Fecal Bacterial Communities as an Indicator of Trophic Interactions Among Anuran Larvae

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FECAL BACTERIAL COMMUNITIES AS AN INDICATOR OF TROPHIC INTERACTIONS AMONG ANURAN LARVAE

by

Steven Everman

A Thesis
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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December 2016
ABSTRACT

Fecal bacterial communities as an indicator of trophic interactions among anuran larvae

by Steven Everman

December 2016

Anurans are mass spawners, often with multiple females spawning together, resulting in thousands of tadpoles sharing a habitat. Such large numbers of tadpoles with limited dispersal can lead to intense competition for resources. Inter and intra-specific competition for food could have negative impacts on the growth and survival of smaller tadpoles. Fecal bacterial communities have the potential to be used as indicators of changes in diet making it possible to determine if tadpoles in the wild are eating the same food or not. After feeding on two prepared diets that differed in the percentage of complex carbohydrates, the fecal bacterial communities of tadpoles were not significantly different. After enclosing small and large southern leopard frog tadpoles at two locations, size and location had significant effects on the composition of the fecal bacterial communities. Location had a significant effect on the composition of the fecal bacterial communities of green tree frog tadpoles. After capturing wild tadpoles, the sequenced fecal bacterial communities were similar at the phylum level between small and large southern leopard frog tadpoles while the bacterial communities of southern leopard frog and green tree frog tadpoles were easily distinguishable at the phylum level. Using the fecal bacterial communities to make inferences about diet selection in wild tadpoles,
small and large southern leopard frog tadpoles avoid competition by eating different things. Additionally, green tree frog and southern leopard frog tadpoles also avoid competition by eating different things.
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TABLE OF CONTENTS

ABSTRACT .............................................................................................................................................. ii

ACKNOWLEDGMENTS ........................................................................................................................ iv

LIST OF ILLUSTRATIONS ................................................................................................................... vii

CHAPTER I - INTRODUCTION ........................................................................................................... 1

CHAPTER II – METHODS AND MATERIALS ...................................................................................... 7

  Study Site ........................................................................................................................................... 7

  Tadpole and Feces Collection and DNA Extraction ................................................................. 8

  HRM .................................................................................................................................................. 8

  PERMANOVA ................................................................................................................................. 10

  NMDS ............................................................................................................................................... 10

   Laboratory Study: Food Preparation ......................................................................................... 11

   Laboratory Study: Housing and Feeding ................................................................................... 11

   Field Study: Bucket Enclosures ................................................................................................. 12

   Field Study: Fecal Bacterial Community Composition of Wild Tadpoles ..... 14

CHAPTER III – RESULTS .................................................................................................................... 16

  Laboratory Study .......................................................................................................................... 16

  Field Study: Bucket Enclosures ................................................................................................ 18

  Field Study: Fecal Bacterial Community Composition of Wild Tadpoles ..... 21

CHAPTER IV – DISCUSSION ................................................................................................................ 24
Laboratory Study ...................................................................................................... 25
Field Study: Bucket Enclosures ............................................................................... 26
Field Study: Fecal Bacterial Community Composition of Wild Tadpoles .......... 28
APPENDIX A - FORMS ......................................................................................... 33
MDWFP Collection Permit ..................................................................................... 33
IACUC Approval Letter ......................................................................................... 36
REFERENCES ........................................................................................................ 37
LIST OF ILLUSTRATIONS

Figure 1. Effect of Diet on Southern Leopard Frog and Green Tree Frog Tadpole Fecal Bacterial Communities. ................................................................. 16

Figure 2. Effect of Diet and Size on Southern Leopard Frog Tadpole Fecal Bacterial Communities................................................................. 17

Figure 3. Effect of Size and Location on Small and Large Southern Leopard Frog Tadpole Fecal Bacterial Communities During Summer 2015. ......................... 18

Figure 4. Effect of Size and Location on Small and Large Southern Leopard Frog Tadpole Fecal Bacterial Communities During Summer 2016. ......................... 19

Figure 5. Effect of Location on Green Tree Frog Tadpole Fecal Bacterial Communities During Summer 2015................................................................. 20

Figure 6. Effect of Location on Green Tree Frog Tadpole Fecal Bacterial Communities During Summer 2016................................................................. 21

Figure 7. Fecal Bacterial Community Composition of Wild Caught Small and Large Southern Leopard Frog Tadpoles at the Phylum Level. ......................... 22

Figure 8. Fecal Bacterial Community Composition of Wild Caught Southern Leopard Frog and Green Tree Frog Tadpoles at the Phylum Level. ......................... 23
CHAPTER I - INTRODUCTION

Anurans are mass spawners, often with multiple females spawning in synchrony. Different species of anurans can also spawn in the same pool of water resulting in thousands of tadpoles. Both factors can lead to intense competition for resources. With thousands of individuals sharing a habitat, one resource developing anurans might compete for is food. Inter and intra-specific competition for food could have negative impacts on the growth and survival of tadpoles (Werner, 1994). Eating as much food as possible, while at the larval stage, would increase growth and size at metamorphosis. Larger size at metamorphosis improves the fitness of anurans making the transition to a terrestrial life (Gosner, 1960).

Lab based competition studies have typically used a single food as a limiting resource and usually larger tadpoles negatively impact smaller tadpoles. Werner (1992) noted in laboratory experiments that larger tadpoles seemed to outcompete smaller tadpoles when food was a limiting resource. Katzmann, Waringer-Löschенkohl, and Waringer (2003) reported that *Bufo* tadpoles were smaller at metamorphosis than the same *Bufo* tadpoles not exposed to larger *Bufo* tadpoles of another species. Boone, Little, and Semlitsch (2004) placed large overwintered American bullfrog tadpoles with southern leopard frog tadpoles. The bullfrog tadpoles reduced the growth of leopard frog tadpoles. The leopard frog tadpoles were smaller at metamorphosis than others not exposed to bullfrog tadpoles and this was presumed to be because of food resource competition. Smith, Dingfelder, and Vaala (2004) observed larger hylid tadpoles
having a negative effect on smaller ranid tadpoles’ growth rates when food was limited.

There is little evidence demonstrating food resource competition among wild tadpoles. Seale (1980) examined the gut contents of four species of tadpoles, collected from the same body of water, which varied greatly in size. The diet of the four species were inconclusive. There was no visual difference in the content of the ingested material. Rossa-Feres, Jim, and Fonseca (2004) visually examined the gut contents of 13 species of tadpoles and found that most of the material ingested was the same, the amount of material differed by tadpole types. Santos, Protázio, Moura, and Juncá (2016) observed similar material in the digestive tracts of two tadpole species collected from a natural habitat concluding that they may be competing for food. Visually examining ingested material for differences in composition is not an effective way to demonstrate food resource competition among wild tadpoles.

It is difficult to determine the diet of wild caught tadpoles by visually examining their gut material or feces because the content is mostly indistinguishable. Most of the material is plant matter and algae (Altig, Whiles, & Taylor, 2002; Seale, 1980). Additionally, it is unclear what has already been digested. Examining their fecal bacterial community however, may be informative because the bacterial species present is likely to reflect their diet (Matijašić et al., 2014). The response of the fecal bacterial communities in tadpoles to changes in diet could be used as an alternative to visually distinguishing the ingested material.
Research on how fecal microbial communities change in response to diet has been studied in other organisms. In a human study, changes in the fecal microbial numbers of individuals were primarily affected by their dietary intake (Simoes et al., 2014). Matijašić et al. (2014) observed differences in the fecal bacterial community of vegans and non-vegans. Ingerslev et al. (2014) noted a difference in the fecal microbial communities of rainbow trout eating different diets. While the effect of diet on the fecal bacterial communities of tadpoles has not been studied the fecal bacterial communities of other organisms respond to changes in diet. Tadpoles do not have the ability to digest the complex carbohydrates found in plant material and could be relying on symbiotic relationships with microbes to digest this material producing short chain fatty acids the host can use as a source of energy (Pryor & Bjorndal, 2005). Additionally, the type of complex carbohydrates available for use by microbes can affect the composition of the fecal bacterial community in humans (Yang, Martínez, Walter, Keshavarzian, & Rose, 2013). Not only would feeding on different substrates effect the fecal bacterial community but feeding in an aquatic environment would also expose tadpoles to a wide range of microbes that may survive passage through the digestive tract and be detected in the feces (Vences et al., 2016). Thus, individuals feeding on the same food resources are expected to have similar fecal bacterial communities.

Several studies have been conducted on gut microbiology of tadpoles. Hird et al. (1983) noted many species of Gram negative bacteria found in the intestines of northern leopard frog tadpoles collected in the wild. Pryor and
Bjorndal (2005) captured scanning electron microscopy images of bacteria lining the colon wall of bullfrog tadpoles. Many species of bacteria are even associated with the intestinal tract of anurans (Fedewa, 2006). Pryor (2008) found that the total number of bacteria increased towards the distal end of the digestive tract in bullfrog tadpoles and the density of the microbiota were similar to other animals that harbored gut microbiota. Kohol, Cary, Karasov, and Dearing (2013) examined the gut microbiota of lab raised northern leopard frog (Lithobates pipens) tadpoles and found that members of the phyla Proteobacteria and Firmicutes were the most dominant. Vences et al. (2016) examined the gut microbiota of wild caught tadpoles from Brazil and Madagascar and found that members of the phyla Firmicutes, Proteobacteria and Synergistetes were the most dominant in tadpoles from both locations. Tadpoles seem to harbor a diverse gut microbiota, and similar to other vertebrates, have microbial communities dominated by members of the phyla Firmicutes and Proteobacteria.

The goal of this research was to determine if wild populations of tadpoles were competing for the same food resource using fecal bacterial communities as an indicator of shifts in diet. Competitive interactions among tadpoles have been observed in lab settings and usually the larger competitor has had negative effects on the smaller competitor. Food in these studies (Boone et al., 2004; Katzmann et al., 2003; Smith et al., 2004; Werner, 1992) has been a limiting resource. However, tadpoles in a natural habitat are not limited to one source of food. In a habitat where food is not a limiting resource are tadpoles competing for the same food? If tadpoles were eating the same thing they could have potential
negative interactions with larger tadpoles. Although several studies have shown
the negative effect large tadpoles have on smaller tadpoles’ growth rates when
competing for limited food resources, studies of whether this competitive
interaction occurs in nature are lacking. Additionally, visually examining the
ingested material of wild tadpoles has shown to be inconclusive because the
material is difficult to distinguish. Can the fecal bacterial community serve as a
reliable alternative for visually examining ingested material for changes in diet? If
so, differences in diet would be reflected by changes in the composition of the
tadpole fecal bacterial community. If tadpoles in a natural setting are avoiding
competition for food by eating different things then the difference in diet would
lead to an observable difference in the fecal bacterial community.

One objective of this research was to determine whether diet affects the
fecal microbial community in tadpoles. I hypothesized that differences in
prepared diets would be reflected by changes in the fecal bacterial community. If
so, the composition of fecal microbial communities of field caught or wild
tadpoles can be used to infer whether tadpoles of different size or species
compete for the same food in their natural habitats. An additional objective was
to determine if competition for food exists between small and large southern
leopard frog tadpoles in natural setting using the fecal bacterial communities of
tadpoles as indicators of differences in diet. I hypothesized that small and large
southern leopard frog tadpoles avoid competition by eating different things.
Another objective was to determine if competition for food exists between larger
southern leopard frog and smaller green tree frog tadpoles in natural setting
using the fecal bacterial communities of tadpoles as indicators of differences in diet. I hypothesized that southern leopard frog and green tree frog tadpoles avoid competition by eating different things. If the fecal bacterial communities can be used as indicators of changes in diet then tadpoles avoiding competition for food would have different fecal bacterial communities. Another objective was to identify members of the fecal bacterial communities collected from wild tadpoles.

In order to test my hypotheses I used a combination of laboratory and field studies. After feeding tadpoles two different diets in the laboratory setting, High Resolution Melting Analysis (HRM) was used to detect differences in the composition of fecal bacterial communities. My hypothesis was that differences in prepared diets would be reflected by changes in the fecal bacterial community. Tadpoles eating the same prepared diet would have similar fecal bacterial communities. To ensure that competing tadpoles in the field studies had access to the same food and to limit their dispersal, they were placed inside enclosures in a natural pond. After feeding for a week, their fecal bacterial communities were compared using HRM and high-throughput sequencing. My hypothesis was that tadpoles avoided intra-specific competition for food by eating different things and therefore would have different fecal bacterial communities. I also hypothesize that tadpoles avoided inter-specific competition for food by eating different things and therefore would have different fecal bacterial communities.
CHAPTER II – METHODS AND MATERIALS

Study Site

Tadpoles used in laboratory studies were collected from a small pond in Hattiesburg, Mississippi, USA (N31°20.821 W089°22.355) Field studies were conducted using the same pond. The tadpoles most commonly found at the study site during the spring are southern leopard frog (*Lithobates sphenopephala*) tadpoles. Typically, eggs are laid in late December to early January and again in late February to early March. When the tadpoles in the second breeding event begin feeding, larger tadpoles are already present and feeding. This provided an opportunity to study intra-specific competition. During early summer months, green tree frog (*Hyla cinerea*) tadpoles dominated the study site. During early summer months tadpoles of both species occupy the pond, with the leopard frog tadpoles being larger, providing an opportunity to study inter-specific competition. Most of the tadpoles found at the study site were always found within one specific area. This area and an additional area where tadpoles were rarely found were chosen as the locations used in the field study. There was an obvious difference in available food items between the two locations so it was expected that tadpole fecal bacterial communities would differ. There was less aquatic vegetation and detritus in the area where tadpoles were rarely found.
Tadpole and Feces Collection and DNA Extraction

Tadpoles were captured at the study site using gloved hands or a small dip net. To collect feces from freshly caught tadpoles, they were placed individually in two stacked polyethylene cups containing approximately 200ml of filtered, aged tap water. The upper cup had a mesh screen at the bottom that allowed feces to fall through to the lower cup undisturbed. After removing the tadpole, fecal samples were collected using a sterile transfer pipette. All fecal samples were collected in the same way. After centrifugation to concentrate the feces in a micro-centrifuge tube, DNA was extracted and purified using the Powersoil DNA Extraction Kit (MoBio, Carlsbad, CA) following the manufacturer’s recommended protocol. Extracted DNA was then quantified using sample absorbance at 260 nm. All DNA extractions and quantification were performed in this manner. Tadpoles that did not produce enough feces were omitted from each analysis. Animal collection and laboratory experiments were approved by the Mississippi Department of Wildlife, Fisheries, and Parks (Permit No. 0623151) and the University of Southern Mississippi IACUC Committee (Protocol No. 13121202), respectively.

HRM

Polymerase chain reaction (PCR) was used to amplify the hypervariable V3 region of the bacterial 16S rRNA gene. Each PCR was performed in a 25ul reaction volume containing 12.5ul of EconoTaq PLUS 2X Master Mix (Lucigen), 10ng extracted DNA, 2.5ul each of 5 uM 341-F (5'-CCTACGGGAGGCAGCAG-3'), and 2.5ul each of 5 uM 805-R (5'-CCGCAATTGCGACCTTGT-3').
3’) and 518-R (5’-ATTACCGCGGCTGCTGG-3’) primers, 1.25ul of 20X EvaGreen (Biotium), 0.5ul of 25mM MgCl2 to adjust the final magnesium concentration to 2mM and DNase/RNase-free water to a volume of 25ul. Initial DNA melting took place at 94°C for 4 min followed by 20 cycles of melting at 94°C for 1 min, annealing at 66°C for 20 s with a 0.5°C decrease in temperature after each cycle and extension at 72°C for 30 s. Touchdown PCR was used to increase the specificity of DNA amplification (Korbie & Mattick, 2008). The initial 20 cycles were followed by 10 cycles of melting at 94°C for 1 min, annealing at 56°C for 20 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min.

To detect differences in fecal bacterial community composition, high resolution melting analysis of the amplified DNA was performed using a Rotor-Gene 6000 thermal cycler (Corbett Life Sciences). Sample fluorescence was acquired from 70°C to 95°C at 0.2°C increments two seconds after each temperature increment had been reached. First derivatives of the change in sample fluorescence over time (dF/dT) at each 0.2°C increment between 75°C and 90°C were calculated using the Rotor-Gene 6000 Series Software version 1.7. All samples were run in technical duplicates and the results averaged using the replicate view function of the Rotor-Gene software. The first derivative values of fluorescence at each 0.2°C temperature increment between 75 and 90°C were calculated for each sample. These values were summed resulting in a total first derivative value of fluorescence for each sample. The first derivative values at each 0.2°C increment were divided by the total first derivative value of each
sample resulting in a relative first derivative value of fluorescence at each temperature increment.

PERMANOVA

A permutational analysis of variance (PERMANOVA) (Anderson, 2001), using Bray-Curtis as the distance metric with 10,000 permutations, was used to test for significant differences among the relative first derivative values at each temperature increment of the HRM melting peak profiles. PERMANOVAs were performed using the adonis function of the vegan package in R (Version 3.2.2). P-values less than 0.05 were considered significant.

NMDS

Relationship among fecal microbial communities were visualized using non-metric multidimensional scaling (NMDS). The relative first derivative values at each temperature increment of the HRM melting peak profiles were used to construct dis-similarity matrices based on the Bray-Curtis metric (Kim and Lee, 2015). NMDS, retaining two dimensions ($k = 2$), was performed on all sets of data using the metaMDS function of the vegan package in R (Version 3.2.2). Bacterial communities more similar in composition lie closer in proximity to one another compared to those that are more dissimilar in composition when visualizing the data points in two dimensions. Data points were exported into Microsoft Excel 2013 to reconstruct figures.
Laboratory Study: Food Preparation

A high protein diet (44% fish meal) was made by mixing 100 g of frog brittle powder (eNasco, Fort Atkinson, WI, USA) with 100 ml of a molten 0.5% agar solution. A diet lower in protein and resembling a more omnivorous diet was made by mixing 50 g of frog brittle powder and 50 g of oven dried, powdered timothy grass pellets (Standlee, Kimberly, ID, USA) with 100 ml of a molten 0.5% agar solution. Each mixture was placed in a plastic bag and thoroughly mixed by hand. The food mixtures were then placed into sterile 50 ml syringes and extruded onto a flat surface into long noodles in a sterile biological safety cabinet. The noodles were dried for 24 hours at room temperature, broken into small pieces and stored at 4°C until used for feeding.

Laboratory Study: Housing and Feeding

To determine whether the bacterial communities in the feces of tadpoles can be used as indicators of differences in diet 12 green tree frog tadpoles and 12 southern leopard frog tadpoles were collected. Six of each species were fed the high protein diet and the others were fed the diet higher in complex carbohydrates. Tadpoles were housed in 1 L polypropylene bowls with approximately 250 ml of aged, filtered tap water. The photoperiod was 14 hours light and 10 hours dark and the temperature was held constant at 28°C. Tadpoles were allowed to feed ad libitum while food remained in the container. After 24 hours, the water was replaced, old food and feces removed and fresh food was given. Tadpoles were allowed to feed for seven full days before
collection of feces took place. Collection of feces, DNA extraction, HRM and NMDS were performed as previously described. A two-factor PERMANOVA including the interaction term was performed to test for the effect of species and diet on the composition of the fecal bacterial communities. This procedure was repeated using 12 small and 12 large southern leopard frog tadpoles. A two-factor PERMANOVA including the interaction term was also performed to test for the effect of size and diet on the composition of the fecal bacterial communities.

Field Study: Bucket Enclosures

To determine if intraspecific and interspecific competition between tadpoles occur under natural conditions, field enclosure studies were conducted. To ensure that tadpoles had restricted access to the same potential food items, bucket enclosures were used. The bottom of each 19 L bucket was removed and a window approximately 8.5 cm by 19 cm was cut into the side. A plastic mesh screen was attached to the window to prevent tadpoles from escaping and to allow water exchange between the bucket and the pond. The buckets were always placed at the two locations previously mentioned within the study site. Once placed in the water, the buckets were pressed into the sediment, sealing the bottom. Window screen was used to seal the top. To reduce the chance of disturbance of experiments by weather or animals at the study site, they were used for one week only.

To determine the effect of tadpole size and habitat on southern leopard frog tadpole fecal bacterial communities, two enclosures were placed at each of
two locations described above. Twelve small and twelve large recently captured southern leopard frog tadpoles were placed in each enclosure. They were allowed to remain, undisturbed for seven days. After seven days, six tadpoles of each size were removed from each enclosure resulting in 24 tadpoles. Collection of feces, DNA extraction, HRM and NMDS were performed as previously mentioned. A two-factor PERMANOVA including the interaction term was performed to test for the effect of size and location on the fecal microbial communities of small and large southern leopard frog tadpoles. This procedure was repeated the following summer.

To determine the effect of habitat on the fecal bacterial community of green tree frog tadpoles, two enclosures were placed at the same two locations. Six recently captured green tree frog tadpoles were placed in each enclosure. They were allowed to remain, undisturbed for seven days. After seven day, three tadpoles were removed from each enclosure resulting in 12 tadpoles. Collection of feces, DNA extraction, HRM and NMDS were performed as previously mentioned. A one-factor PERMANOVA was performed to test for the effect of location on the fecal bacterial communities. This procedure was also repeated the following summer. Attempts to study inter-specific competition using the bucket enclosures failed due to non-overlapping larval periods during the summers these field studies were conducted.
Field Study: Fecal Bacterial Community Composition of Wild Tadpoles

To identify members of the fecal bacterial communities of wild tadpoles, three small and three large southern leopard frog tadpoles were collected from the location at the study site where tadpoles were always found high in abundance. Three green tree frog and three southern leopard frog tadpoles were also collected from the same location. Summer 2014 was the only summer where larval periods of both species overlapped. They were immediately brought back to the laboratory where fecal collection and DNA extraction took place. Instead of utilizing HRM, next generation sequencing was performed to compare the composition of fecal bacterial communities. The fecal bacterial community of each tadpole was assessed by sequencing the V3-V4 region of the bacterial 16S rRNA gene. Sequencing was performed by the core sequencing facility at the University of Mississippi Medical Center, Jackson MS in both directions using Illumina MiSeq. The following forward and reverse primers were used to amplify the V3-V4 region:

16S-F:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S-R:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Only full length, quality filtered (q25) and overlapped reads were used in the sequencing data analysis. Using the FASTX-Toolkit (Version 0.0.14), 
reads were trimmed to contain only the sequence of the hypervariable V3-V4 region. A minimum similarity threshold of 97% was used in assigning OTUs and for parsing out chimeric sequences using USEARCH Version 8.1 (Edgar, 2013). Sequences were classified at the genus level by comparison to reference sequences in the Ribosomal Database Project (RDP) (version 11.4) (Wang, Garrity, Tiedje, & Cole, 2007) with default parameters. Classified reads were organized at the phylum level and then exported into Microsoft Excel 2013 to construct bar graphs of relative abundances of 16s rRNA genes.
CHAPTER III – RESULTS

Laboratory Study

Diet did not have a significant effect on the fecal bacterial communities of southern leopard frog and green tree frog tadpoles \((df = 1, \text{pseudo-}F = 1.36 \text{ and } p = 0.2369)\), however species had a significant effect on the fecal bacterial communities of southern leopard frog and green tree frog tadpoles \((df = 1, \text{pseudo-}F = 6.09 \text{ and } p = 0.0068)\). The NMDS ordination plot of the HRM data \((\text{stress} = 0.072)\) did not reveal distinct clusters containing fecal bacterial communities associated with either species or diet (Figure 1). The species showed general separation on the second axis.

Figure 1. Effect of Diet on Southern Leopard Frog and Green Tree Frog Tadpole Fecal Bacterial Communities.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while...
Diet had a significant effect on the fecal bacterial communities of small and large southern leopard frog tadpoles \((df = 1, \text{pseudo-}F = 4.15 \text{ and } p = 0.0132)\). Additionally, size did not have a significant effect on the fecal bacterial communities of small and large southern leopard frog tadpoles \((df = 1, \text{pseudo-}F = 1.95 \text{ and } p = 0.1211)\). The NMDS ordination plot of the HRM data (stress = 0.089) revealed clusters containing fecal bacterial communities associated with diet (Figure 2).

**Figure 2.** Effect of Diet and Size on Southern Leopard Frog Tadpole Fecal Bacterial Communities.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while retaining two dimensions. SLF = southern leopard frog, FB = frog brittle and FB/TG = frog brittle and timothy grass (1:1).
Field Study: Bucket Enclosures

The fecal bacterial communities of small and large southern leopard frog tadpoles enclosed at the two locations during summer 2015 were significantly different ($df=1$, $pseudo-F = 78.52$ and $p < 0.05$) and were significantly affected by size ($df=1$, $pseudo-F = 9.60$ and $p = 0.0023$). The interaction term was also significant ($df=1$, $pseudo-F = 4.12$ and $p = 0.0395$), indicating the effect of size was not the same between the two locations. The NMDS ordination plot of the HRM data (stress = 0.042) revealed distinct clusters containing fecal bacterial communities associated with each location and the size of tadpoles (Figure 3).

**Figure 3.** Effect of Size and Location on Small and Large Southern Leopard Frog Tadpole Fecal Bacterial Communities During Summer 2015.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while retaining two dimensions. SLF= southern leopard frog.
The fecal bacterial communities of small and large southern leopard frog tadpoles enclosed at the two locations during summer 2016 were significantly different ($df = 1$, \textit{pseudo-}F = 7.29 and $p = 0.0007$) and were not significantly affected by size ($df = 1$, \textit{pseudo-}F = 0.089 and $p = 0.9821$). The NMDS ordination plot of the HRM data (stress = 0.1003) revealed clusters containing fecal bacterial communities associated with each location (Figure 4).

\textbf{Figure 4.} Effect of Size and Location on Small and Large Southern Leopard Frog Tadpole Fecal Bacterial Communities During Summer 2016.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while retaining two dimensions. SLF= southern leopard frog.

The fecal bacterial communities of green tree frog tadpoles enclosed at two separate locations during summer 2015 were significantly different ($df = 1$, \textit{pseudo-}F = 6.57 and $p = 0.0147$). The NMDS ordination plot of the HRM data
(stress = 0.040) revealed distinct clusters containing fecal bacterial communities associated with each location (Figure 5).

Figure 5. Effect of Location on Green Tree Frog Tadpole Fecal Bacterial Communities During Summer 2015.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while retaining two dimensions. Blue = location 1 and red = location 2. GTF = green tree frog.

The fecal bacterial communities of green tree frog tadpoles enclosed at two separate locations during summer 2016 were significantly different (df = 1, pseudo-$F$ = 7.33 and $p = 0.0057$). The NMDS ordination plot of the HRM data (stress = 0.0003) revealed distinct clusters containing fecal bacterial communities associated with each location (Figure 6).
Figure 6. Effect of Location on Green Tree Frog Tadpole Fecal Bacterial Communities During Summer 2016.

Non-metric multidimensional scaling ordination plots of fecal bacterial communities based on high resolution melting analysis of the hypervariable V3 region of the bacterial 16S rRNA gene. Distance measure used was Bray-Curtis while retaining two dimensions. An HRM control sample (black) was included in the ordination because the within location variation was too small to be observed in the original figure. GTF = green tree frog.

Field Study: Fecal Bacterial Community Composition of Wild Tadpoles

A total of 72,678 full length, overlapping sequences were obtained (12,113 ± 4,291 sequences per sample) from wild caught small and large southern leopard frog tadpoles, containing 701 OTUs (229 ± 29 OTUs per sample). The composition of fecal bacterial communities of wild caught small and large southern leopard frog tadpoles were similar at the phylum level (Figure 7). Both were mostly dominated by *Fusobacteria* (19.4 % and 32.9 % for small and large tadpoles, respectively), *Bacteroidetes* (25.9 % and 24.1 %) and *Firmicutes* (19.3
% and 14.7 %). Bacteria in the phylum *Verrumicrobia* were more abundant in small tadpoles (4.0 %) compared to the larger conspecifics (1.2 %).

Figure 7. Fecal Bacterial Community Composition of Wild Caught Small and Large Southern Leopard Frog Tadpoles at the Phylum Level.

Bar plot of the relative abundance of bacterial sequence reads identified at the phylum level in wild caught small and large southern leopard frog tadpole fecal bacterial communities. A number identifying individual tadpoles of each size was used.

A total of 57,605 full length, overlapping sequences were obtained (9,600 ± 3,443 sequences per sample) from wild caught southern leopard frog and green tree frog tadpoles, containing 668 OTUs (179 ± 48 OTUs per sample). The relative percent abundance of sequencing reads obtained from fecal bacterial communities of wild caught southern leopard frog and green tree frog tadpoles were distinguishable at the phylum level (Figure 8). Both species were mostly dominated by members of the phyla *Fusobacteria* (54.2 and 13.3 % for southern leopard frog and green tree frog tadpoles, respectively) and *Firmicutes* (16.59
and 52.74 % for southern leopard frog and green tree frog tadpoles, respectively), Bacteria in the phylum *Verrumicrobia* were more abundant in southern leopard frog (2.57 %) than in green tree frog (0.78 %) tadpoles. Bacteria in the phylum *Fusobacteria* were highly dominant in southern leopard frog tadpole feces (54.21 %) while *Firmicutes* dominated the bacterial communities of green tree frog tadpoles (52.74 %). Additionally, *Proteobacteria* were more abundant in southern leopard frog tadpole feces (6.09 %) than in green tree frog tadpole feces (3.86 %).

*Figure 8. Fecal Bacterial Community Composition of Wild Caught Southern Leopard Frog and Green Tree Frog Tadpoles at the Phylum Level.*

Bar plot of the relative abundance of sequence reads identified at the phylum level in wild caught southern leopard frog and green tree frog tadpole fecal bacterial communities. SLF = southern leopard frog tadpole and GTF = green tree frog tadpole with a number identifying individual tadpoles of each species.
CHAPTER IV – DISCUSSION

Competition for food resources among tadpoles has been studied in the lab and in the field. However in lab settings, tadpoles were usually limited to a single food source and a larger competitor reduced the smaller tadpole’s growth (Boone et al., 2004; Katzmann et al., 2003; Smith et al., 2004; Werner, 1992). Field studies aimed at competitive feeding interactions among tadpoles are lacking and most accounts are based on the visual examination of partially digested, indistinguishable material (Rossa-Feres et al., 2004; Santos et al., 2016; Seale, 1980). Tadpoles of a larger size might have a competitive advantage over smaller tadpoles in a natural setting if they were eating the same thing. Tadpoles suffering from a negative interaction with other tadpoles may be smaller at metamorphosis, decreasing fitness (Gosner, 1960). The first research objective was to determine whether the bacterial communities associated with the feces of tadpoles eating the same food, became similar. By feeding tadpoles prepared laboratory diets, it was expected that the fecal bacterial communities of tadpoles eating the same food would be similar. The second objective was to determine if competition for food exists among tadpoles in a natural setting using the fecal bacterial communities of tadpoles as indicators of differences in diet. By allowing tadpoles to feed in their natural habitat it was expected that the fecal bacterial communities would be similar among tadpoles of different size, species and location. Differences observed among the fecal bacterial community of tadpoles from the field were presumed to be the result of changes in diet.
Ultimately, I expected to ascertain whether or not tadpoles in their natural habitat were competing for the same food resources.

Laboratory Study

After feeding on two prepared diets that differed in the percentage of complex carbohydrates, the fecal bacterial communities of southern leopard frog and green tree frog tadpoles eating the same diet were significantly different (Figure 1), rejecting my hypothesis. Additionally, the variation among the fecal bacterial communities explained by species was significant. Tadpoles used for this particular lab study were collected from four different locations. The two species never occupied the same body of water at the study site due to evaporation of the pond. The fecal bacterial communities of these tadpoles were expected to be different when the lab study began, given that they were collected at different locations. Given additional time to feed, the effect of diet on the fecal bacterial communities may have been more noticeable. Also, tadpoles of one species may have never been exposed to the bacteria residing in the gut of the other species. Prepared diets may enrich certain bacteria, but only those already present in the gut. Without prior exposure to the same bacteria, the effect of a specific diet on fecal bacterial community composition would differ.

After feeding on two prepared diets that differed in the percentage of complex carbohydrates, the fecal bacterial communities of small and large southern leopard frog tadpoles eating the same diet were not significantly different (Figure 2). Additionally, the variation among the fecal bacterial
communities explained by size was not significant. Unlike the tadpoles used previously, these tadpoles were collected at two different locations. However, there was a period where they all occupied the same body of water and were exposed to the same bacteria. It is likely that these tadpoles entered the laboratory already harboring the bacteria enriched by the two prepared diets.

Field Study: Bucket Enclosures

After enclosing small and large southern leopard frog tadpoles at two locations during summer 2015, the fecal bacterial communities were significantly affected by size and location (Figure 3), both of which were expected. The interaction term was also significant; indicating the effect of size, while significant, was dependent on location. Clear differences between the fecal bacterial communities of small and large southern leopard frog tadpoles at both locations indicate that the tadpoles were not eating the same thing and avoiding a potentially negative interaction. Additionally, the difference between small and large southern leopard frog tadpole fecal bacterial communities at the location where tadpoles were commonly found indicates little overlap in diet. With an abundance of food items, little overlap in tadpole diet at this location would support greater numbers of tadpoles because competitive interactions are not occurring. This may explain why tadpoles at the study site preferred this location over all others.

After enclosing small and large southern leopard frog tadpoles at two locations during summer 2016, the fecal bacterial communities were significantly
affected by location, which was expected. The variation explained by size was not significant, which was unexpected. Clear differences between the fecal bacterial communities of small and large tadpoles at the different locations indicate that the tadpoles were feeding on different things (Figure 4). Unlike the previous observation however, small and large leopard frog tadpoles' fecal bacterial communities were not significantly different at each location indicating that the tadpoles were feeding on similar things. The location where tadpoles were commonly found had been altered by heavy machinery between summer 2015 and summer 2016. As a consequence, it appeared food availability at this location was drastically reduced. Without being able to choose from the diverse array of potential food items like before, tadpoles were likely forced to eat what little was available to them.

After enclosing green tree frog tadpoles at two locations during summer 2015, the fecal bacterial communities were significantly different. Like before, there were significant differences based on location (Figure 5), indicating that they were feeding on different things. The fecal bacterial communities of green tree frog tadpoles enclosed at the two locations were also significantly different during summer 2016 (Figure 6). The green tree frogs were to be placed in enclosures with southern leopard frog tadpoles but during the summers of 2015 and 2016 their larval periods did not overlap as they did during summer 2014.

The consistent and significant effect of location on the fecal bacterial communities of tadpoles used in the field study was not surprising given that food
availability appeared to differ between the two locations. There was aquatic vegetation, overhanging vegetation, detritus and algae highly abundant in this area. Tadpoles were always sampled from the location where they were always abundant and taken to the other location. Assuming tadpoles from the original location had similar fecal bacterial communities to those enclosed there, a change in diet was reflected by the fecal bacterial communities after just seven days of being moved to another location.

Field Study: Fecal Bacterial Community Composition of Wild Tadpoles

After capturing wild small and large southern leopard frog tadpoles at the location where they were commonly found, the sequenced fecal bacterial communities were similar at the phylum level (Figure 7). There were noticeable differences in the relative abundance of reads belonging to the phylum *Verrumicrobia*, with smaller tadpoles having a larger proportion. This phylum has been recently established and members are most commonly associated with soil and aquatic environments. The relative abundance of reads belonging to the phylum *Firmicutes* were lower in small tadpoles. A majority of the members of this phylum were identified as un-cultured *Clostridium spp.* Additionally, the large tadpoles had a higher proportion of reads belonging to the phylum *Fusobacteria*. The observed differences between the proportions of reads identified at the phylum level indicate that the tadpoles were feeding on different things.

After capturing wild southern leopard frog and green tree frog tadpoles at the location where they were commonly found, the sequenced fecal bacterial
communities were easily distinguishable at the phylum level (Figure 8). Southern leopard frog tadpoles had higher proportions of reads belonging to the phyla *Verrumicrobia, Proteobacteria, Fusobacteria* and *Actinobacteria*. Green tree frog tadpoles had a higher proportion of reads belonging to the phyla *Firmicutes*. The observed differences between the proportions of reads identified at the phylum level indicate that wild southern leopard frog and green tree frog tadpoles were feeding on different things.

Only one study identifying the gut microbiota of wild tadpoles has been published to date. Vences et al. (2016) identified the bacteria present in the midgut of numerous tadpole species from both Brazil and Madagascar. We sampled feces while Vences et al. (2016) sampled a portion of the digestive tract with partially digested material still inside. The difference in sampling of the bacterial communities makes it difficult to compare the results as they concluded that the majority of the bacterial DNA that was sequenced belonged to the true gut microbiota that line the digestive tract. As we collected feces, it was presumed that members of the true gut microbiota were outnumbered by microbes that survived passage through the digestive tract. Although bacteria found in the feces may not represent the true gut microbiota, the presence of certain bacteria in the feces can be used to make inferences about diet selection in wild tadpoles.

Most reads belonging to the phylum *Fusobacteria* were identified as a single species of an un-cultured member of the genus *Cetobacterium*. The only
two known members of this genus produce short chain fatty acids as a metabolic by product which may benefit a host by providing nutrition (Finegold et al., 2003). Given that tadpoles have been known to ingest indigestible material, there is a lack of published accounts of symbiotic fermentation by bacteria in the digestive tract of tadpoles.

Future studies could be directed towards determining if the mucosal associated bacterial community in tadpoles differ from the fecal bacterial community. According to Vences et al. (2016) the tadpole gut microbiota may not be represented by the fecal bacterial community. Additionally, Pryor (2008) captured scanning electron microscopy images of mucosal associated bacterial lining the digestive tract of tadpoles after the gut content was removed. Tadpoles seem to harbor resident bacteria and bacteria associated with the ingested material. If the bacterial communities associated with the feces are simply passing though the digestive tract, these bacterial communities may not be impactful to a tadpole. However, the effects of diet on the resident bacterial community could have significant impacts on tadpole health and nutrition.

These tadpoles contain bacteria known to produce short chain fatty acids and while most of the material found in tadpole digestive tracts is plant material (Arias, Peltzer, & Lajmanovich, 2002; Diaz-Paniagua, 1985; Jenssen, 1967; Seale, 1980) it is unclear if tadpoles are actually benefiting from the breakdown of this material by microbes. Another study that could be performed is to examine the response of tadpole growth rates, after feeding on a diet high in complex
carbohydrates, with and without resident bacterial communities. There are no published accounts of symbiotic fermentation of indigestible material by bacteria in tadpoles however, Pryor and Bjorndal (2005) anaerobically incubated the contents of bullfrog colons and found that the concentration of short chain fatty acids increased over time. If tadpoles are relying on bacteria to break down indigestible material then tadpoles without a resident gut microbiota would show decreased growth rates when compared to those with a resident bacterial community.

In conclusion, fecal bacterial communities of tadpoles eating prepared food in a lab setting are not indicators of shifts in diet, but fecal bacterial communities of wild caught tadpoles can serve as indicators of shifts in diet. Food in a lab is prepared relatively microbe free while tadpoles in the wild constantly ingest bacteria that survive passage through the digestive tract. Prepared diets only enrich bacteria that are already present in the gut. The lack of microbes in the prepared diets mean that resident bacteria were most likely shed in the feces. Tadpoles entering the lab setting with differences in their resident bacteria would have differences in their fecal bacterial communities when eating the same thing. Additionally, smaller southern leopard frog tadpoles are avoiding potential competitive interactions in their natural habitat by eating different things than larger southern leopard frog tadpoles. However, when food resources are limited small and large tadpoles appear to be eating things more similar in composition which could have a negative impact on the smaller
tadpoles. Smaller green tree frog tadpoles also are avoiding potential competitive interactions in their natural habitat by eating different things than larger southern leopard frog tadpoles. The fecal bacterial communities of wild tadpoles are complex, with multiple phyla represented, but the role of these bacteria are unknown. Additional research is needed to better understand the relationship between tadpoles and the bacterial communities that reside in their digestive tracts.
APPENDIX A - FORMS

MDWFP Collection Permit

MISISSIPPI
DEPARTMENT OF WILDLIFE, FISHERIES, AND PARKS

Sam Polles, Ph.D.
Executive Director

2 December 2014

ADMINISTRATIVE SCIENTIFIC COLLECTION PERMIT NUMBER 0623151

TO WHOM IT MAY CONCERN:

Permission has been granted to:

Steven Evermann
Department of Biological Sciences
University of Southern Mississippi
118 College Drive Box 5018
Hattiesburg, MS 39406,

to collect eggs and tadpoles of the Southern Leopard Frog (Rana sphenocephala) and Green Tree Frog (Hyla cinerea) in South Mississippi. In addition, tissue samples may be collected from non-listed (federal or state, see attached list) frogs in Mississippi. Frogs must be released otherwise unharmed after tissue collection.

This permit is valid from 2 December 2014 to 1 December 2015.

SPECIFIC CONDITIONS AND RESTRICTIONS

1.) Collections will be made using dip nets and by hand.

2.) Any tadpoles used in experiments must be euthanized at the conclusion of the experiments.

GENERAL CONDITIONS AND RESTRICTIONS:

1) Specimens retained after collection must be placed in a public museum or collection where they will be available for examination by the scientific community. The Mississippi Museum of Natural Science (MMNS), 2148 Riverside Drive, Jackson, MS 39202-1353, ph: (601) 576-6000, is the principal repository of terrestrial and freshwater vertebrates, freshwater mollusks, and crayfish collected in Mississippi, and welcomes additional specimens. Unless alternative arrangements are made with the MMNS Collections manager (Scott Peyton, 601-576-6000) or curatorial staff at the MMNS,
all collections of federally listed and state listed species will be deposited at the Mississippi Museum of Natural Science.

2) This permit does not authorize the taking of any federally threatened or endangered species or any state endangered species (list attached), unless otherwise specified in this permit.

3) All wildlife, including fish and invertebrates, collected under the permit are considered to be a natural resource of the State of Mississippi. Collected specimens should be handled humanely, and live, uninjured specimens not needed for permanent collections should be returned to appropriate habitat at the capture locality when no longer required. Specimens that die incidental to collection activities or which are intentionally preserved must be maintained in a scientifically acceptable fashion in a study/research collection where they will be available for examination by the general scientific community, or should be offered to a museum. The intent of the scientific collecting permit is to encourage meaningful study and to discourage the loss of specimens and information.

4) The issuance of a permit does not authorize trespass by the permittee. Permit is also void if permittee has not obtained other necessary permissions/permits for collection activities on public lands.

5) Collection of migratory birds, their nests, or eggs, collection of federally listed endangered species, and collection of federally listed threatened species (when the collector is not an agent of the State of Mississippi) requires a federal permit in addition to a state permit.

6) Copies of publications, survey reports, and other printed materials prepared as a result of this collection should be sent to the Mississippi Museum of Natural Science (Attn: Scientific Collection Permit Review Committee) 2148 Riverside Dr., Jackson, MS 39202.

REQUIRED COLLECTING PERMIT REPORTS

1) A collecting permit report using format described below must be filed within 15 days of the expiration of the permit. A new permit will not be issued until the report has been received. Collection reports should list taxa collected, number of individuals of each, exact collection locality and date of collection. Locality information must include the county of collection, and it is preferred that precise locality information be provided in latitude/longitude (GPS) or in the township, range, and section (TRS) system. If the TRS system is used, precise location within a section should be indicated (e.g.: NW4 of SE4 of Sec 11), if possible. If GPS or TRS information is not provided, include instead a clear and precise description of the location of the collection site relative to the nearest named or numbered road, town, intersection, and/or other feature(s) likely to be mapped on a USGS quad map. For aquatic species, provide the name of the stream in which collections were made.
Instructions for completing Scientific Collections Report

Below is a list of information that should be included in scientific collecting reports, if it applies to the activities covered by the collecting permit. Because of the broad spectrum of activities covered by collecting permits, individual reports may require an altered format or other information not described below. If possible, reports should be submitted electronically in a spreadsheet format (preferably in Excel or Access). A blank spreadsheet with the requested fields can be provided to you by Email. Please include the following fields in the spreadsheet, if they apply to the work conducted under the permit. If you cannot provide an electronic version of the collections report, a blank hard copy of a collections report form can be provided to you. If you have any questions, please contact Scott Peyton at 601-576-6000 or collections.manager@mmns.state.ms.us.

A. SPECIES - species name (scientific name), or lowest taxonomic description possible, for each collected taxon.
B. SACRIFICED - If specimens were killed for vouchers or other scientific purposes, indicate the number taken.
C. NUMBER - total number of each species collected or handled. Include both the number taken and the number released in this total.
D. DATE - specific date of each collection.
E. COUNTY - county where each collection occurred.
F. COORDINATES (X) - latitude/longitude, UTM coordinates
G. COORDINATES (Y) - latitude/longitude, UTM coordinates
H. UTM ZONE - UTM coordinates only
I. TRS - Township, Range and Section (optional, but please include if possible)
J. LOCALITY - brief description of locality, e.g. Chickasawhay River 100m upstream from HWY 84 bridge.
K. COLLECTOR(S) - person or persons who made the collection.
L. TISSUE - Indicate the number of specimens from which tissue samples were taken for genetic analysis or other purposes. If no tissue samples were taken, this column can be omitted.
M. DISPOSITION - For sacrificed specimens or tissue samples, list institution(s) where specimens/samples were deposited. For specimens released, indicate where the specimens were released.
N. TEMP EXP or TEMP PROP - If specimens are held in captivity temporarily for experimental purposes or for propagation and later released, a field should be included to capture this information.
O. TAGGED - If specimens are marked or tagged and released, a field should be included to capture this information.
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

NOTICE OF COMMITTEE ACTION

The proposal amendment noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 13121202
PROJECT TITLE: Gut microbiota in southern leopard frogs
PROPOSED PROJECT DATES: 02/2015 - 09/2017
PROJECT TYPE: Modification
PRINCIPAL INVESTIGATOR(S): Shiao Wang
DEPARTMENT: Biological Sciences
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Full Committee Approval
PROTOCOL EXPIRATION DATE: September 30, 2017

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Frank Moore, PhD
IACUC Chair

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Date

02/13/2015
REFERENCES


