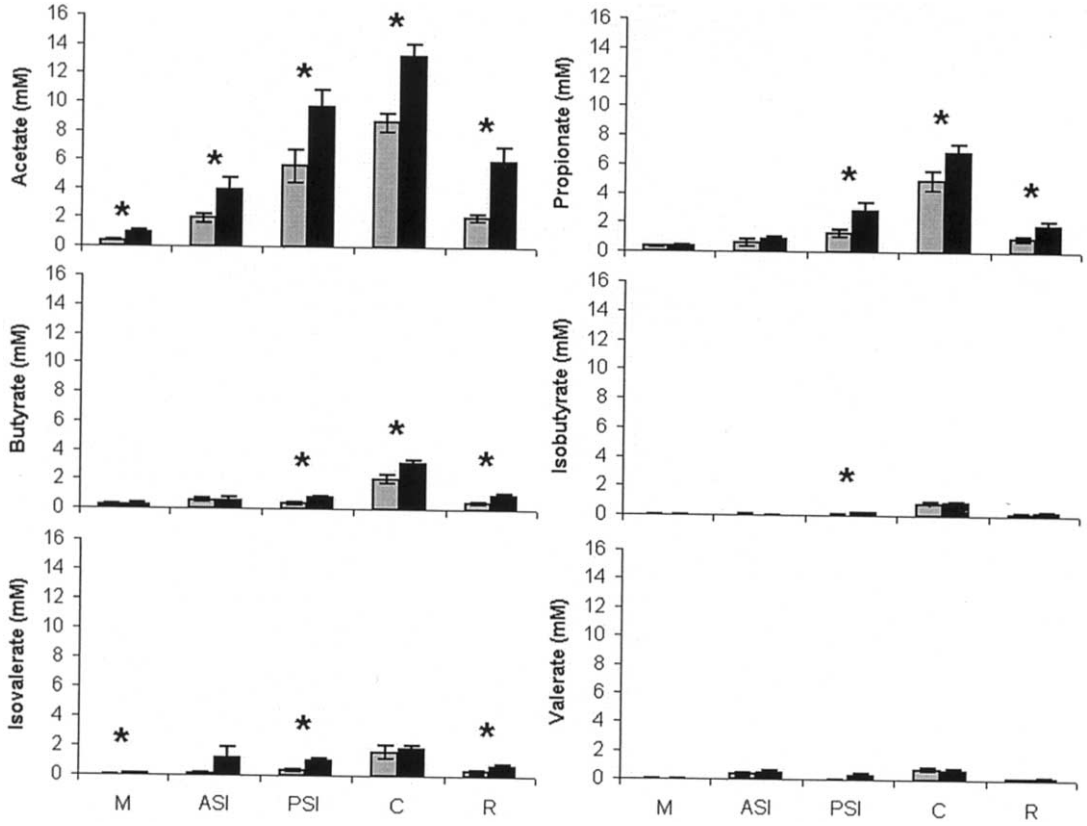

Figure 7. Concentrations of short-chain fatty acids (SCFAs) acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate in distinct regions of the bullfrog tadpole gastrointestinal tract. Gut regions: *M* = manicotto; *ASI* = anterior small intestine; *PSI* = posterior small intestine; *C* = colon; and *R* = rectum. Concentrations expressed as mean molarity of SCFAs in gut fluid, in millimoles per liter (mM). Error bars represent 1 SE. Light bars represent samples at time zero, and dark bars represent samples after 1 h in vitro fermentation (for each set of samples, $N = 5$ pooled samples per gut region). Significant differences between time-zero and 1-h in vitro samples for a given gut region are indicated by asterisks (*t*-tests; $P < 0.05$), indicating active fermentation.

tain gut symbionts in this region. The distinct symbiotic distribution observed in the bullfrog tadpole colon (i.e., localized in a mucous matrix lining the epithelial surface) has also been reported in other species of tadpoles (Battaglini and Boni 1967; Viertel and Richter 1999) and a herbivorous fish (Fishelson et al. 1985) and may represent another mechanism by which symbionts are maintained in the gut.

The pH and the endemic symbiotic community of the bullfrog tadpole colon are also similar to those of other hindgut fermenters. The colonic pH is near neutral, and there are elevated levels of SCFAs in this region relative to other sections of the gut. Fermentative gut regions in other herbivores are also maintained around a neutral pH (Table 1; reviewed in Stevens and Hume 1995). A complex endemic symbiotic community, including host-specific ciliates and nematodes, is widespread among ectothermic herbivores and has been described in herbivorous iguanid lizards (ciliates: Iverson 1982; Garza and Hernandez 1986; nematodes: Nagy 1977; Iverson 1982), turtles (ciliates and nematodes: Bjorndal 1997), and fish (ciliates: Fishelson et al. 1985; Clements et al. 1989; Clements 1997; nematodes: T. Platt and D. Brooks, personal communication). The function of nematodes inhabiting the fermentative gut regions of ectothermic herbivores remains undescribed, but several researchers have suggested a mutualistic relationship between these symbionts and their hosts (Nagy 1977; Iverson 1982; Bjorndal 1997; Pryor and Greiner 2004).

Transit times of digesta in bullfrog tadpoles in this study (6 h at 29°C) were similar to those reported for *Rana temporaria* tadpoles (4.75–8 h at 16°–18°C; Savage 1952) and those reported previously for bullfrog tadpoles (4.25–10 h at an unspecified temperature; Wassersug 1975). However, transit times in our study were much longer than those reported by Altig and McDearman (1975) for bullfrog tadpoles (1.68 h at 22°C and 0.65 h at 30°C). Steinwascher (1978*a*) suggested that the digesta passage reported in the latter study was underestimated due to the methods employed. Indeed, the very rapid passage reported in that study may have been due to dosing techniques: markers were not ingested in a food bolus but were orally

filtered from an aqueous suspension by the tadpoles. In addition, Altig and McDearman (1975) did not specify whether the tadpoles consumed any food or if their feeding behavior was altered after ingesting the markers. The influence of feeding regime and environmental factors on passage can be considerable. For example, Wassersug (1975) showed that digesta passage in well-fed bullfrog tadpoles (4.25–10 h) is much shorter than in tadpoles fed on an irregular basis (44 h). Warkentin (1992) also demonstrated significant variation in digesta passage among *Rana clamitans* tadpoles in response to microhabitat differences. Such variation was observed in both wild and laboratory settings.

Savage (1952) discounted the possibility of symbiotic fermentation in tadpoles because he considered their digesta passage prohibitively rapid. However, similarly rapid overall digesta passage has been described for other herbivorous hindgut fermenters such as emus (*Dromaius novaehollandiae*: MRT = 6 h; Herd and Dawson 1984), pheasants (*Phasianus colchicus*: MRT = 5 h; Duke et al. 1968), ptarmigans (*Lagopus mutus*: MRT = 2 h; Gassaway et al. 1975), rabbits (*Oryctolagus cuniculus*: transit time = 5 h; Garcia et al. 2002), hamsters (*Mesocricetus auratus*: MRT = 9 h; Sakaguchi et al. 1987), five species of small mammals (MRT = 1.5–5 h; Karasov et al. 1986), and many species of herbivorous fish (2–10 h; reviewed in Clements 1997). In these herbivorous species, retention times similar to those of tadpoles have not precluded active symbiotic fermentation and the maintenance of a fermentative symbiotic community.

When interpreting digesta passage, the influence of body size should also be considered (Meienberger et al. 1993; Clements 1997; McNab 2002). Despite rapid overall digesta passage in tadpoles, their mass-specific digesta passage is extremely slow compared to most herbivorous vertebrates that have been examined (Table 3). Unfortunately, body mass data are not available for the other species of tadpoles for which digesta passage has been investigated (Savage 1952; Altig and McDearman 1975; Wassersug 1975). Therefore, comparisons between the mass-specific digesta passage of bullfrog tadpoles and tadpoles with other feeding strategies await the results of further research.

Active symbiotic fermentation in bullfrog tadpoles in this study was indicated by relatively high levels of SCFAs, elevated in vitro 1-h fermentation yields, and the presence of fermentative symbionts in the posterior small intestine, colon, and rectum. This is the first account of gastrointestinal fermentation in the class Amphibia. Concentrations of SCFAs in the bullfrog tadpole colon match or exceed those of some herbivorous mammals, birds, reptiles, and fish (Table 1).

Fermentation in bullfrog tadpoles is not limited to the colon but also occurs in the posterior small intestine and rectum. Similarly, in Florida red-bellied turtles (*Pseudemys nelsoni*), significant energetic contributions are made by an extensive gastrointestinal fermentation that spans from the small intestine to the posterior colon (Bjorndal and Bolten 1990). In bullfrog tadpoles, evidence of active fermentation in the posterior small intestine and rectum included higher concentrations of SCFAs in these gut regions relative to the manicotto and anterior small intestine. However, because high concentrations of SCFAs in the posterior small intestine and rectum could represent reduced absorption rates, rather than increased fermentation activity, the in vitro rates of SCFA production among gut regions must be considered. The 1-h fermentation yields indicated increased fermentation in the posterior small intestine and rectum relative to the manicotto and anterior small intestine, independent of SCFA absorption rates. The occurrence of ciliates and nematodes—symbionts associated with the fermentative gut regions of other herbivores—in the posterior small intestine and rectum of bullfrog tadpoles also supports the assertion that fermentation is not restricted to the colon. The energetic contributions of fermentation in the posterior small intestine, colon, and rectum of bullfrog tadpoles equal 20% of their daily energy requirements, an estimate in the middle of the range reported for mammalian hindgut fermenters (i.e., 5%–39%: Parra 1978; Stevens and Hume 1995; Hume 1997), higher than the range for avian hindgut fermenters (i.e., 4%–11%: Stevens and Hume 1995), and at the low end of the range reported for reptilian hindgut fermenters (i.e., 15%–100%: Bjorndal and Bolten 1990; Stevens and Hume 1995).

The molar proportion of the three predominant SCFAs (acetate : propionate : butyrate) produced in the bullfrog tadpole colon (56 : 31 : 13) is near that of ruminants fed high-quality, concentrate-based diets (50 : 40 : 10, Owens and Goetsch 1988; 53 : 34 : 13, Bergman 1990). Compared to the SCFA ratios in various species of mammalian hindgut fermenters (70 : 20 : 10, Bergman 1990) and in ruminants fed forage-based diets (65 : 25 : 10, Owens and Goetsch 1988; 69 : 20 : 11, Bergman 1990), the molar proportion of SCFAs in bullfrog tadpoles is shifted toward an increased production of propionate. In ruminants, such shifts result from the proliferation of different symbiotic populations that occur in response to altered diets, digesta passage, pH, food particle size, and other factors (Owens and Goetsch 1988; Van Soest 1994). Elevated gastrointestinal propionate levels are generally associated with a reduction in dietary cellulose and hemicellulose and a corresponding increase in highly fermentable carbohydrates (Owens and Goetsch 1988; Bergman 1990; Van Soest 1994). Relatively high propionate production is energetically advantageous to the host because propionate is the only major SCFA that is glucogenic (Bergman 1990).

An important behavioral trait—coprophagy—is also shared between bullfrog tadpoles and many small-bodied hindgut fermenters. Prevention of coprophagy in some herbivores results in reduced weight gains and major changes in their gastrointestinal symbiont populations (reviewed in Hornicke and Bjornhag 1980). In accord with these observations, Steinwascher (1978*b*) showed that bullfrog tadpoles are copropha-

gous and exhibit reduced growth rates when denied access to their feces. In *Rana pipiens* tadpoles, not only was growth inhibited by prevention of coprophagy, but feces deposited after reingestion had lower energy content than feces deposited after a single passage through the gut (Gromko et al. 1973). These studies indicate that herbivorous tadpoles, like many other hindgut fermenters, benefit from nutritional gains associated with coprophagy. Whether tadpoles produce distinct fecal pellets for reingestion (e.g., cecotrophs; Stevens and Hume 1995) has yet to be examined.

Conclusions

The morphological, physiological, and behavioral characteristics of herbivorous bullfrog tadpoles parallel those of a diverse assemblage of other herbivores. The active hindgut fermentation we described in these tadpoles is the first such account for the class Amphibia. As observed in other ectothermic herbivores (Nagy 1977; Iverson 1982; Bjorndal 1997), bullfrog larvae harbor host-specific nematodes in their fermentative gut regions. Further research is needed to elucidate the roles of these symbionts in the digestive processing of their herbivorous hosts.

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