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Effects on Campostoma oligolepis Digestive Morphology and Gut Microbiota Composition across a Gradient of Urbanization

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Effects on *Campostoma oligolepis* Digestive Morphology and Gut Microbiota Composition across a Gradient of Urbanization

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ABSTRACT

Campostoma oligolepis, the Largescale Stoneroller, is a species of minnow native to northwest Georgia. This study investigated the impacts of urbanization on gut length and gut microbiota community composition in *C. oligolepis* from four small watersheds within the Etowah River drainage basin with different levels of impervious surface coverage (ISC). Individuals were collected from each sample site in the summer of 2016 between late July and early September. A second round of sampling was conducted in the fall between mid-October and mid-November. The gut length to total length ratio was calculated for each individual to allow for an unbiased comparison of gut lengths between individuals of varying sizes. Additionally, gut microbiota communities from summer individuals' fore- and hindguts were identified using 16S metagenomic sequencing on an Ion Torrent platform. Summer individuals from watersheds with lower ISC had significantly longer gut lengths than individuals from watersheds with higher ISC. However, no significant difference in relative gut length was found between sample sites for the individuals collected in fall. A significant decrease in relative gut length of individuals from sites with lower ISC was observed from summer to fall. Sequence data from the summer individuals revealed the presence of 36 bacterial phyla and 1,008 genera in *Campostoma* gut contents. Overall, Proteobacteria and Firmicutes accounted for the greatest percentage of the attributable reads. *Pseudoalteromonas*, *Acinetobacter*, and *Blautia* were the three most prevalent genera across sites and between gut regions. Nonmetric multi-dimensional scaling analysis using the 22 bacterial genera that accounted for the highest proportion of sequences across all sites revealed some grouping of samples based on site. However, distinct patterns in bacterial composition according to gut region or ISC by site were not apparent. These findings suggest that *Campostoma* may not rely on symbiotic bacteria to aid in digestion.

BACKGROUND

Impacts of Urbanization on the Stream Environment

Anthropogenic effects on the environment like urbanization alter lotic freshwater systems. Reduction of the riparian zone and increased levels of impervious surface coverage (ISC) within urban watersheds impact stream temperature, substrate composition, resource availability, and nutritional quality of resources (Paul and Meyer 2001). Habitat alteration in urban streams reduces biodiversity, including the diversity of fishes. Sensitive species with specific habitat requirements tend to be reduced or eliminated entirely from a system, while more tolerant species and habitat generalists persist.

One effect of urbanization is the alteration of epilithic microbial communities in streams (Paul and Meyer 2001, Lear and Lewis 2009), which causes a change in food composition and availability for grazers in these systems. Bacteria, specifically coliform, nitrifying, and iron-oxidizing bacteria, tend to be much more abundant in urban streams compared to rural streams (Paul and Meyer 2001). Increased bacterial growth at urban sites can be attributed in part to increased stream temperature. Reduction of the riparian zone reduces shaded areas within the stream and runoff from impervious surfaces directly into the stream both increase overall water temperature in impacted systems (Paul and Meyer 2001).

Algal composition can also be altered within urbanized systems. Accumulation of silt reduces available stable substrate necessary for algal growth by covering and replacing existing substrate like gravel, cobble, or even boulder and bedrock. Reduction of available stable substrate, along with increased turbidity, has been shown to limit epilithic accumulation of algae (Paul and Meyer 2001). In contrast, reduced canopy cover and increased nutrients in urban streams can result in increased epilithic biomass with reduced short chain fatty acid (SCFA)

content (Cashman et al. 2013, Guo et al. 2015). Although there might be more biomass present for consumption by grazers in urban streams where stable substrates are not reduced, the resources that are available are of a lower nutritional quality.

Campostoma oligolepis *and Urbanization*

Urbanization alters fish assemblages favoring more tolerant species. *Campostoma oligolepis*, the Largescale Stoneroller, and other species within the genus *Campostoma* are considered tolerant (Roy et al. 2005). As such, *Campostoma* are prevalent in both urban and rural streams. Despite being a tolerant species, there are a number of aspects of *C. oligolepis* life history that should be negatively impacted by urbanization. Boschung and Mayden (2004) describe *C. oligolepis* as needing shallow, clear, gravel-bottomed streams for spawning activities like nest-building. Additionally, *C. oligolepis* has been characterized as an herbivorous grazer feeding on the epilithic algal and bacterial populations growing on the surface of stable substrates (Etnier and Starnes 1993, Boschung and Mayden 2004).

With these aspects of *Campostoma* life history and ecology in mind, it is surprising that *C. oligolepis* are successful in impacted systems where stable substrate and suitable nesting habitat are reduced. However, *Campostoma* seem to thrive in these conditions. Recent studies investigating the life history and digestive morphology of *C. oligolepis* suggest that increased ISC is linked to distinct shifts in digestive morphology and growth rates of *C. oligiolepis* in the Etowah drainage basin of northern Georgia (South and Ensign 2013, Mutchler et al. 2014).

South and Ensign (2013) found that urban *C. oligolepis* have an extended spawning season with spawning in urban streams beginning a month earlier than in rural systems. This study also found that urban *Campostoma* had higher growth rates. Due to earlier spawning and increased growth in urban systems, urban young-of-year (YOY) reach a larger size by the onset

of winter as compared to rural YOY. These findings suggest that *Campostoma* YOY in the urban systems have a much better chance of surviving through the winter.

Mutchler et al. (2014) found that *C. oligolepis* in watersheds with higher levels of ISC had significantly shorter digestive tracts than their rural counterparts. Differences in fish intestinal length have been used as indicators of dietary quality (Elliot and Bellwood 2003, Wagner et al. 2009). Longer gut lengths are linked to diets of low nutritional quality, while shorter gut lengths are tied to diets of increased nutritional quality (Ribble and Smith 1983, Horn 1989, Elliot and Bellwood 2003, Wagner et al. 2009). This suggests a difference in resources quality for *C. oligolepis* from urban and rural sites. Specifically, the shorter gut lengths observed in urban individuals suggest that individuals in watersheds with increased ISC have access to resources that are of a higher nutritional quality and are easier to digest.

Overall, urban *Campostoma* have increased growth rates and increased survival through the winter as compared to rural *Campostoma*. Differences in digestive morphology between urban and rural individuals also suggests that urban *Campostoma* have access to resources that are of a higher nutritional quality. I will be investigating these observed differences between urban and rural *C. oligolepis* by looking at differences in gut length as well as differences in gut microbiota composition across a gradient of urbanization within the Etowah River drainage basin. Gut microbiota composition like gut length can give some insight into differences in diet and resource availability (Clements et al. 2014, Larsen et al. 2014a, Li et al. 2014). Investigating differences in gut microbiota will also allow for a rough environmental assessment of the microbes present in the different streams. By looking at both gut length and gut microbiota composition, I hope to further our understanding of the differences in biology and digestive morphology observed in urban and rural *C. oligolepis*.

CHAPTER I: Digestive Morphology and Condition of *Campostoma oligolepis* across Gradient of Urbanization

INTRODUCTION

Campostoma oligolepis, a species of freshwater minnow, has been classified as an herbivorous grazer (Etnier and Starnes 1993, Boschung and Mayden 2004). Using its cartilaginous lower lip, *C. oligolepis* feeds on epilithic communities growing on stable substrates in streams. *C. oligolepis* possess comparatively long digestive tracts consisting of an undifferentiated intestine coiled around the swim bladder. This elongated intestine increases retention time of digesta to maximize digestive efficiency. However, distinct differences in digestive morphology have been noted between *Campostoma* from sites with high and low levels of impervious surface coverage (ISC). Individuals from urban sites have significantly shorter gut lengths than rural individuals (Mutchler et al. 2014). One potential explanation for this observed difference in gut length is that *Campostoma* from urban and rural sites have access to food sources that differ in nutritional quality.

Nutritional Quality of Food Sources and Impacts on Gut Length

Nutrient composition, energy content, and digestibility determines the overall quality of a food source (Wagner et al. 2009), and thus dictate how the resources should be processed by the organism. Overall quality can be described in a number of ways including elemental composition (Sterner and Elser 2002), refractory material content (Karasov and Douglas 2013), and fatty acid content (Cashman et al. 2013, Guo et al. 2015). Elemental ratios, specifically the ratio of C:X where X is a specific element of interest (usually nitrogen or phosphorus), represent the nutrient content of a given food source. Higher portions of X indicate higher nutrient quality (Sterner and Elser 2002). The amount of refractory material present in the diet also impacts overall quality.

Refractory materials are resilient to digestion via enzymatic activity making them difficult to process (Karasov and Douglas 2013). Food sources with reduced levels of refractory material, like animal material, are more easily digested and are therefore considered to be of a higher nutritional quality (Karasov and Douglas 2013). Finally, nutritional quality can be assessed by looking at the concentration of short chain fatty acid (SCFA) which are required for animal growth and reproduction (Cashman et al. 2013, Guo et al. 2015). Foods with increased SCFA content are considered to be of a higher nutritional quality.

Animal material requires less digestive effort than plant material due to its increased nutritional quality, decreased refractory material, and increased fatty acid concentrations (Horn 1989, Wagner et al. 2009, Karasov and Douglas 2013). These differences in nutritional quality require different digestive approaches. As such, fishes with differing dietary preferences have differing gut morphologies that best suit digestion of their primary food source (Horn 1989). Individuals that consume foods with high fiber and low nutrients, like *C. oligolepis*, have longer intestinal tracts to optimize digestion and assimilation of nutrients (Horn 1989). In contrast, carnivorous species tend to have shorter intestines due to their high quality, easy-to-digest diets with higher concentrations of proteins and fats (Horn 1989, Elliot and Bellwood 2003, Karasov and Douglas 2013). Many studies have investigated the effects of diet on intestinal length in fishes and found that herbivorous and omnivorous species have longer gut lengths than carnivorous species (Ribble and Smith 1983, Horn 1989, Elliot and Bellwood 2003, Wagner et al. 2009). Further, Elliot and Bellwood (2003) concluded that intestinal length in fishes was a reliable indicator of dietary quality.

Working under the assumption that gut lengths are different in response to dietary nutritional quality, we can go beyond comparing herbivorous and carnivorous diets. Variation in

gut length occurs within groups of herbivores based on the relative nutritional quality of available food sources. Wagner et al. (2009) found that the intestinal length in a species of algal-browsing cichlid varied greatly among populations. These differences in gut length were well correlated with the algal nutrient content. A C:N ratio was used as the measure of nutritional quality with higher portions of N correlating to a higher quality food source (Wagner et al. 2009). While all of the cichlid populations were herbivorous, the nutritional quality of the algae available to specific populations shifted resulting in different nutrient availability between populations. Populations in areas with algae of higher nutritional quality were found to have shorter gut lengths (Wagner et al. 2009). The results from this study are consistent with the Elliot and Bellwood (2003) conclusion that intestinal length is a useful indicator of dietary quality.

We can use this information and apply it to what has been observed in urban and rural *C. oligolepis* gut length. Urban *Campostoma* have significantly shorter gut lengths than rural individuals (Mutchler et al. 2014). A follow-up study conducted in 2013 also found significantly shorter gut lengths in *C. oligolepis* from more urbanized watersheds (Schooling and Ensign 2014). These findings suggest that urban *Campostoma* have access to resources that are of a higher nutritional quality compared to rural *Campostoma*. I will be comparing the gut lengths of *C. oligolepis* collected from four watersheds with varying levels of ISC. I predict that individuals collected from sites with increased ISC (urban sites) will have significantly shorter gut lengths than individuals from watersheds with lower ISC (rural sites). Further, if shorter gut lengths are indicative of increased quality of diet, I predict that individuals with shorter gut lengths will be in better condition as measured by their condition factor (K) (William 2000).

METHODS

Sample Sites

Campostoma were collected from four small watersheds (wastershed area <15km^2) within the Etowah River drainage basin in northern Georgia with varying levels of impervious surface cover (ISC). One urban site, two intermediate sites, and one rural site were chosen for this study. The sites span from Cobb County, GA, just north of Atlanta to Paulding County, GA, west of Atlanta (Figure 1.1).

The urban collection site is a tributary of Noonday Creek in Cobb County, Georgia. This site, from here on referred to as Rockbridge Creek (34.0217N, 84.5442W), has ISC in 29.84% of its total area. The first of the intermediate collection sites, Lawrence Creek (33.9638N, 84.8356W), is a tributary of Little Pumpkinvine Creek in Paulding County, Georgia. Approximately 5.98% of its area is covered by impervious surface. It should also be noted that this site is located on the property of a water treatment facility. The second intermediate collection site, Possum Creek (34.0122N, 84.7910W), is also a tributary of Little Pumpkinvine. It is located in Paulding County, Georgia with 6.91% of its area covered by impervious surface. Finally, the rural site selected for this study is Raccoon Creek (33.9689N, 84.9315W). Located in Paulding County, Georgia, this site has impervious surface covering 0.64% of its total area.

Sample Collection

Campostoma were sampled from each sample site using a backpack electroshocker between mid-July and early September 2016. A second round of sampling took place in fall 2016 between mid-October and mid-November at the Rockbridge, Possum, and Raccoon Creek sites. At least ten individuals were collected from each sample site during each sample period.

Campostoma were transported alive back to the lab. Fish were euthanized in lab with an overdose of tricaine methanesulfonate (Larsen et al. 2014a, Li et al. 2014). Individuals were

weighed and their total body length (TL) measured. The whole, intact gut of each individual was then removed aseptically, and the length of the gut was measured to the nearest mm.

Data Analysis

In order to allow for an unbiased comparison of *Campostoma* gut lengths for fish of different sizes, relative gut length ratios were calculated by dividing gut length by TL. Kruskal-Wallis tests were then run in R to compare mean relative gut length among sites for the summer and fall data. Post-hoc Nemenyi-tests for multiple comparisons with Tukey-distribution approximation of independent samples were also conducted in R. Additionally, 2-tailed t-tests assuming equal variance were conducted to compare relative gut length from each site between summer and fall ($\alpha = 0.05$).

Condition factor (K) was calculated for each individual using the following formula (William 2000):

$$K = (10^5)\left(\frac{weight}{TL^3}\right)$$

Condition factors were compared between sites for summer and fall using Kruskal-Wallis tests in R with post-hoc Nemenyi-tests. In order to assess if any significant shift in condition occurred from summer to fall at any of the sites, 2-tailed t-tests assuming equal variance were conducted ($\alpha = 0.05$).

An additional Kruskal-Wallis test with post-hoc Nemenyi-tests were run in R to compare the relative gut lengths from the 2013 study to the summer data collected for this study. The 2013 study was also conducted in the summer at sites with similar watershed area and ISC to the sites used for this study. The three watersheds used in the 2013 study were Little Noonday, Little Pumpkinvine, and Upper Raccoon. Little Noonday was the most impacted site and is comparable

to Rockbridge Creek. Little Pumpkinvine serves as an intermediate site and is comparable to Lawrence and Possum Creek. The least impacted site, Upper Raccoon, is most comparable to Raccoon Creek. It should also be noted that summer 2016 data from Lawrence and Possum were pooled together to form the "Pumpkinvine" data set. This consolidation allowed for better comparison to the 2013 study.

RESULTS

Gut Length Comparison

Mean total length (TL) and weight for summer individuals was 106.1 mm and 9.9 g, respectively (Table 1.1). Fall individuals were found to be smaller in both length and weight, with a mean TL of 84.7 mm and weight of 5.3 g. Mean gut length (GL) for summer individuals was 417.2 mm. Fall individuals also had shorter gut lengths with a mean GL of 322.7 mm (Table 1.2). It should also be noted that there was a wider range of relative gut lengths for summer individuals as compared to fall individuals.

A significant difference was observed between the relative gut lengths for the summer collection period (KW test, $p = 0.00045$, Figure 1.2a). Individuals from Possum Creek, one of the intermediate sites, were shown to have significantly longer guts relative to body size than both Rockbridge (Nemenyi test, $p = 0.0017$), the most impacted site, and Lawrence, the other intermediate site (Nemenyi test, $p = 0.0122$). Individuals from Raccoon Creek, the least impacted site, also had significantly longer gut lengths than individuals from Rockbridge Creek (Nemenyi test, $p = 0.0285$).

While significant differences in relative gut length between sites were observed for the summer individuals, no significant difference was observed between any of the sample sites for

the fall sampling period (Figure 1.2b). In fact, there was a significant decrease in relative gut lengths of individuals in Possum and Raccoon from the summer to the fall (Table 1.3).

The relative gut lengths from the summer collections were found to be fairly consistent with the 2013 study (Figure 1.3). The 2013 data found significantly longer relative gut lengths in Little Pumpkinvine and Upper Raccoon, the intermediate and rural sites, as compared to Noonday, the most impacted site. Additionally, no significant differences were observed between the most comparable sites from the 2013 and 2016 studies. Rockbridge and Noonday, Pumpkinvine and Little Pumpkinvine, Raccoon and Upper Raccoon all had very similar relative gut lengths.

The condition factors for individuals across sites were fairly consistent for the summer (Figure 1.4a) and fall samples (Figure 1.4b). The only exception was the Possum summer sample which was significantly greater than all other sample sites ($p<0.0005$). There is also a significant decrease in K for Possum between summer and fall (Table 1.3). Overall, K values for fall sampling seemed to be more uniform overall, whereas the K values for summer showed more variation between sites.

DISCUSSION

The summer data were consistent with previous studies and suggested that *C. oligolepis* from more impacted sites have significantly shorter gut lengths than *C. oligolepis* from rural sites. However, this trend appears to hold true only for the summer months as the fall data were not consistent with the summer data or with previous studies conducted in the summer. In fact, a significant decrease in gut length was observed in Raccoon and Possum from summer to fall. This seasonal shift in gut length could be due to changes in resource availability and

composition. Changes in digestive morphology in response to seasonal shifts in resource availability is a phenomenon known as adaptive modulation (Whelan et al. 2000).

Adaptive Modulation of Digestive Morphology

Variation in gut length in accordance with the nutritional quality of a food sources is necessary for efficient digestion (Horn 1989). Digestive tissues are one of the most energetically demanding types of tissue, thus maintaining unnecessarily high levels of digestive tissue can result in an energy deficit (Cant et al. 1996). Fishes have adapted to keep the energetic demands of digestion in balance by shortening or lengthening their gut in response to shifts in nutrient availability and quality (Horn 1989, Whelan et al. 2000).

The seasonal shifts in *Campostoma* gut length from summer to fall suggest that the resources available to them are more easily digestible or of a higher nutritional quality in the fall. With the exception of Rockbridge Creek which showed no significant change, there were significant decreases in the relative gut length from the summer to fall at all sites. A potential explanation for this change could be increased microbial activity in the streams with the increased input of organic matter from falling leaves. The increased rate of decomposition as a result of microbial activity could have provided the *Campostoma* with food sources that were already partially processed, thus making them easier to digest (Paul and Meyer 2001).

Condition Factor and Digestive Morphology

Despite the seasonal discrepancies in gut length, there was a significant difference in gut length observed between urban and rural *Campostoma* for the summer sampling period. These findings suggest that *C. oligolepis* in more urbanized environments have access to food that is of a higher quality and is more easily digestible (Elliot and Bellwood 2003, Wagner et al. 2009). In conjunction with access to higher quality food, higher condition factors were expected for

Campostoma in more urban systems. However, this was not reflected in the findings. No significant difference in condition factor was observed between any of the sites with the exception of Possum Creek which had a significantly higher K value than any other site. This significant difference in K between Possum Creek in summer and individuals from the other three sites is likely due to differences in the time of capture. Possum summer individuals were collected in early September so they had more time to recover from the spawning season. Individuals from other sites were captured closer to the spawning season. Similarly, the reduced amount of variation in K values in the fall might also be attributed to the time of capture. The fall individuals were collected within one month of one another, between mid-October and mid-November. Summer individuals were collected between mid-July and early September. As such, fall individuals were collected from sites under similar environmental conditions whereas there would have been more variation in environmental conditions for the summer collections.

Genetic Considerations and Phylogenetic Constraints on Adaptive Modulation

Although the differences in gut length observed in *C. oligolepis* might point to differences in resource availability, genetic and phylogenetic influences need to be considered. Adaptive modulation is one potential explanation for this difference, but it is possible that these differences are being observed due to differences in the populations' genetic makeup. Differences in gut length between urban and rural populations might be a result of genotypic differences resulting in a difference in phenotype.

It should also be noted that organism morphology is constrained by phylogeny. Even if there is a significant shift in resource availability for a population, whether it is seasonal or something more permanent, overall gut morphology will remain relatively constant—it cannot change completely due to a sudden lack or abundance of nutrients. A number of studies have

found that phylogenetic constraints influence the level and significance of adaptation in fishes' guts, even when the fishes are faced with a significant shift in diet (Elliot and Bellwood 2003, German and Horn 2006, German et al. 2010a). This is not to say that no adaptation or shift in gut morphology can be observed. In fact, there is a surprising amount of phenotypic plasticity demonstrated in gut morphology of fishes (Elliot and Bellwood 2003, Wagner et al. 2009, German et al. 2010a, German et al. 2010b). Due to these phylogenetic constraints and genetic considerations, gut morphology might not tell us everything we need to know about nutritional quality of food sources being exploited by *Campostoma* though it gives some indication of what differences might exist. Investigating other aspects of *C. oligolepis* digestion like gut microbiota composition and nutritional quality of food sources may help increase our understanding of why we observe these differences in relative gut length.

CHAPTER II: Gut Microbiota Composition as an Indicator of Dietary Differences in *C. oligolepis*

INTRODUCTION

Herbivorous organisms face difficulties in breaking down and absorbing nutrients from their food sources. These difficulties can be attributed in large part to an increased abundance of refractory materials in their diet. Refractory materials, like cellulose and chitin, are compounds that cannot be broken down by endogenous enzymes (Karasov and Douglas 2013). Foods with higher abundance of refractory materials are consider to be of a lower quality as they are difficult to process and digest. Herbivores employ a number of different strategies in order to efficiently digest lower quality food sources. These strategies include but are not limited to having increased gut length, increased retention time of digesta, increased reaction rates of digestive enzymes, and increased diversity and abundance of symbiotic gut microbiota (Karasov and Douglas 2013). While all of these components are necessary for efficient digestion, this chapter will focus specifically on gut microbiota.

Many herbivores, including some fishes, house extensive communities of gut microbes that produce enzymes which aid in the breakdown of refractory materials (Karasov and Douglas 2013, Clements et al. 2014). These gut microbial communities are generally housed in the hindgut, the distal-most region of the gut (Karasov and Douglas 2013, Clements et al. 2014). Further, specific feeding habits of fishes influence the specific bacterial genera present in these communities. For example, some herbivorous species have been found to have increased levels bacterial genera like *Aeromonas*, *Clostridium*, *Acinetobacter*, and *Pseudomonas* which are known enzyme-producers, as well as *Brevundimonas*, *Leuconostoc*, and *Anoxybacillus* which are cellulolytic (Li et al. 2014).

Campostoma oligolepis is an herbivorous species of freshwater minnow that is native to northwestern Georgia (Etnier and Starnes 1993, Boschung and Mayden 2004). *C. oligolepis* feed on the epilihtic communities of algae, bacteria, and fungi that grow on the surface of stable substrates in streams utilizing their cartilaginous lower lip (Etnier and Starnes 1993, Boschung and Mayden 2004). As such, *C. oligolepis* employ some of the herbivore strategies discussed above to maximize digestive efficiency. *Campostoma* have long guts that coil around the swim bladder. However, studies have shown that *C. oligolepis* in urban streams with higher levels of impervious surface coverage (ISC) have significantly shorter gut lengths than individuals from rural sites with lower levels of ISC (Mutchler et al. 2014). Like gut microbe community composition, gut length can be used as an indicator of dietary nutritional quality (Elliott and Bellwood 2003, Wagner et al. 2009). A longer gut length is associated with a lower quality diet while a shorter gut length is associated with higher quality diet (Horn 1989, Elliot and Bellwood 2003, Wagner et al. 2009, Karasov and Douglas 2013). Due to their significantly shorter gut lengths, urban *C. oligolepis* seem to have access to food sources that are of a higher nutritional quality than rural *C. oligolepis*. If this this the case, one might expect decreased diversity and abundance of gut microbiota in urban *Campostoma* whose diet does not seem to require the same level of digestive effort. Further, specific cellulolytic and enzyme-producing bacterial genera might be more abundant in rural *Campostoma* in response to their lower quality diet.

Gut microbial analysis has never been conducted on *C. oligolepis* but based on previous studies and observed differences in gut length I made the following prediction: rural *C. oligolepis* have access to lower quality food sources, thus requiring greater gut microbiota diversity and abundance than urban *C. oligolepis*. Further, I expected to see a greater proportion of specific bacterial genera like *Aeromonas*, *Clostridium*, *Pseudomonas*, and *Acinetobacter* that

are known cellulose-degraders (Li et al. 2014). These genera would serve to aid in the breakdown of refractory materials present in rural *Campostoma* diet. Bacterial diversity and abundance found in urban and rural *C. oligolepis* guts can be used to infer what differences in dietary preference or nutritional quality might exist between systems with high and low ISC without relying exclusively on digestive morphology.

METHODS

Sample Sites

Campostoma were collected from four small watersheds (wastershed area < 15 km^2) within the Etowah River drainage basin in northern Georgia with varying levels of impervious surface cover (ISC). The sites span from Cobb County, GA, just north of Atlanta to Paulding County, GA, west of Atlanta (Figure 1.1). Rockbridge Creek was the most impacted site with 29.84% impervious surface coverage. The two intermediate sites selected for this study were Lawrence and Possum Creek with 5.98% and 6.91% ISC, respectively. Raccoon Creek was the least impacted site with 0.64% ISC.

Sample Collection

Campostoma were sampled from each sample site using a backpack electroshocker between mid-July and early September 2016. At least ten individuals were collected from each sample site during each sample period.

Campostoma were transported alive back to the lab. Time between capture and transport back to the lab never exceeded three hours to ensure the fish did not undergoing prolonged stress that might alter gut microbe composition (Larsen et al. 2014a, Li et al. 2014). Fish were euthanized in lab with an overdose of tricaine methanesulfonate (Larsen et al. 2014a, Li et al. 2014). The whole, intact gut of each individual was removed aseptically. The contents of the

foregut (the anterior half of the intestine) and the hindgut (the posterior half of the intestine) were emptied into separate sterile vials (Larsen et al. 2014b). The entire gut content was not sampled together to avoid homogenization of symbiotic and ingested bacterial populations (Clements et al. 2014). Gut content samples were immediately subjected to DNA extraction using a PowerFecal® DNA Isolation Kit (MoBio, Inc, Carlsbad, CA, USA) (Larsen et al. 2014b).

High-Throughput Metagenomic Sequencing

After the DNA was extracted from the gut content, DNA samples were prepped for sequencing using the Ion 16S Metagenomics Kit (Thermo Fisher, Inc, Waltham, MA, USA) following the manufacturer's instructions. This kit allowed for PCR amplification of the 16S V2-V9 hypervariable regions of bacterial DNA. The resultant PCR products were run on 3% agarose gel in order to verify DNA fragment lengths. Fore- and hindgut samples for each of the four study sites were then pooled together yielding eight sets of amplicons. Barcoded DNA libraries were then built using Ion Xpress Barcode Adapters (Thermo Fisher, Inc, Waltham, MA, USA) and the eight sets of pooled PCR product. The eight barcoded libraries were then sent PrimBio Reasearch Institute, LLC in Exton, PA, USA for sequencing on an Ion Torrent PGM using an Ion Torrent 318 chip.

Sequence Data Analysis

The sequence data from the fore- and hindgut DNA samples were returned as separate FASTQ files according to barcode with both the sequence and quality scores. The FASTQ files were processed using Trimmomatic-0.36 using a 4:15 sliding window and minimum sequence length of 200 base pairs (Larsen et al. 2014b, Li et al. 2014). The resultant number of reads for the eight files after filtering and trimming ranged from 58,894 to 284,343 reads. FASTQ files were then converted to FASTA files using Mothur v.1.38.1. The FASTA files were uploaded to

Taxonomer™ to sort and classify reads into OTUs. Taxonomer™ bases bacterial classification on a marker gene approach utilizing the 16S rRNA gene and the Greengenes database (Flygare et al. 2016).

Relative abundance of bacterial phyla were compared between the fore- and hindgut and among all sample sites. Nonmetric multidimensional scaling (NMDS) of the top ten most commonly detected genera from each site, 22 genera in total, was conducted in R. The decision to use only the ten most common genera for the NMDS analysis was established by constructing a genus accumulation curve that plotted the cumulative percentage of reads of the most common genera per sample (Figure 2.3, Appendix). Utilizing only the ten most commonly detected genera per sample allowed for 68% to 83% coverage of all the sequence data in the NMDS analysis. Additionally, paired t-tests were conducted to determine if a significant difference in the proportion of enzyme-producing bacteria existed between the foregut and hindgut at each site. The enzyme-producing bacteria used for the fore- and hindgut comparison were *Aeromonas*, *Clostridium*, *Pseudomonas*, and *Acinetobacter*. These genera have been found in the gut microbiota of other freshwater fishes (Larsen et al. 2014a, Li et al. 2014).

RESULTS

Gut Microbiota Composition

On average, 23% of the reads from each sample site were classified as ambiguous meaning they could not be attributed to any specific classification (i.e. bacterial, fungal, human). The remaining 77% of the reads were classified as bacterial. Of those attributable reads, an average of 81% could be classified to genus.

A total of 36 bacterial phyla were identified across all samples and sites with a mean of 26 phyla per sample. The Proteobacteria and Firmicutes phyla represented the largest proportion

of reads across sites in both the fore- and hindgut. Proteobacteria accounted for 69% of the foregut sequences and 57% of the hindgut sequences. Firmicutes accounts for 18% of the foregut sequences and 27% of the hindgut sequences (Figure 2.1).

Proteobacteria also accounted for the highest number of reads across all of the sites, ranging from 52% to 74% of the attributable reads (Figure 2.2). The phylum Firmicutes accounted for the second highest proportion of reads with 13% to 34% of the total attributable reads (Figure 2.2). Within the Proteobacteria, 41% to 58% of the reads fall under the Gammaproteobacteria subphylum. Gammaproteobacteria was followed by Alphaproteobacteria with 2% to 12% of the attributable reads within Proteobacteria.

Figure 2.2 shows the relative proportions of each phyla between fore- and hindgut samples as well as among sites. Samples from Lawrence Creek had the greatest diversity with 30 phyla (678 genera) in the foregut and 31 phyla (520 genera) in the hindgut. Possum Creek had the lowest diversity overall with 26 phyla (368 genera) in the foregut and 23 phyla (323 genera) in the hindgut. The most impacted site, Rockbridge, had the most consistent phyla composition between the fore- and hindgut samples. Also, foregut samples for Lawrence, Possum, and Raccoon have a higher proportion of Proteobacteria and a lower proportion of Firmicutes than the hindgut samples from the same site. In addition to having similar phyla composition, the paired t-tests revealed no significant difference in the proportion of *Aeromonas*, *Clostridium*, *Pseudomonas*, and *Acinetobacter*, all known cellulolytic genera, in the foregut versus the hindgut.

Within the 36 bacterial phyla, 1,008 unique genera were identified across all sites. The most dominant genera were *Pseudoalteromonas*, *Acinetobacter*, *Blautia*, and *Aneuribacillus*. For each sample, three out of these four genera represented the greatest number of attributable reads

with the exception of the Raccoon hindgut sample. The Raccoon hindgut sample was dominated by *Blautia*, *Pseudoalteromonas*, and *Chromatocurvus*.

Pseudoalteromonas accounted for most of the attributable reads accounting for 19% of the reads on average. However, *Pseudoalteromonas* accounted for 44% and 35% of the Possum foregut and hindgut sequences, respectively. The second most dominant genera, *Acinetobacter*, made up 16% of the reads on average. The proportion of sequences attributed to *Acinetobacter* ranged from 5% in the Possum foregut sample and 26% in the Rockbridge hindgut sample. *Blautia* was the third most dominant genera on average making up 13% of reads on average across sample sites. When looking at the percent coverage of genera diversity across samples, the cumulative percent of reads began to level off after the ten genera with the greatest proportion of reads at each site were accounted for (Figure 2.3). Percent coverage of sample diversity increased only marginally with the addition of each new genus after the top ten genera. Figure 2.3 also illustrates some trends in genera composition within each sample site. With the exception of Rockbridge, all of the hindgut samples initially trail the foregut samples in cumulative percent coverage. Rockbridge fore- and hindgut samples follow one another very closely suggesting similar evenness of genera composition. The Raccoon samples are the most divergent from one another. The first three genera account for about 50% of the Raccoon foregut sample reads, while the hindgut sample does not reach that level of coverage until the sixth genus.

A total of 22 genera were used for non-metric multidimensional scaling (NMDS) analysis, shown in Figure 2.4. The 22 genera selected for this analysis account for the ten genera in each sample that make up the greatest proportion of attributable reads. Based on the NMDS, both Rockbridge samples fall very close to one another suggesting strong similarities in

composition. Relatively higher proportions of *Flammeovirga* and *Bacteroides* seem to distinguish the Rockbridge samples from other sites. The Possum samples also seem to be more similar to one another than they are to other sites. Both are strongly associated with *Pseudoalteromonas* and *Faecalibacterium*, while the other sites have significantly lower proportions of these genera.

The results from the Lawrence and Raccoon samples are more complicated. Neither Raccoon nor Lawrence foregut and hindgut samples group very closely together suggesting that the bacterial composition between the fore- and hindgut is more variable at these sites. Reads attributed to *Chitinibacter*, *Cetobacterium*, and *Aquaspirillum* are proportionally much greater in the Raccoon foregut sample as compared to all other samples including Raccoon hindgut. Higher proportions of *Mycoplasma*, *Sporosarcina*, and *Patulibacter* seem to easily distinguish the Raccoon hindgut. For the Lawrence foregut, *Shewanella*, *Vibrio*, and *Chromatocurvus* are driving the observed differences from other samples, while the Lawrence hindgut falls very close to the center of the plot.

DISCUSSION

Microbiota Composition in the Foregut and Hindgut

No marked difference was observed in the composition of gut microbiota between the foregut and hindgut overall (Figure 2.1). Additionally, there was no significant difference in the proportion of known cellulolytic genera in the foregut versus the hindgut. There was also no significant difference in the proportion of cellulolytic/enzyme-producing bacteria between urban and rural systems. If the *Campostoma* were using microbiota to specifically aid in digestion, I would expect increased levels of *Aeromonas*, *Clostridium*, *Pseudomonas*, and *Acinetobacter* in the hindgut of individuals from rural systems. I expected to find these genera in higher

abundance in those specific samples because symbiotic bacteria are generally housed in the hindgut (Clements et al. 2014), and rural *Campostoma* have significantly longer gut lengths suggesting a lower quality diet that might benefit more from the digestive aid of microbes. These findings suggest that *Campostoma* may not be actively housing communities of symbiotic bacteria.

Although no significant differences were observed in the proportions of these specific genera, many were prevalent throughout the guts of individuals from different sites. Both *Acinetobacter* and *Aeromonas* were observed in high proportions, accounting for 16% and 3% of reads on average at each site. *Clostridium* and *Pseudomonas* were also found in relatively high proportions. *Clostridium* represented an average of 0.9% of the total attributable reads and *Pseudomonas* represented 0.7% of reads. Despite the lack of differentiation in the composition between gut regions, the present enzyme-producing bacteria might be providing some type of digestive aid. It is also possible that the method of sampling the gut contents could have inadvertently allowed for homogenization of symbiotic and ingested bacterial populations. Future studies should sample an even more distal portion of the intestine to represent the hindgut instead of dividing the intestine in half to designate fore- and hindgut. However, considering the similarities in foregut and hindgut samples across all of the samples collected in this study, it is likely that little differentiation in bacterial community composition exists from fore- to hindgut.

Microbiota Composition across Sites

Rockbridge Creek, the most urbanized site for this study, had 515 genera in the foregut and 406 genera in the hindgut. Overall, the composition of the Rockbridge fore- and hindgut was the most uniform. This uniformity is represented in the relative proportions of phyla (Figure 2.2), the similar shapes of the genera percent accumulation curves (Figure 2.3), and the close grouping

of the two samples on the NMDS plot (Figure 2.4). All of these data suggest that there is little to no differentiation between the foregut and hindgut samples from Rockbridge. However, the Rockbridge samples fall in a separate quadrant of the NMDS plot separate from other samples suggesting that they are distinct from other sample sites.

Lawrence Creek, one of the intermediate sites, had the greatest diversity with 687 genera represented in the foregut and 520 genera in the hindgut. Increased diversity was expected as this collection site was in close proximity to a wastewater treatment plant. I also expected higher proportions of *Blautia*, *Escherichia*, and other genera associated with fecal waste. However, no substantial increase in these genera were found in either sample from Lawrence Creek. There was also not a close grouping of the Lawrence foregut sample with the hindgut sample in the NMDS analysis (Figure 2.4). This tends to suggest that the composition between the two regions is different. The Lawrence hindgut sample fell very close to the center of the plot suggesting it had high proportions of genera commonly found in samples from the other sites.

Possum Creek, the second intermediate site, had the lowest overall diversity with 368 genera identified in the foregut and 323 in the hindgut. Like the Rockbridge site, the foregut and hindgut samples from the Possum site grouped very closely together on the NMDS plot (Figure 2.4). This grouping is attributed in large part to the high proportion of *Pseduoalteromonas* and *Faecalibacterium* relative to other sites. However, there are some differences between the fore- and hindgut bacterial composition. There is an increase in both the phylum Firmicutes and Bacteroidetes from the foregut to the hindgut (Figure 2.2). There is also a decrease in the relative proportion of Proteobacteria. A potential explanation for this pattern, which is also observed in the Lawrence and Raccoon samples, is that Proteobacteria are very common in the environment. Bacteroidetes and Firmicutes have been shown to be proportionally more abundant in the human

gut as compared to Proteobacteria (Hong et al. 2011). As such, the foregut could be inundated with environmental Proteobacteria. Levels of environmental bacteria should decrease further along the intestine allowing resident symbionts to be detected at a higher frequency in the hindgut. However, it should also be noted that the shift in the proportions of Proteobacteria, Firmicutes, and Bacteroidetes from the foregut to hindgut are marginal at best.

Raccoon Creek, the most rural site, had 448 genera detected in the foregut and 393 genera detected in the hindgut. The fore- and hindgut samples for Raccoon Creek, like Lawrence, did not group closely together in the NMDS plot (Figure 2.4). Of all the sample sites, the Raccoon Creek fore- and hindgut samples seem to be the most divergent. The shapes of the genera percent accumulation curves for the Raccoon samples are noticeably different from one another (Figure 2.3). The hindgut sample lags behind the foregut sample. This suggests that the Raccoon hindgut sample is more even in its genus composition than the foregut sample.

Based on the data, it appears that the most urban site had the least amount of differentiation between the fore- and hindgut, while the most rural site had the most differentiation. Whether this differentiation is due to symbiotic microbes being housed in the hindgut is unclear, though that does not appear to be the case due to the relatively low magnitude of differences detected.

Properties and Ecological Roles of Prevalent Genera

Pseudoalteromonas, the genus with the highest proportion of reads overall, is an important component of some epilithic biofilms (Bowman 2007). *C. oliglepis* eat epilithic communities that accumulate on stable substrate within streams (Etnier and Starnes 1993, Boschung and Mayden 2004). As such, I predicted that *Pseudoalteromonas* would be most prevalent in the foregut of *Campostoma* and that it would be more abundant in rural systems as

compared to urbanized systems. Urban systems tend to have reduced stable substrate which reduces the ability of biofilms to accumulate (Paul and Meyer 2001). Although we did not see a difference in the relative proportion of *Pseudoalteromonas* between the fore- and hindgut, this genus was least prevalent at the most impacted site, Rockbridge Creek. However, in addition to being a component of biofilms members of the *Pseudoalteromonas* genus have been shown to produce agarases, enzymes that aid in the breakdown of algal cell walls (Holmstrom and Kjelleberg 1999). As a result, this genus has been found in symbiotic association with eukaryotic organisms (Holmstrom and Kjelleberg 1999). Although it is unclear given the data from this study, *Pseudoalteromonas* might be aiding in the breakdown of algae consumed by *C. oligolepis*.

Many of the most commonly observed bacterial genera across all sites are closely associated with the human gut microbiome. This finding is not surprising as runoff and underground seepage from human waste receptacles might introduce these gut-associated bacteria into streams (Paul and Meyer 2001). For example, high proportions of *Blautia* were observed across all sites. *Escherichia* and *Clostridium* were present in relatively high proportions, accounting for around 1% of attributable reads on average. The genus *Faecalibacterium* was found in relatively high proportions at Possum and Rockbridge sites. Rockbridge also had higher proportions of *Bacteroides*. While all of these genera are known symbionts in the human gut, it is possible that they are acting as symbionts in the *Campostoma* gut. For example, *Clostridium* has been isolated from fishes' guts in previous studies and is a well-known cellulose-degrader (Larsen et al. 2014a, Li et al. 2014). These bacteria could be playing a similar role in *Campostoma* as they do in humans.

Dietary Variability in C. oligolepis *and Other Considerations*

The diet of *C. oligolepis* is very complex and should not be over simplified. While *Campostoma* consume large amounts of algae, they also consume the fungus, bacteria, diatoms, and detritus that make up the epilithic communities in the stream. German et al. (2010a) found almost equal proportions of algae, detritus, and diatoms in *C. oligolepis* gut contents. It is also not unlikely that *Campostoma* consume microscopic invertebrates living on or near the epilithon. The composition and relative proportions of each of these components can fluctuate not only from stream to stream but from rock to rock within a stream. With this in mind, future studies should look to not only assess gut microbiota composition, but the composition of epilithic samples from the stream as well. This assessment would allow for a clearer indication of which symbiotic bacteria would most likely be aiding in digestion.

While many enzyme-producing and cellulolytic genera were identified as being present in the gut of *Campostoma*, it is unclear whether or not the sampled bacteria are resident symbionts or ingested from the environment. Bacterial composition from the fore- to hindgut was uniform overall without any observed significant differences. There were also no significant observed differences in the composition of the microbiota across sites with similar genera making up a bulk of the reads from each site. Additionally, *C. oligolepis* has been shown to have low fermentation potential in the gut as compared to other herbivorous fishes (German et al. 2010a). With all of these considerations in mind, it does not appear that *Campostoma* house symbiotic gut microbes. It is likely that many of the bacteria observed in this study were ingested as food from the environment rather than being resident.

Despite this lack of clarity regarding whether or not specific bacteria are resident or from the environment, many studies suggest that bacteria detected in the gut are a component of the

fish's microbiota simply on the grounds that they are present (e.g. Larsen et al. 2014a, Li et al. 2014, Ye et al. 2014). Further, these studies assert that certain genera are aiding in the digestive process if they are known to produce certain enzymes while genera that are not enzyme-producers are said to have been ingested (Li et al. 2014, Ye et al. 2014). These distinctions of what is environmental and what is resident appear to be based only on whether or not the bacteria are known to produce enzymes. However, there is no evidence beyond the capacity of the genera to produce enzymes that would suggest that these bacteria are resident while others are ingested. Given the relative consistency of enzyme-producing genera from foregut to hindgut in *Campostoma*, the data from this study do not support the assertion that enzyme-producing genera are resident. Future studies investigating the gut microbiota of organisms should use caution when claiming specific bacteria are a part of the organism's flora.

Conclusions

Overall, the results from this study were consistent with previous studies. *Campostoma* captured during the summer months did display a significant difference in relative gut length as hypothesized: urban *C. oligolepis* had significantly shorter guts than rural individuals. However, a significant decrease in the relative gut length of *Campostoma* from Possum and Raccoon was observed in the fall sample. This shift in gut length might be attributed to adaptive modulation in response to changes in the composition of resources available. In order to investigate this seasonal phenomenon, future studies should look to test the composition and/or the nutritional quality of the epilithic communities present at each site. Investigating potential differences in resource nutritional quality and monitoring any seasonal changes might help explain the observed differences and shifts in *C. oligolepis* gut length.

The bacterial groups identified were fairly consistent with previous studies investigating the composition of gut microbiota in fishes (Larsen et al. 2014a, Li et al. 2014, Ye et al. 2014). No evidence for differentiation in gut microbe composition between gut regions (fore- versus hindgut) or among sites was found. This lack of differentiation makes it impossible to establish whether these individuals were resident symbionts or were ingested from the environment. However, it does suggest that *Campostoma* do not rely on bacterial symbionts to aid in the digestion of their food sources. Additionally, the lack of a significant pattern across urban and rural sites suggests that gut microbiota are not playing a significant role in the shift in *Campostoma* gut length. Assessing seasonal microbiota composition could help verify that this is the case. DNA was extracted from the gut contents of individuals collected in the fall. Sequencing this DNA to look for differences in gut microbe composition from summer to fall could offer new insight.

Integration of Thesis Research

This study integrated a number of techniques from a wide array of biological disciplines. How urbanization impacts *C. oligolepis* morphology and digestion is foundationally an ecological question but it relies on the investigation of animal biology and physiology. Additionally, aspects of molecular and microbiology were employed to further investigate this seemingly ecological or physiological question. Ecological techniques were utilized to collect the individuals needed for the study. Specifically, *Campostoma* were collected in the field by backpack electroshocking, a technique commonly used in aquatic ecology studies. Aspects of animal biology and physiology were used in dissecting the captured individuals. Aseptic technique, a practice commonly utilized in microbiology, was also implemented in the dissection process to reduce the likelihood of bacterial contamination of the gut content samples. Once the gut contents were collected, molecular techniques were utilized including DNA extraction, PCR, and DNA library preparation and barcoding. High-throughput metagenomic sequencing, a technique relating to both molecular biology and genetics, was then utilized to identify the bacteria present in the gut contents. Processing the data output from the sequencing definitely fell under the discipline of bioinformatics and biostatistics with the utilization of programs like Trimmomatic, Mothur, Taxonomer, and R. Interpreting the results from the sequencing required a combination of both microbiology and ecology. The bacteria needed to be identified but their ecological niche also needed to be investigated so that their specific role in both the fish gut and in the stream itself might be better understood. Overall, techniques from aquatic ecology, animal biology/physiology, microbiology, molecular biology, computer science, and biostatistics were integral to the completion of this research.

Acknowledgements

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Tables and Figures

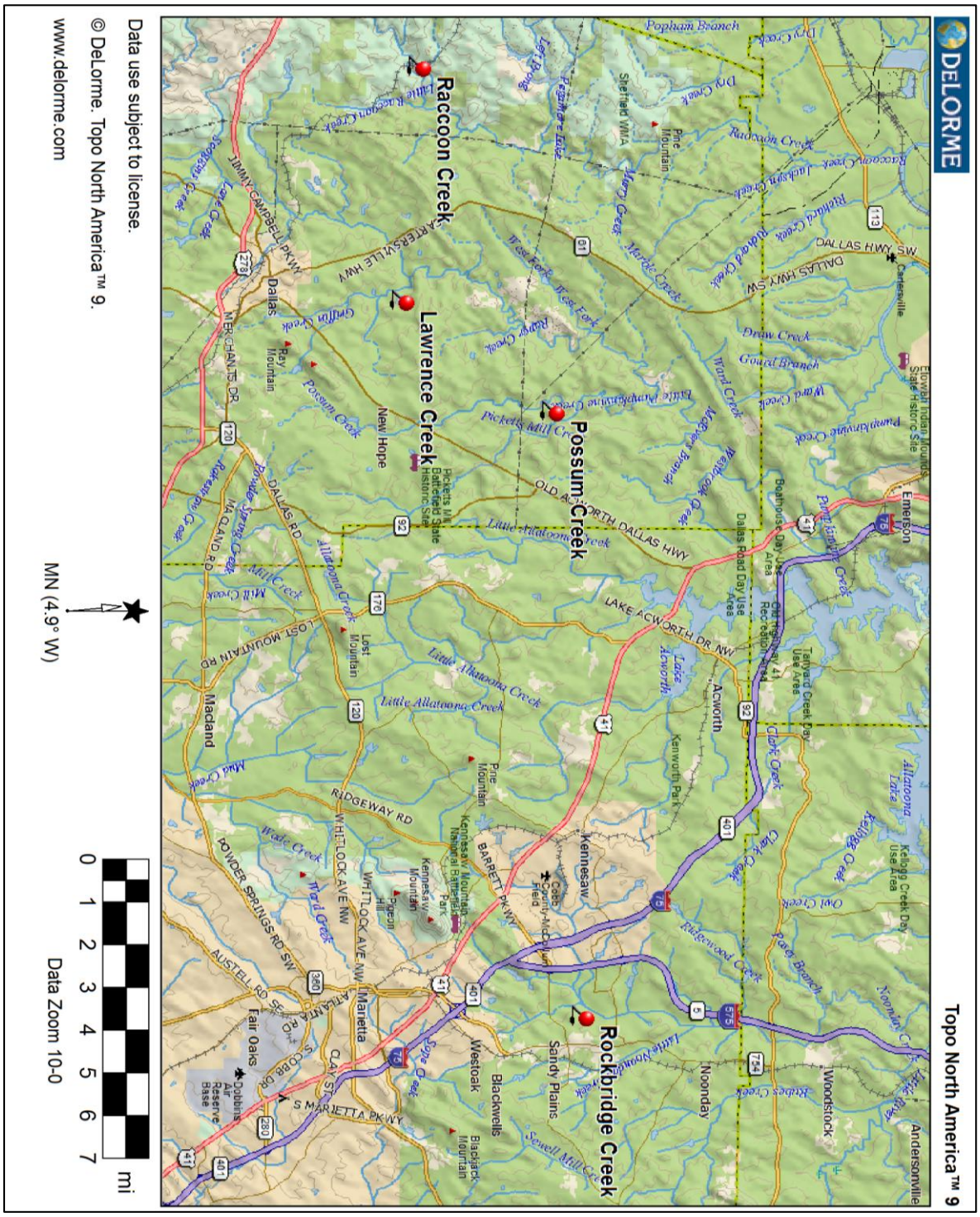

Figure 1.1. Map of sample sites with Raccoon Creek being the least impacted site, and Rockbridge Creek being the most impacted site.

Table 1.1: Mean total length (mm), weight (g), and gut length (mm) for *C. oligolepis* collected from each sample site during the summer collection period.

	Rockbridge (n = 9)	**Lawrence (n = 14)**	**Possum (n = 10)**	**Raccoon (n = 11)**
Mean Total Length (mm)	106.1	103.4	113.6	101.1
Mean Weight (g)	8.9	8.7	14.0	8.0
Mean Gut Length (mm)	340.9	370.6	526.7	430.4
Mean Relative GL	3.21	3.58	4.64	4.26

Table 1.2: Mean total length (mm), weight (g), and gut length (mm) for *C. oligolepis* collected from each sample site during the fall collection period. Collections were not obtained from Lawrence Creek during the fall sampling period (NC).

	Rockbridge (n = 19)	**Lawrence**	**Possum (n = 14)**	**Raccoon (n = 16)**
Mean Total Length (mm)	87.9	NC	81.1	85.1
Mean Weight (g)	5.6	NC	4.6	5.8
Mean Gut Length (mm)	353.4	NC	319.7	295.1
Mean Relative GL	4.02	NC	3.94	3.47

Table 1.3: Seasonal comparisons of relative gut length (GL/TL) and condition factor (K) at the Rockbridge, Possum, and Raccoon sites. Summer to fall comparisons look at differences in GL/TL and K between the two seasons at each site. P-values were calculated using 2-tailed t-tests assuming equal variance ($\alpha = 0.05$). Significant differences between the summer and fall data at a specific site are denoted with gray shading.

Site	**Summer to Fall**	
	Relative Gut Length	**Condition Factor**
Rockbridge	p = 0.0737	p = 0.7432
Possum	p = 6.5476E-05	p = 0.00098
Raccoon	p = 0.00196	p = 0.7083

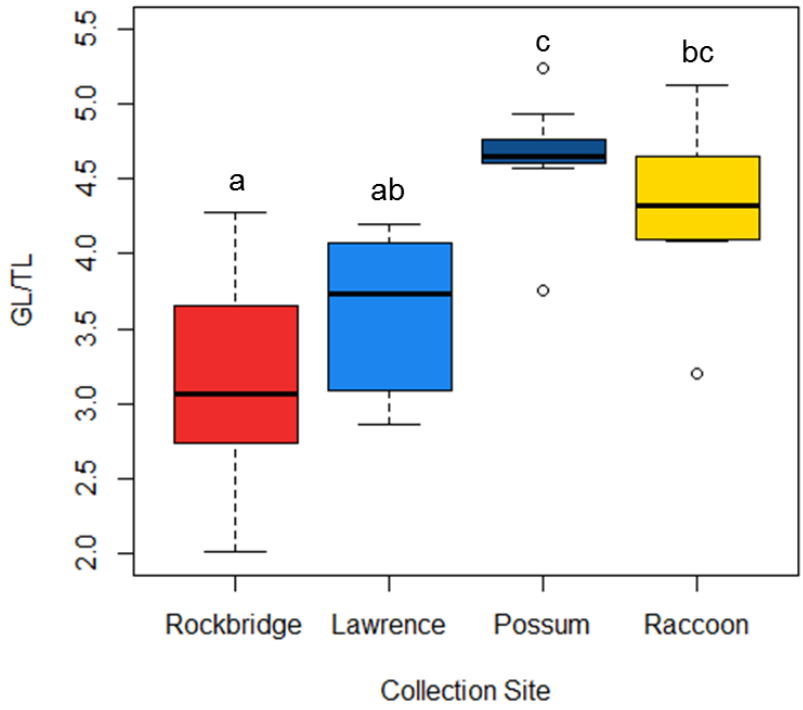

Figure 1.2a. Summer relative gut lengths of *Campostoma* sampled from each collection site. Noted differences between sample sites are based on a Kruskal-Wallis test with post-Hoc Nemenyi-tests for multiple comparisons with Tukey-distribution approximation of independent samples. Samples that do not share a letter in common were significantly different from one another.

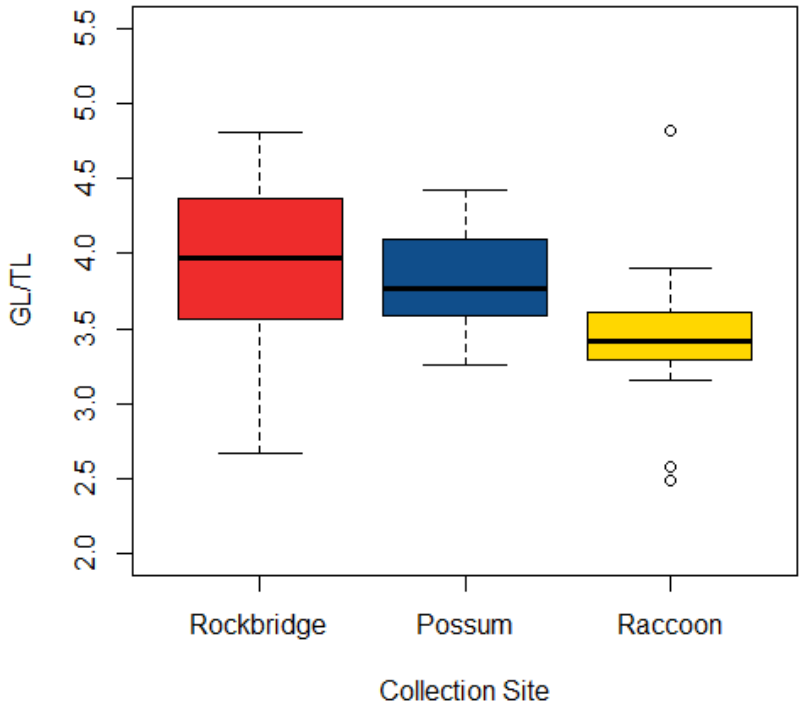

Figure 1.2b. Fall relative gut lengths of *Campostoma* sampled from Rockbridge, Possum, and Lawrence Creek. No differences in gut length were observed between the sites for the fall sampling period.

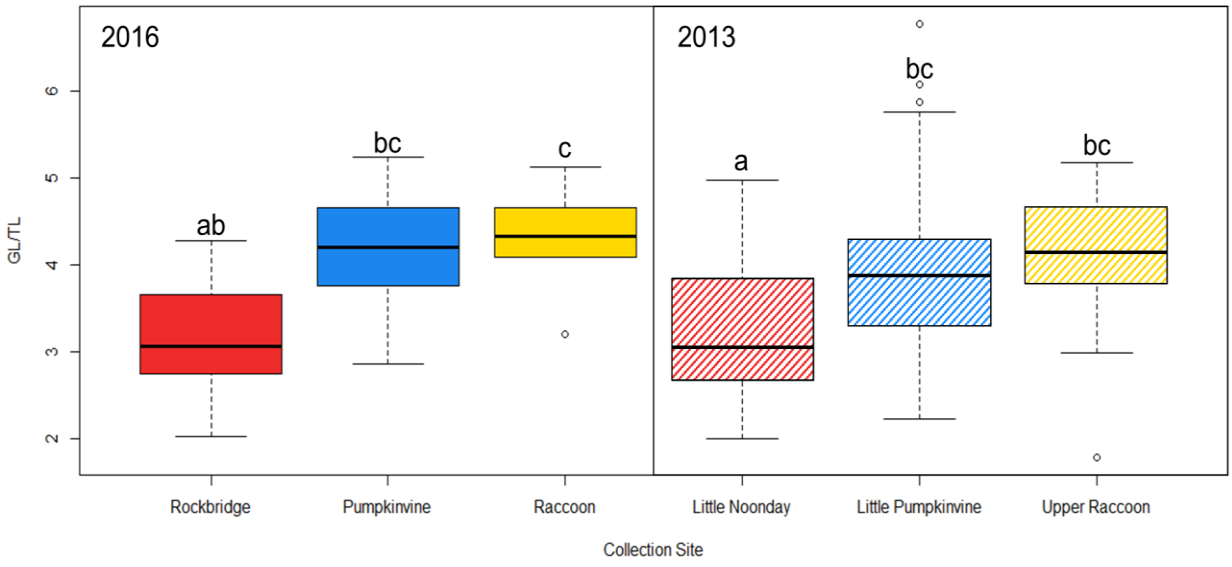

Figure 1.3. Relative gut lengths of *C. oligolepis* collected for this study (2016) and for the 2013 study (Schooling and Ensign 2013). The relative gut lengths between the 2016 and 2013 studies were similar with no differences were observed between the two impacted (red) sites, the three intermediate (blue) sites, or the two rural (yellow) sites.

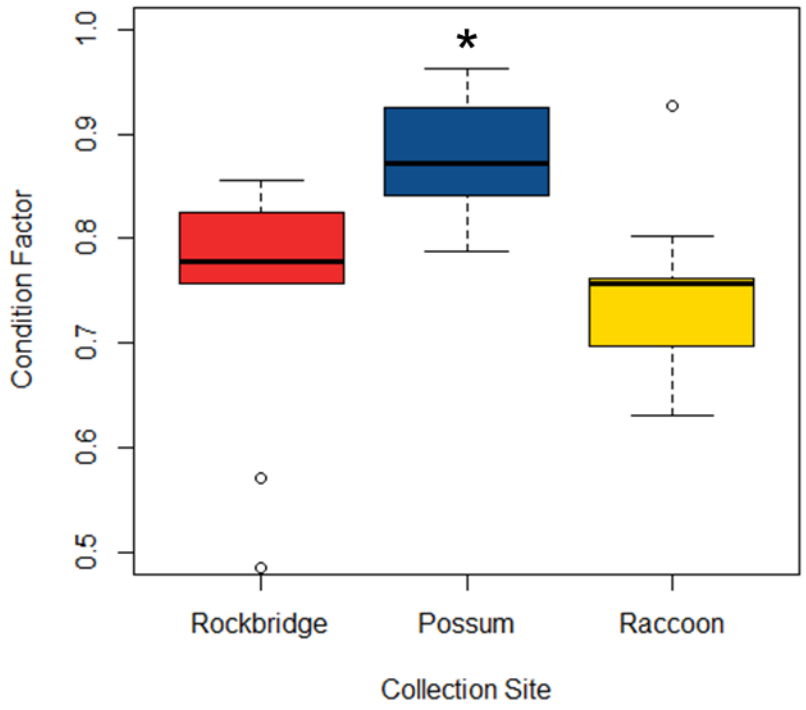

Figure 1.4a. Summer condition factors (K) of *Campostoma* from Rockbridge, Possum, and Raccoon. Possum had significantly higher K values than Rockbridge (Nemenyi test, p = 0.0254) and Raccoon (Nemenyi test, p = 0.0022).

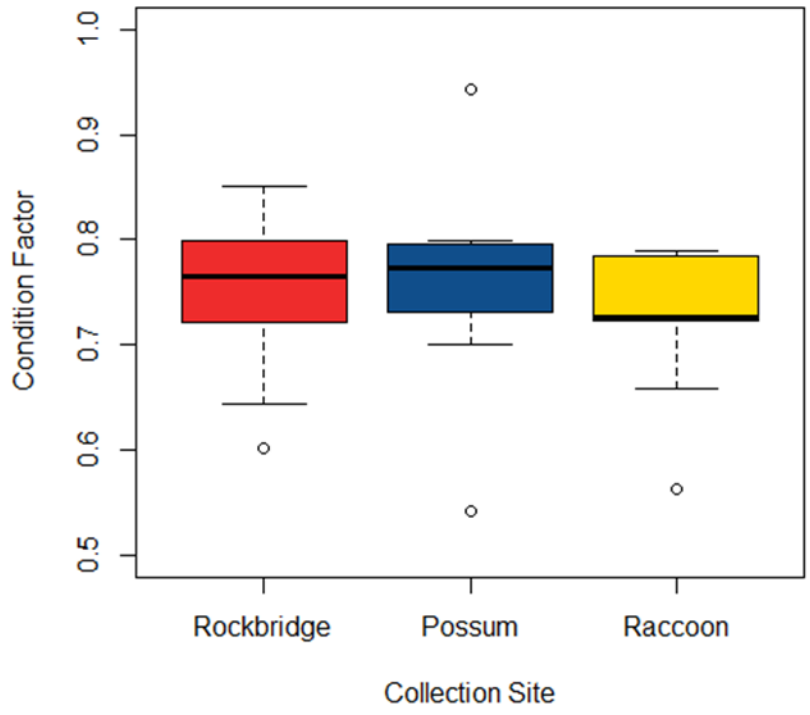

Figure 1.4b. Fall condition factors (K) of *Campostoma* from Rockbridge, Possum, and Raccoon. No significant difference was observed between any of the sites.

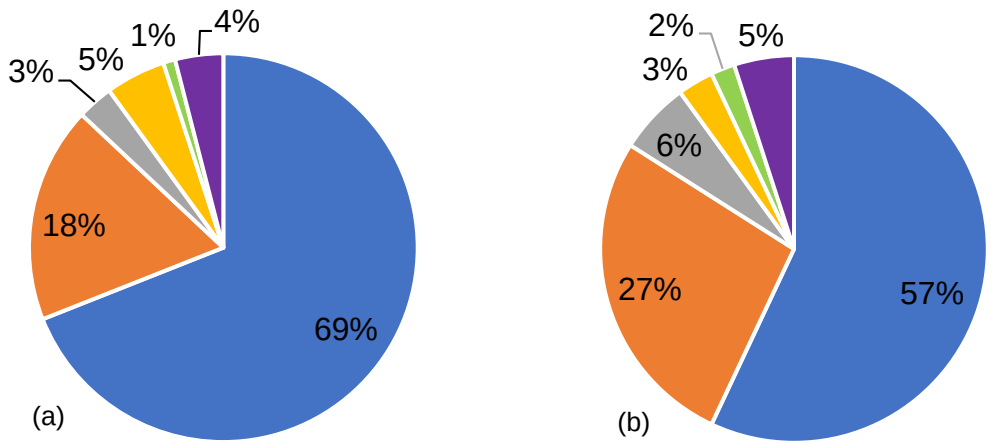

Figure 2.1. Percent distribution of most commonly detected phyla across all sites in *Campostoma* foregut (a) and hindgut (b). Phyla that made up less than one percent of the sequences are grouped into "Other". (■) Proteobacteria, (■) Firmicutes, (■) Bacteroidetes, (■) Actinobacteria, (■) Cyanobacteria, (■) Other.

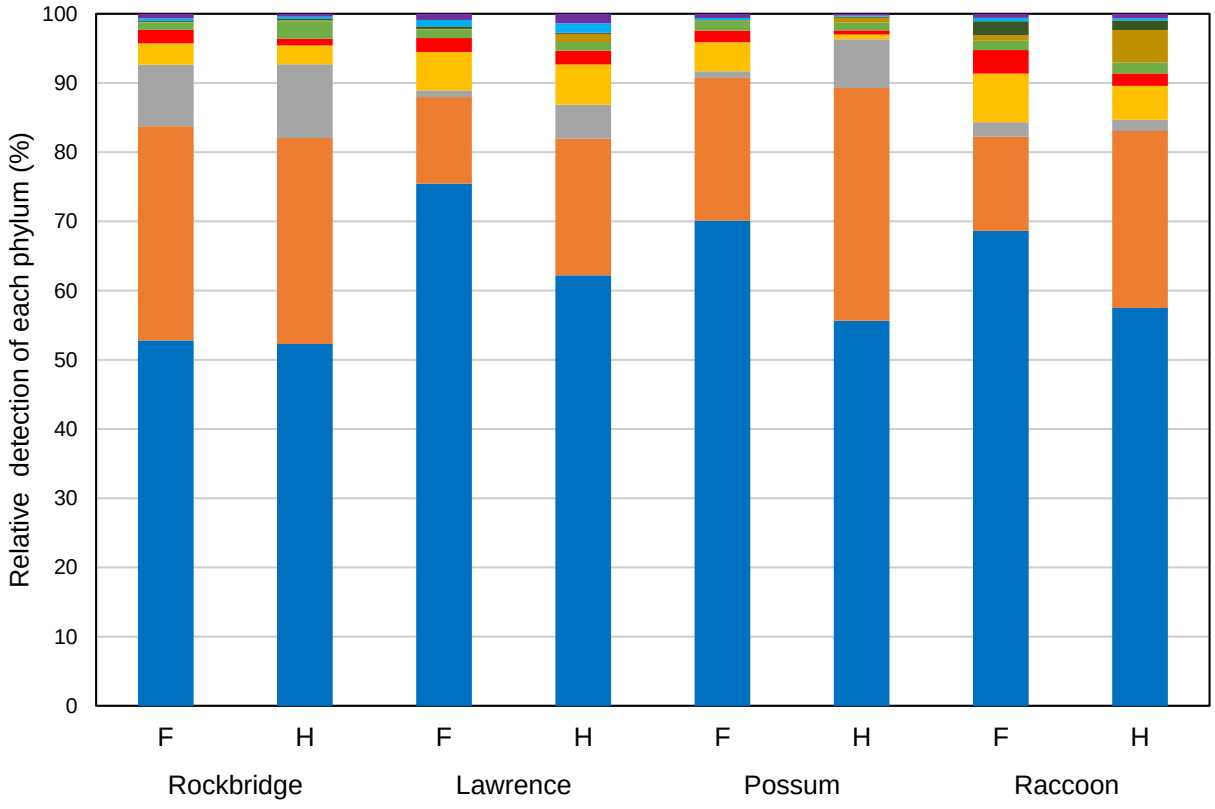

Figure 2.2. Composition of the most commonly detected bacterial phyla in *Campostoma* foregut (F) and hindgut (H) from all sample sites. (■) Proteobacteria, (■) Firmicutes, (■) Bacteroidetes, (■) Actinobacteria, (■) Planctomycetes, (■) Cyanobacteria, (■) Tenericutes, (■) Fusobacteria, (■) Acidobacteria, (■) Other.

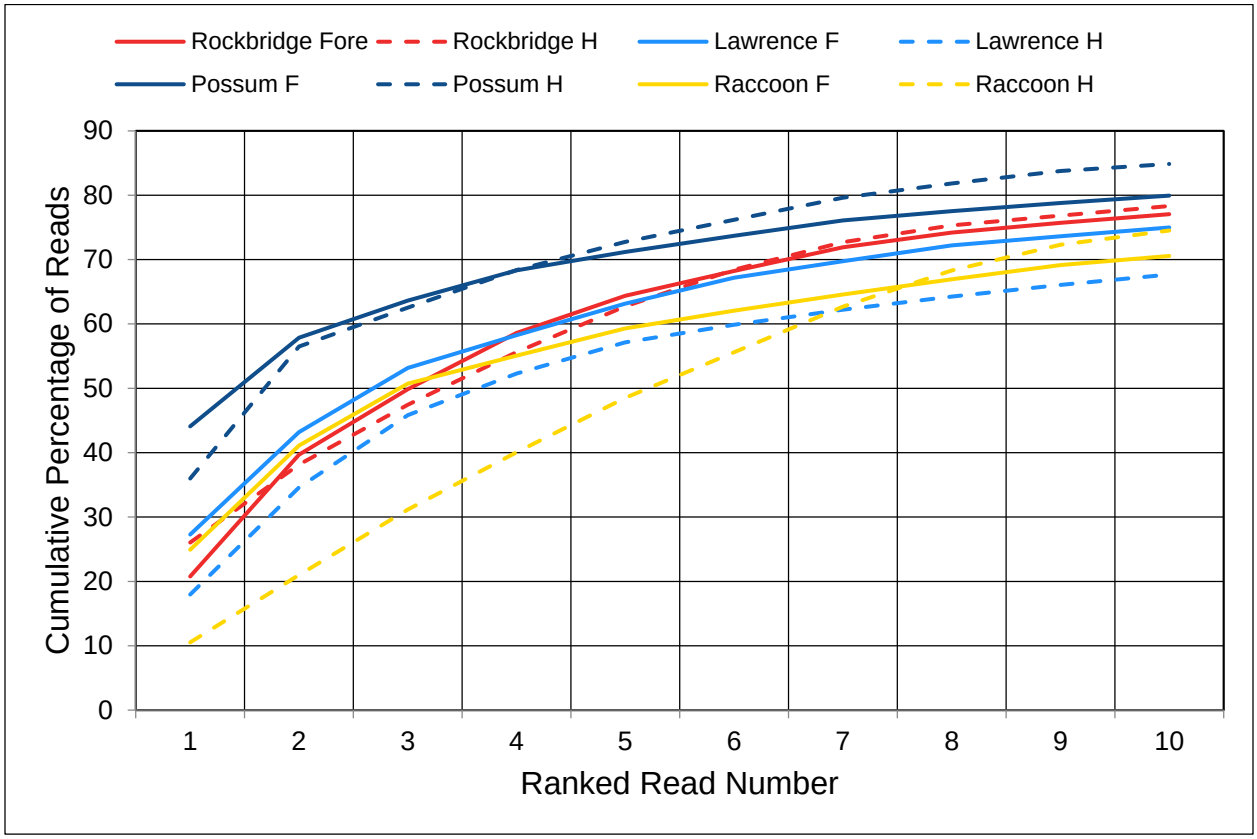

Figure 2.3. Cummulative percent coverage of reads and sample diversity. The top ten genera that accounted for the highest proportion of reads from each sample were used for this accumulation curve (Appendix). These ten genera per sample account for 68% to 83% of sequences. All curves plateau with only gradual increases after this point.

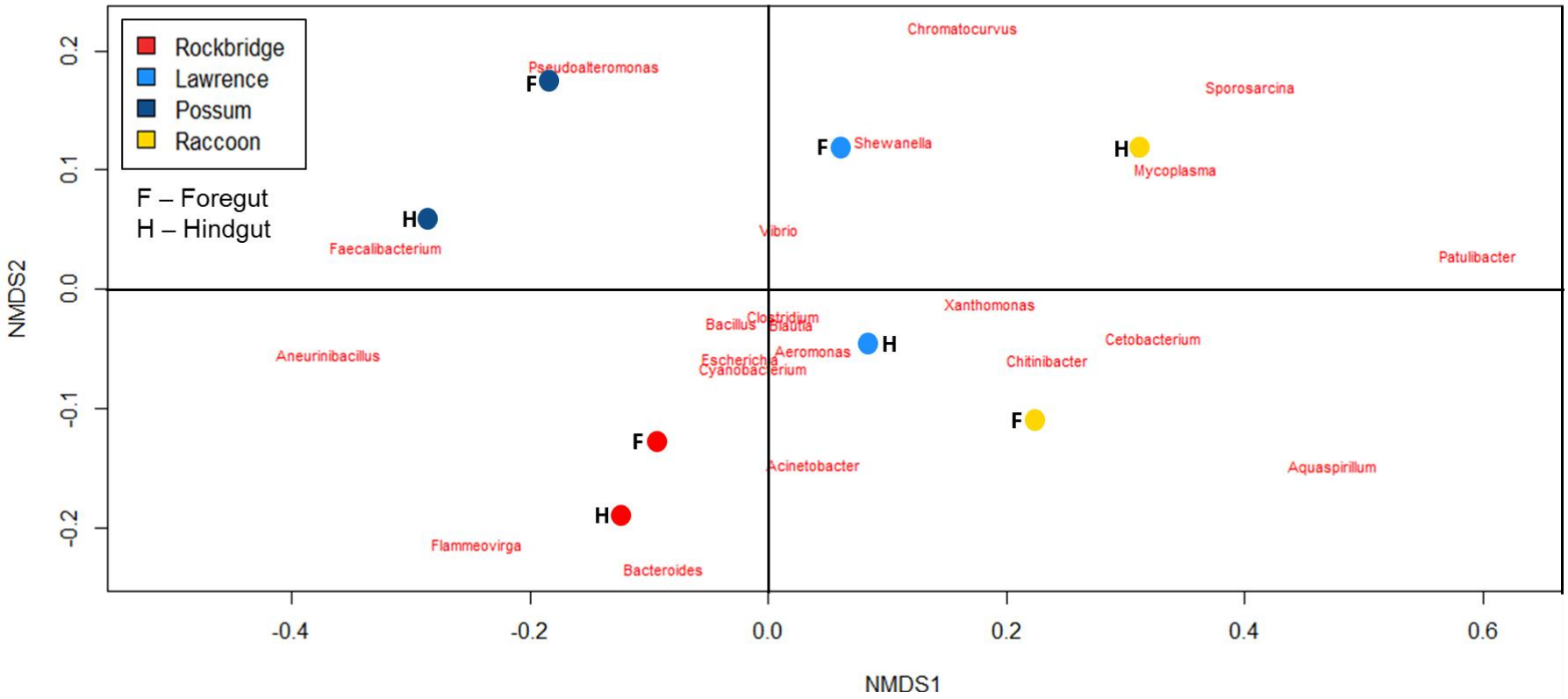

Figure 2.4. Nonmetric multi-dimensional scaling of fore- and hindgut samples from each site. A total of 22 genera were used for this analysis to include the 10 genera that account for the highest proportion of attributable reads at each site.

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Appendix – The tables below show the percentage of reads (% reads) accounted for by each genus along with the corresponding rank number for genus by sample. Genera highlighted in gray were within the ten most common genera detected in each sample. The highlighted genera were used to construct the accumulation curve (Figure 2.3). All 22 genera listed were used to conduct the NMDS analysis.

	Rockbridge Creek				Lawrence Creek			
	Fore		Hind		Fore		Hind	
Genus	**Rank**	**% reads**	**Rank**	**% reads**	**Rank**	**% reads**	**Rank**	**% reads**
Pseudoalteromonas	4	8.746	5	7.121	1	27.286	3	11.253
Acinetobacter	1	20.772	1	26.039	2	15.897	1	17.966
Blautia	2	18.909	3	9.306	3	9.976	2	16.621
Aneurinibacillus	3	10.178	2	12.108	20	0.566	10	1.622
Bacillus	7	3.675	4	8.167	7	2.553	8	2.021
Chromatocurvus	19	0.561	21	0.472	5	4.901	13	1.528
Aeromonas	6	3.855	8	2.611	8	2.467	5	4.845
Shewanella	10	1.336	9	1.514	4	5.101	9	1.824
Xanthomonas	9	1.533	12	1.343	6	4.030	4	6.457
Flammeovirga	5	5.764	7	4.274	39	0.208	6	2.741
Vibrio	11	1.231	16	0.773	9	1.410	18	0.986
Escherichia	12	1.218	11	1.435	10	1.365	16	1.227
Clostridium	17	0.654	14	0.968	11	1.003	7	2.341
Bacteroides	8	2.288	6	5.676	21	0.434	17	1.075
Cyanobacterium	24	0.441	10	1.509	53	0.129	45	0.192
Chitinibacter	35	0.203	37	0.157	16	0.780	11	1.577
Faecalibacterium	15	0.828	13	1.199	36	0.257	31	0.355
Cetobacterium	42	0.158	29	0.241	22	0.425	49	0.168
Mycoplasma	63	0.089	56	0.083	229	0.012	12	1.558
Sporosarcina	51	0.127	61	0.074	229	0.012	14	1.449
Aquaspirillum	16	0.015	108	0.028	51	0.126	36	0.256
Patulibacter	64	0.086	145	0.019	131	0.032	70	0.089

	Possum Creek				Raccoon Creek			
	Fore		Hind		Fore		Hind	
Genus	**Rank**	**% reads**	**Rank**	**% reads**	**Rank**	**% reads**	**Rank**	**% reads**
Pseudoalteromonas	1	44.097	1	35.998	3	9.625	2	10.466
Acinetobacter	4	4.700	3	6.021	1	24.935	4	8.910
Blautia	2	13.764	4	5.845	2	16.167	1	10.538
Aneurinibacillus	3	5.757	2	20.508	73	0.087	14	1.444
Bacillus	8	1.439	7	3.422	22	0.543	6	7.098
Chromatocurvus	6	2.524	8	2.230	9	2.231	3	10.161
Aeromonas	5	2.865	11	1.080	4	4.354	11	2.213
Shewanella	7	2.387	9	1.944	7	2.497	7	7.066
Xanthomonas	15	0.778	21	0.535	5	4.224	9	3.994
Flammeovirga	24	0.286	5	4.369	46	0.190	28	0.333
Vibrio	9	1.269	10	1.087	13	1.135	13	1.520
Escherichia	17	0.655	16	0.648	16	0.923	21	0.531
Clostridium	13	0.798	26	0.233	28	0.440	22	0.504
Bacteroides	38	0.171	12	0.864	31	0.407	15	1.034
Cyanobacterium	18	0.566	17	0.621	18	0.852	16	1.030
Chitinibacter	38	0.171	34	0.103	8	2.362	23	0.499
Faecalibacterium	10	1.146	6	3.439	77	0.081	36	0.238
Cetobacterium	76	0.055	28	0.169	6	2.763	12	1.628
Mycoplasma	76	0.055	15	0.748	15	0.993	8	5.627
Sporosarcina	112	0.027	20	0.542	36	0.326	5	8.438
Aquaspirillum	366	0.000	200	0.003	10	1.406	33	0.283
Patulibacter	220	0.007	324	0.000	14	1.118	10	2.226