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Does Gut Microbiota Affect the Diet Preference In Anurans?

Jordan A. Rice
University of Southern Mississippi

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Does gut microbiota affect the diet preference in anurans?

by

Jordan Rice

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
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Approved by

_____

Shiao Wang, Ph.D., Thesis Advisor
Professor of Biological Sciences

_____

Shiao Wang, Ph.D., Chair
Department of Biological Sciences

_____

Dr. Ellen Weinauer
Dean of Honors College

Abstract

Gut microbiota is a community of bacteria that live in the digestive track of a
host. These microbes assist in the breakdown of indigestible materials as food passes
through the alimentary canal. Metabolites from bacteria may play a role cell to cell
communication with their host and thus gut microbiota may affect the diet preference of
the animal host. Southern Leopard frogs (*Rana sphenocephala*) and Green Tree frogs
(*Hyla cinerea*) were used as focal species to test whether gut microbiota affect their diet
preference. Three groups of tadpoles were tested. The control group was fed a
commercial diet called Frog Brittle which contains essential vitamins and nutrients for
the tadpole. The second diet contained Frog Brittle with the addition of Timothy grass
which has high cellulose content. The third diet contained Frog Brittle with the addition
of chitin. Both cellulose and chitin are generally considered indigestible by animals.
Therefore, my hypothesis is that gut microbes that benefit from cellulose or chitin would
produce chemical cues that influence diet choice among tadpoles with gut microbiota
adapted to diets containing either Timothy grass or chitin.

The gut microbiota was analyzed by amplifying the V3 region of the 16S rDNA
using DNA extracted from tadpole feces. The amplified DNA was analyzed using
Denaturing Gradient Gel Electrophoresis (DGGE) and/or High Resolution Melt (HRM)
Analysis. Diet preference tests were conducted using diets containing fluorescent
microspheres as a tracer.

My results using both DGGE and HRM showed that diet composition affected the
gut microbiota in tadpoles with certain groups of bacteria being more dominant in a diet

dependent manner. However, diet preferences ranged from 0.6443-0.8888 and were

insufficient to support the hypothesis that gut microbiota effects diet preference.

Keywords: Tadpoles, Gut Microbiota, Diet Preference

Dedication

I would like to dedicate this thesis to my parents Jonathan and Angie Rice for always supporting my endeavors whether it is my interest in reptiles, amphibians, or my decision to attend The University of Southern Mississippi. Also, I would like to thank my uncle Patrick Myers for giving me basic knowledge about amphibians and reptiles.

Acknowledgements

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Chapter 1. Introduction

Have you ever thought about why you enjoy eating certain foods? Some people crave chocolate while others do not even like the taste. Many people may think it is because the food tastes good, but there may be another contributor to why people have cravings. In humans there are a myriad of bacteria that live inside and on our bodies. In our intestine alone the bacteria genes outnumber human's genes by 100:1 (Alcock et al., 2014). These bacteria may have an impact on our cravings. Alcock et al. (2014) suggested that people with chocolate desires show differences in their microbial metabolites compared to those who do not care for chocolate.

This role of microbiota in the gut has been an interest for scientists in recent research. Research studies have been done on microbiotas' impact on the immune system. Mueller et al. (2012) presented the impacts of the human immune system when microbiota in the gut becomes unbalanced. These unbalanced communities have been suggested to be the cause of cancer, Crohn's disease, obesity, and diabetes (Mueller et al., 2012). This topic of microbiota can be studied over different fields of research. Techniques such as Illumina Next Generation Sequencing, Polymerase Chain Reactions, and other genetic technology have helped to advance the study of gut microbiomes.

In this project I used Southern Leopard Frog (*Rana sphenocephala)* and Green Tree Frog (*Hyla cinerea)* tadpoles. Diet plays a critical role in the growth and development of anuran larvae as they approach metamorphosis. Research has shown that diets high in protein promote development, and diets high in carbohydrates promote growth (Richter-Boix et al., 2006). Ingested food must be digested in the gut where

communities of microbes live and break down materials that the body is unable to digest such as cellulose and chitin.

Tadpoles diets consist of mainly plant material; however, once they metamorphose into frogs they become mainly insectivores (Kohl et al., 2013). With this transition there must be a change in their diet preference that occurs. The gut microbiota may have an influence on diet preference in tadpoles as they undergo metamorphosis into frogs. The question that will be examined in this project is whether gut microbiota affect tadpoles diet preference.

Chapter 2. Literature Review

What is microbiota? Microbiota is a collection of microorganisms that reside in and on a host's body (Stilling et al., 2014). Microbial communities in the gut have been a current topic in scientific research and have been shown to have profound effects on a wide range of different behaviors in the host. Specifically, research has shown that microbial cell to cell communication with the host's cells has occurred in the intestinal tract of humans causing changes in mood or behavior (Stilling et al., 2014). Other studies have shown specific signaling from microbiota that influences diets of the hosts (Alcock et al., 2014).

Microbiota in the gut helps breakdown indigestible materials consumed by the host. Different communities of bacteria function in the breakdown of carbohydrates, dietary fiber, and some fats (Alcock et al., 2014). When these materials are broken down by bacteria in the gut, they produce metabolites that control levels of amino acids, such as GABA and tryptophan, and monoamines, such as serotonin, histamine and dopamine which aid in neurotransmission in the host (Stilling et al., 2014). These signals to the brain can create responses that trigger mood and behavior such as cravings (Alcock et al., 2014).

One might wonder how microbiota is established in hosts. During birth babies already begin to establish microbiota communities similar to their mothers (Califf et al., 2014). Microbial communities have been found to be personalized for each individual (Califf et al., 2014). Each portion of the body contains different communities of bacteria that are always changing (Califf et al., 2014). These changes can come from an introduction to a new pet, or moving to a new location such as urban to rural, or even a

change in diet.   All of these unique communities of bacteria that are personalized for each individual seem to provide great predictions for a person's health (Califf et al., 2014). Califf (et al., 2014) explains that most healthy adult humans contain the same phyla of bacteria in their gut, but are found in different proportion from individual to individual. Studying these microbiomes is a great way to learn more effective methods for treating diseases for individuals because different people may have different responses to antibiotics or probiotics (Stilling et al., 2014).

Diet has an effect on the composition of the microbes found in the gut. Different balances of carbohydrates, proteins, and fats in the diet of the hosts play a critical role in the communities of microbes (Scott et al., 2012). Geographical regions play little to no effect on whether gut microbes differ; only dietary differences are what make these changes occur. Scott (et al., 2012) and her team found that microbial communities of humans' gastrointestinal tracts were similar in North America and Europe; however differed drastically in North America and South America due to extreme differences in their diet composition (Scott et al., 2012).

Tadpoles have a diet that changes during the course of their development. The diet of the tadpole is generally rich in carbohydrates and switches to protein later in life (Richter-Boix et al., 2006). In an experiment with the diet of anurans, during development scientists noticed how tadpoles' diets changed as stresses in the ecosystem were added during metamorphosis. Stressors included competitors such as larger tadpoles of another species, simulation of ponds drying up causing overcrowding, and food availability (Richter-Boix et al., 2006). Also, diet is very important for how the thyroid is able to function, which has control of how fast the tadpoles go through

development. It has been shown that diets high in protein produce higher levels of thyroxin, which is a hormone that controls the rate of development (Kupferberg, 1997). This brings up the question of whether the environmental stressors cause competition in the gut microbiota in the tadpole and cause them to affect the host's diet, or does the diet actually affect the microbiota in the gut?

To have a basic understanding of the gut in anurans we have to look closely into specific changes in their gut as they metamorphosis from frogs to tadpoles. The gut in anuran larvae undergoes rapid changes in the structure as they undergo metamorphosis. The stomach goes from a non-acidic stomach with a small hindgut to a stomach that is acidic with an enlarged hindgut (Kohl et al., 2013). These changes in acidity cause changes in the gut's pH levels, which alter microbiota communities found in the gut. One interesting connection is that the microbial communities in the guts of tadpoles are very similar to fishes' guts compared to the guts of the adult frog, which tend to be closer to the amniotes (Kohl et al., 2013). The diversity of the gut microbiota is noticeably higher in tadpoles than in the adult frogs seen in Figure 1 (Kohl et al., 2013).
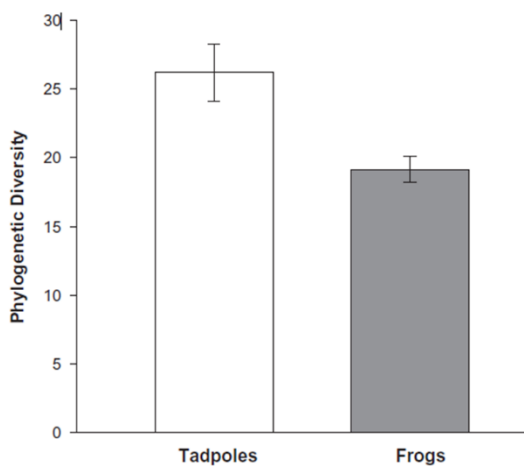
Figure 1: Phylogenetic Diversity of Microbiota in Tadpoles and Frogs (Kohl et al., 2013)

Diets can be traced in frogs by using microsphere beads that are approximately

112 µm in length (Pryor and Bjorndal, 2005). The digestive tract in the species *Rana*

catesbeianus (Bronze Frog) takes approximately 6 hours for the food to leave the system

(Pryor and Bjorndal, 2005). The digestive tract is divided into six regions as seen in

Figure 2 below.

Figure 2: Bullfrog tadpole gastrointestinal tract.

Gut regions:

M=manicotto glandularae (storage compartment); ASI= Anterior small intestine; INF=

inflection region; PSI= posterior small intestine; C=colon; and R= rectum. Illustration by

(Pryor and Bjorndal, 2005).

Chapter 3. Materials and Methods
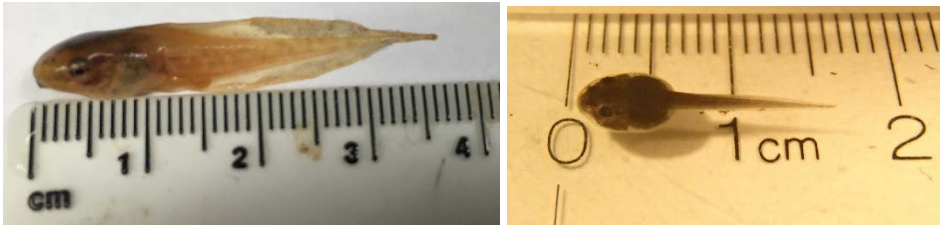
Figure 3: *Hyla cinerea* tadpole (Left)   *Rana sphenocephala* tadpole (Right)
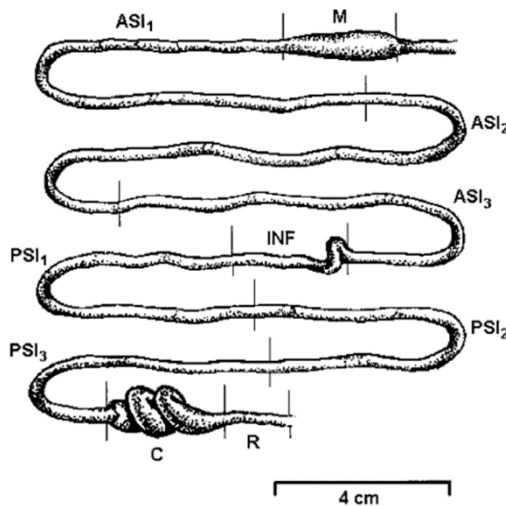
Collection of Focal Species

  The frogs that I worked with in my research project were the Southern Leopard Frog *Rana sphenocephala* and the Green Tree Frog *Hyla cinerea*. More specifically, I worked with tadpoles of these species seen in Figure 3. These frogs are found throughout the Southeastern United States and are not threatened or endangered. *Rana sphenocephala* breeds primarily in the winter and spring, it sometimes breeds in the fall and thus is abundant approximately year round. *Hyla cinerea* breeds at the end of spring and early summer. I confined egg clutches in mesh cages as seen in Figure 4 in a small shallow pond with a mud bottom next to Lake Sehoy, Hattiesburg, Mississippi during mid-February and early March when reproduction was at its peak. The purpose of the cages was to obtain a cohort of tadpoles that were genetically similar with guts inoculated with natural microbiota.

Figure 4: Mesh cage at the pond near Lake Sehoy (Hattiesburg, MS)

When tadpoles reached Gosner stage 25, the stage when tadpoles begin feeding, they were collected from the mesh cages using a net and transferred back to the lab in plastic containers size 34.6cm X 20.3cm X 12.7cm.

Tadpole Maintenance in the Lab

Five tadpoles were kept in each plastic bin with the same dimensions mentioned above and filled with 4-5cm of artificial pond water seen in Table 1. They were kept in an incubator at 25° Celsius. Water was changed on a daily basis to ensure that ammonia levels did not rise. Lighting was used in the incubator and set on a timer to turn on for 12 hours and turn off for 12 hours.

Table 1 shows the composition of artificial pond water used in this study. These compounds were mixed with ten liters of water to form the solution. This solution was used to fill the containers that the tadpoles occupied during the study.

Compound	Amount needed in grams
NaCl	0.625g
$NaHCO_3$	0.096g
KCl	0.030g
$CaCl_2$	0.015g
$MgSO_4$	0.075g
$CaSO_4$	0.060g

Table 1: Composition of Artificial Pond Water

Diet Using Agar as the Binder

Tadpoles were separated into three groups. Group 1, the control, was fed a commercial diet, Frog Brittle (Diet A-1). A second group was fed Frog Brittle mixed with dried Timothy Grass (Diet B). The third group was fed Frog Brittle and chitin (Diet C). Each of the three diets were made by suspending the dry ingredients in molten agar that served as a binder and allowed to solidify in petri dishes. Each diet contained 1.5 % agar and approximately 40% other dry ingredients. Diets B and Diet C contained Frog Brittle and either Timothy Grass or chitin at a ratio of 3:1. Timothy Grass and chitin were chosen because these two food items cannot be digested by the tadpoles alone. Tadpoles need the help of microbiota in the gut to digest these diets. Fresh feed was prepared every seven days and stored at 4°C until use.

Diet Using Gelatin as the Binder

The second experimental diets consisted of minor changes to allow the tadpoles to differentiate between the different types of food. Also, the binding agent was changed as well. The control group, (Diet A-2), consisted of frog brittle and 5% gelatin solution was used as the binder. Group 2, (Diet D), consisted of frog brittle, 5% gelatin solution, Spirulina , and chitin. Group 3, (Diet E), consisted of frog brittle, 5% gelatin solution, Chlorella, and timothy grass. Group 4, (Diet F), consisted of frog brittle, 5% gelatin solution, Spirulina , and Timothy Grass. Group 5, (Diet G), consisted of frog brittle, 5% gelatin solution, Chlorella, and chitin. The addition of Spirulina and Chlorella were used to give the tadpoles a way to identify spirulina with the Timothy Grass and Chlorella with chitin, or vice versa. Diets A, D, and E can be seen in Figure 5.

Figure 5: Diets A, D, and E with gelatin binder

DNA Extraction, Amplification, and Gel Electrophoresis

To determine whether tadpole gut microbiota is diet-dependent, I compared the microbial community of tadpoles fed on various diets. I collected individual fecal samples from each tadpole with a pipette. This was done by placing each tadpole into individual cups with a mesh bottom. The fecal matter sunk through the mesh into another

cup underneath. Then, the pipette collected the fecal matter and was placed in a 2ml

collection tube to be prepared for DNA extraction. DNA from the tadpole's feces was

extracted using the PowerSoil DNA Isolation Kit. Then DNA was amplified using the

polymerase chain reaction (PCR) where DNA was amplified along the 16S Ribosomal

V3 region. Primers 341 and 518 and the enzyme taq polymerase were used during the

PCR reaction and run through a 30 cycle DGGE program. After a PCR was performed

an agarose gel was stained with dye to visualize the amplification PCR products under a

UV transilluminator as seen in Figure 6 below.

Figure 6 DNA amplified from fecal DNA from *Rana clamitans* collected from Lake

Sehoy Hattiesburg, MS

The amplified DNA was visualized by staining a Denaturing Gradient Gel after the DNA

was separated and then viewed under the UV transilluminator. Also, High Resolution

Melt graphs were created by adding EvaGreen dye to the PCR products that fluoresces during the PCR reaction. The fluorescence readings were measured at every 0.2˚C. Then, dendrograms were created to show how each samples microbiota communities related to one another.

Diet Preference Tests

To determine whether gut microbiota affects the dietary preference of tadpoles, I began to introduce multiple diets for the tadpoles to choose from. These diets contained microspheres that were 112μm. The beads made up approximately 0.2% of the diet mixture. The microspheres were used as tracers to determine which foods the tadpoles consumed based on two different colors of beads such as red and green. These microspheres were collected in the tadpole's feces and then the samples underwent sonication where all organic material was broken into fine pieces. Ratios of the green:red beads were determined by counting the amount of microspheres found in each fecal sample under a florescent microscope and comparing the values for each diet that the tadpole consumed to the actual amount of beads found in the diets per gram of dry food. If a preference was detected an antibiotic would be administered to sterilize the digestive tract. This would remove bacteria in the gut. The diets would be offered again to see if a preference still existed. This was done to ensure that it was the microbiota in the gut causing the preference and not any other factor.

Chapter 4. Results

Diet Experiment 1

These groups had different gut microbiota communities that had been established over the course of four weeks. This can be seen in the DGGE gel seen in Figure 7. After the differences in gut microbiota communities were established the choice of two diets were given and the amount of beads found in each sample created ratios that could be used to determine if preference exists. In Figures 8-12 the amount of beads from each diet that were consumed are shown below. The red beads represent diet choice B (Timothy Grass + Frog Brittle + Agar), and the green beads represent diet choice C (Chitin + Frog Brittle + Agar). The amount of beads found in the tadpoles fecal samples represents how much of each diet the individuals consumed. There were three individual tadpoles from Group 1, Group 2, and Group 3 that were gave the option between Diets B and C. During day 1 most of the individuals showed a small preference towards the chitin enriched diet. During day 2 the individuals began to show small random preferences towards both diets. During day 3 individuals from Group 1 showed a slight preference towards the Timothy Grass diet and individuals from Group 3 showed a slight preference towards the chitin diet. Group 2 had individuals who gave no fecal. Day 4 shows a similar trend with the addition of Group 2 having a small preference towards the chitin diet. After 4 days the results were totaled and very little pattern of preference was established.

To look at the percentile of each preference a mean value was calculated by looking at the number of beads found in each of the foods dry weight and a ratio was calculated by dividing the number of green beads found in the diet by the number of red

beads found in the diet. The mean value for the ratio of beads in the diet was 1.33. After

the mean was determined each individual was assigned a z-score and then a percentile

was charted. This information can be seen in Table 2. In Table 2 the percentiles for

preference were valued between 0.5438 and 0.8944. These values show that there is only

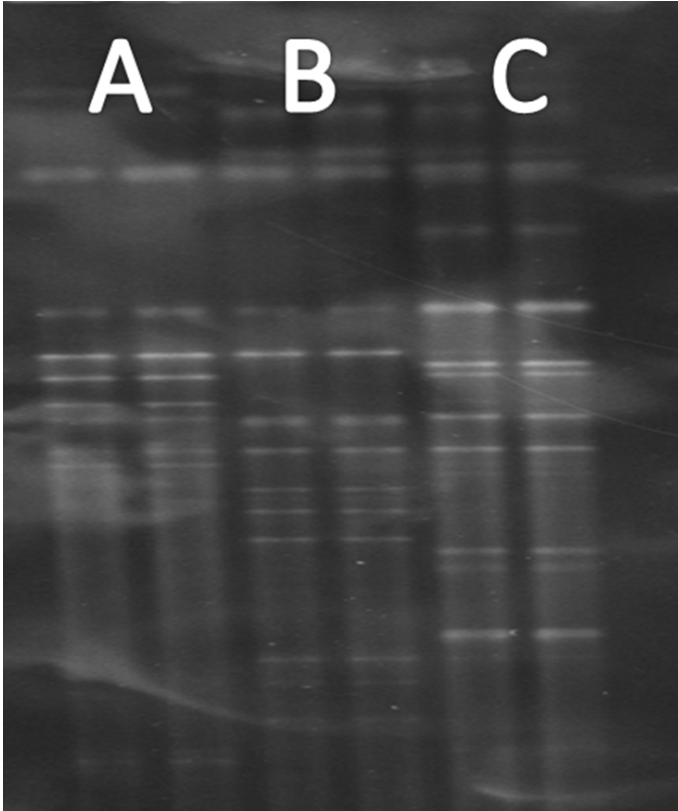a 54.38%- 89.44% chance that a preference is existent.

Figure 7: DGGE for Groups 1 (A), 2 (B), and 3 (C).

Each group contained two samples which have identical banding patterns. Differences in

the banding patterns occur between groups and represent different bacteria communities

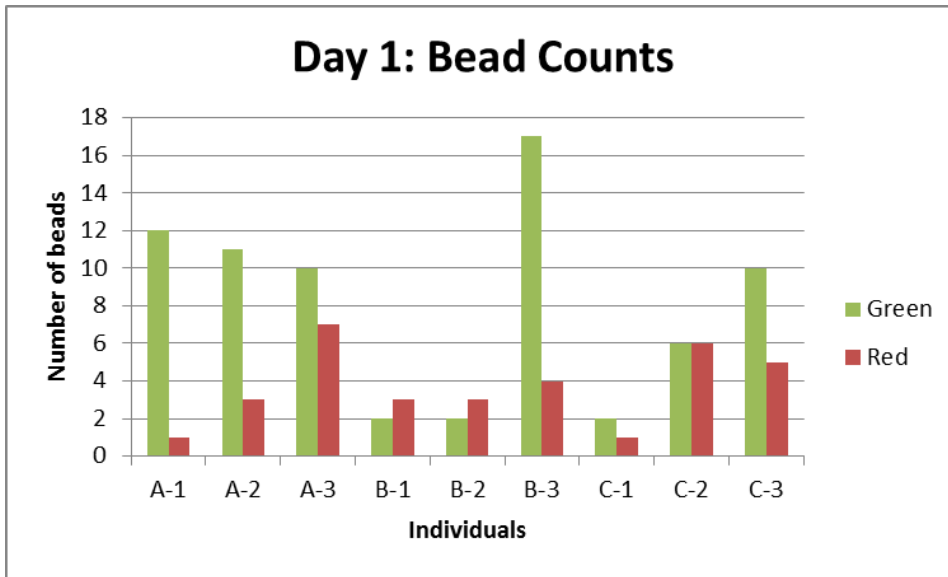that are present between the three groups.

Figure 8: Diet preference of tadpoles on Day 1 of feeding trial using agar as the binder.

Feed trial took place after 14 days of feeding to stabilize the microbial community in tadpole guts in the laboratory. A: tadpoles fed a control diet; B: tadpoles fed diet enriched with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers represent individual tadpoles. Green: food containing chitin. Red: food containing cellulose.

Figure 9: Diet preference of tadpoles on Day 2 of feeding trial using agar as the binder.

Feed trial took place after 14 days of feeding to stabilize the microbial community in tadpole guts in the laboratory. A: tadpoles fed a control diet; B: tadpoles fed diet enriched with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers represent individual tadpoles. Green: food containing chitin. Red: food containing cellulose.
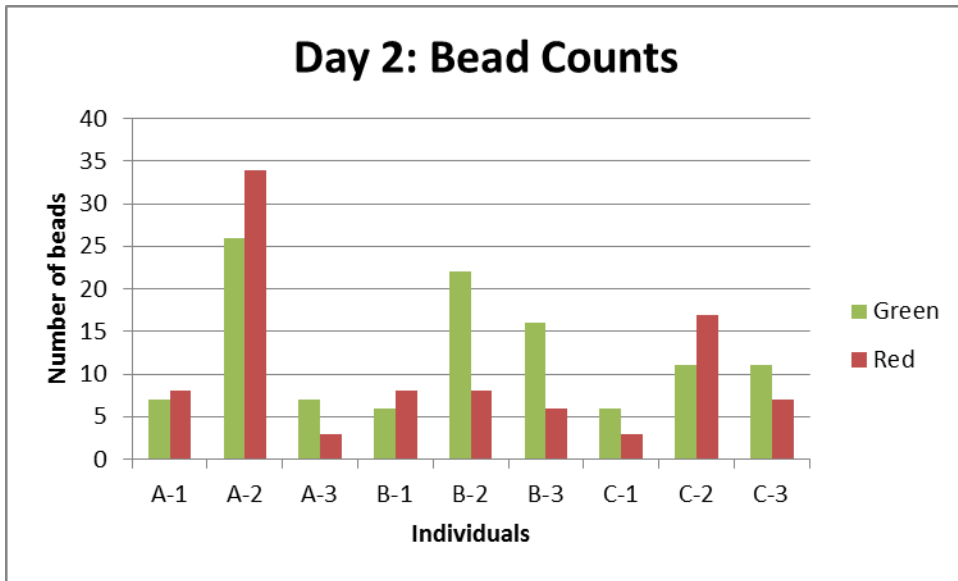
Figure 10: Diet preference of tadpoles on Day 3 of feeding trial using agar as the binder.

Feed trial took place after 14 days of feeding to stabilize the microbial community in tadpole guts in the laboratory. A: tadpoles fed a control diet; B: tadpoles fed diet enriched with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers represent individual tadpoles. Green: food containing chitin. Red: food containing cellulose. Tadpole A-3, B-2, and B-3 did not produce feces.
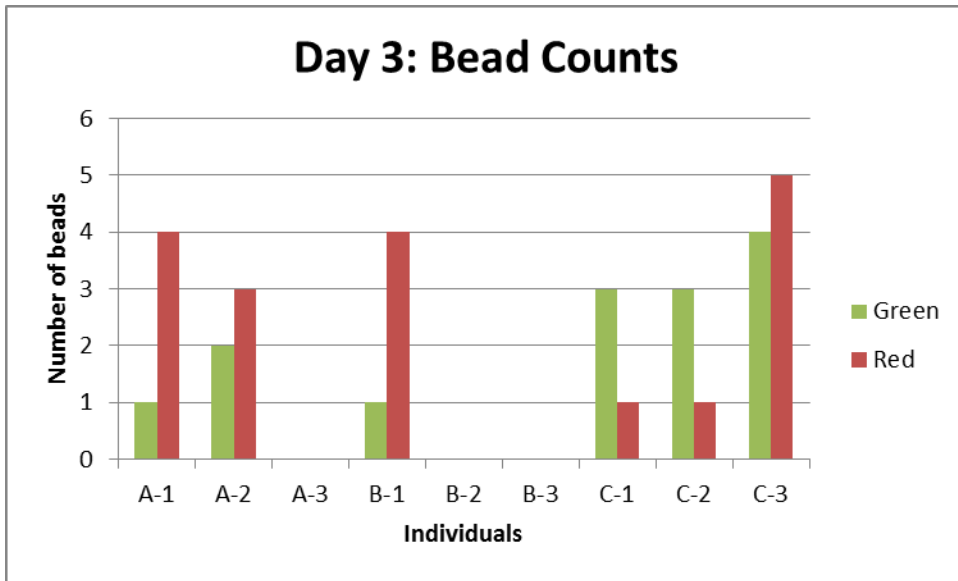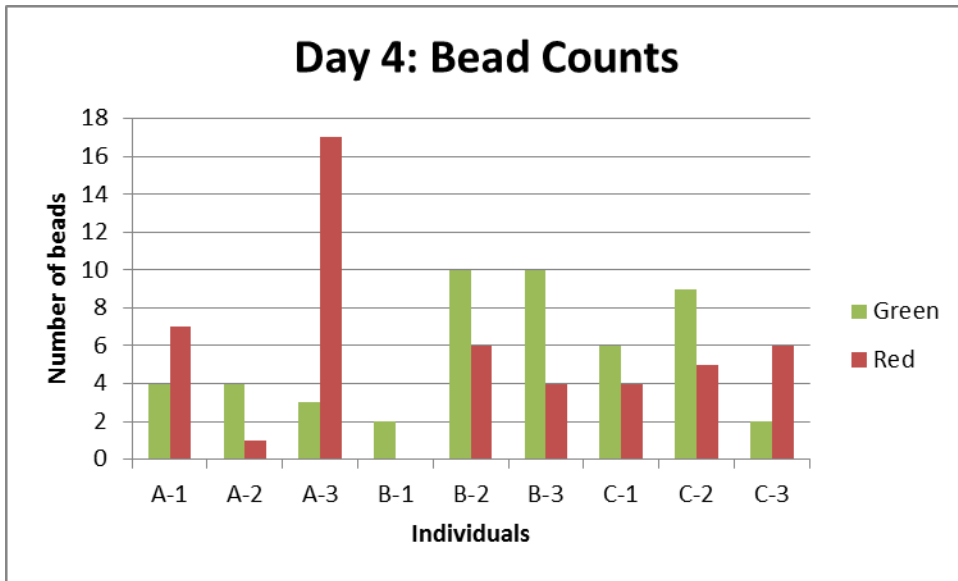
Figure 11: Diet preference of tadpoles on Day 4 of feeding trial using agar as the binder.

Feed trial took place after 14 days of feeding to stabilize the microbial community in

tadpole guts in the laboratory. A: tadpoles fed a control diet; B: tadpoles fed diet enriched

with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers

represent individual tadpoles. Green: food containing chitin. Red: food containing

cellulose.

Figure 12: Cumulative bead counts over four days of testing.

The tadpoles had been feeding for 14 days in the laboratory. A: tadpoles fed a control diet; B: tadpoles fed diet enriched with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers represent individual tadpoles. Green: food containing chitin. Red: food containing cellulose.

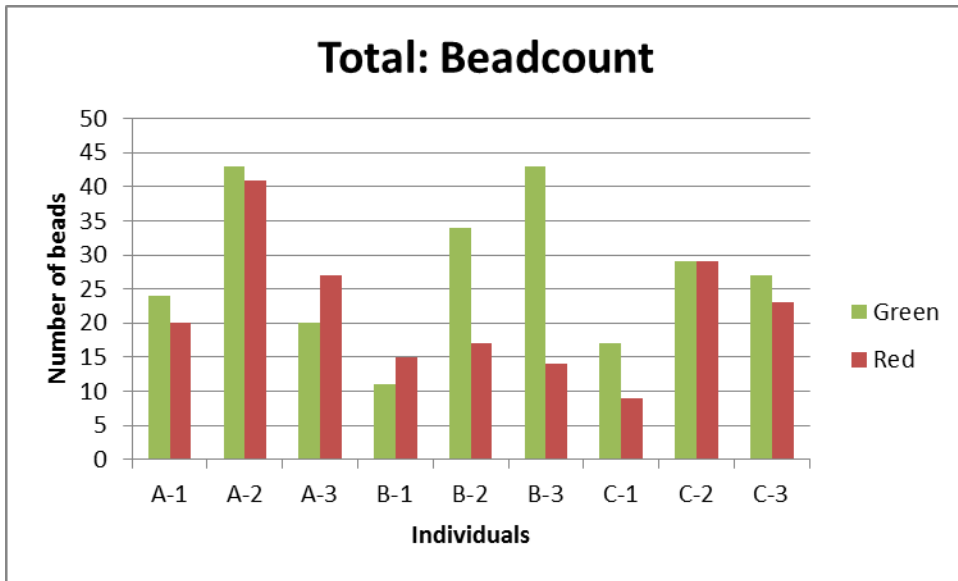| Individual	Ratio G:R	$(x-\mu)^2$	σ	z-score	p-value
A-1	1.2	0.017	0.47	-0.277	0.6064
A-2	1.05	0.078	0.47	-0.596	0.7224
A-3	0.741	0.347	0.47	-1.25	0.8944
B-1	0.733	0.356	1.39	-0.429	0.6628
B-2	2	0.449	1.39	0.482	0.6844
B-3	3.07	3.03	1.39	1.25	0.8944
C-1	1.89	1.89	1.42	0.394	0.6517
C-2	1	1	1.42	-0.232	0.591
C-3	1.17	1.17	1.42	-0.113	0.5438

Table 2: Z-score statistics of food preference by tadpoles.

A: tadpoles fed a control diet; B: tadpoles fed diet enriched with cellulose (Timothy grass); C: tadpoles fed diet enriched with chitin. Numbers represent individual tadpoles. Ratio G:R is the ratio of green beads (that indicated preference for food containing chitin) to red beads (that indicated preference for food containing cellulose). The green to red bead ratio in the test diet was 1.33.

Diet Experiment 2

In this diet preference test the binder was changed from agar to gelatin and also the addition of chlorella and spirulina algae was introduced to allow the tadpoles to distinguish between the diets. Differences in the gut microbiota were observed after two weeks of feeding. The HRM curves and dendrogram are shown below in Figure13-14. The HRM curves show Groups 2 (D) and 4 (F). These two groups were used as an example because they had the most differences according to the dendrogram.

The bead count data is shown below in Figures 15-19. On Day 1 two individuals from Group D preferred the Timothy Grass and Chlorella enriched diet, while two individuals from Group E preferred the Chitin and Spirulina diet. This trend continued on day 2; however on days 3 and 4 all seemed to prefer the Timothy Grass and Chlorella diet. Diet D represents the red beads and consists of Gelatin+ Frog Brittle+ Chitin+ Spirulina. Diet E represents the green beads and consists of Gelatin + Frog Brittle + Timothy Grass + Chlorella. The same method of using the ratio of beads found in the dried diet was used to calculate a mean. The mean for this test was 0.824. In this experiment the values for the percentiles in Table 3 ranged from 0.6443-0.8888. The data for the individual preference percentiles can be seen below in Table 3.
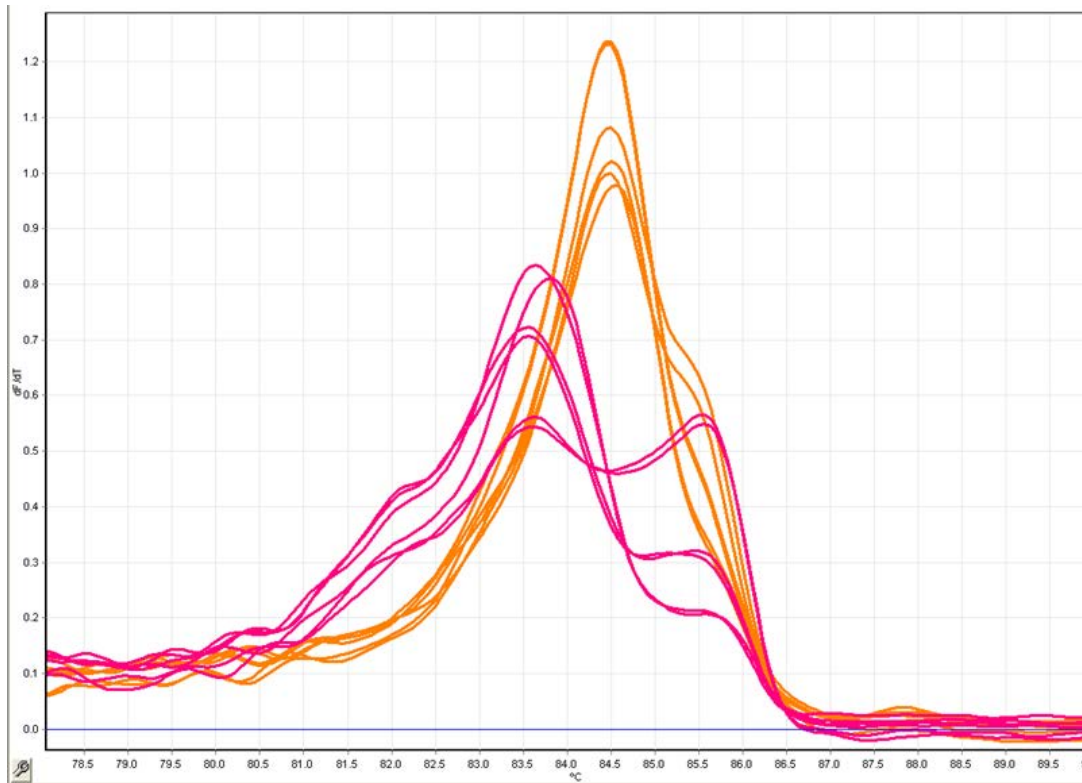
Figure 13: Representative HRM melt curves of amplicons representing gut microbiota

communities.

There are three samples from tadpoles fed each diet and each sample was analyzed in duplicates. The pink curves represent the gut microbiota of tadpoles fed cellulose and spirulina. The orange curves represent the gut microbiota of tadpoles fed Frog Brittle, the control diet.
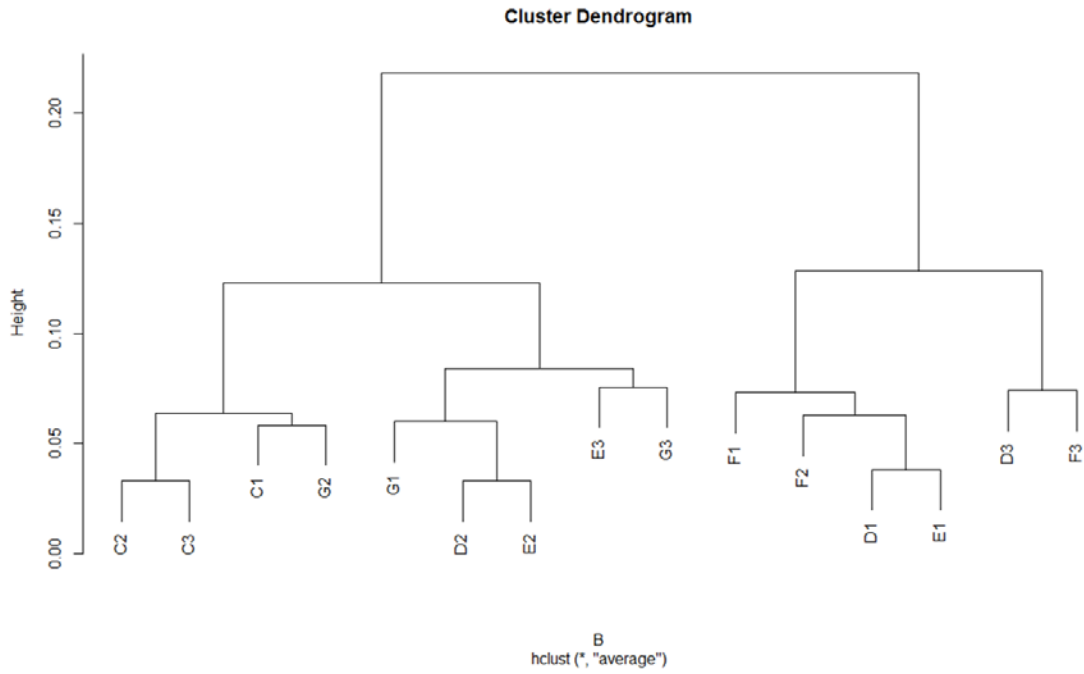
Cluster Dendrogram

Figure 14: Dendrogram from cluster analysis of HRM melt profiles.

Different letters represent gut microbial communities from tadpoles fed different diets.

The numbers represent individual tadpoles.

Figure 15: Diet preference of tadpoles on Day 1 of feeding trial using gelatin as the

binder.

Feed trial took place after 14 days of feeding to stabilize gut microbiota in the laboratory.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a

diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles.

Green: food containing cellulose (Timothy grass). Red: food containing chitin.
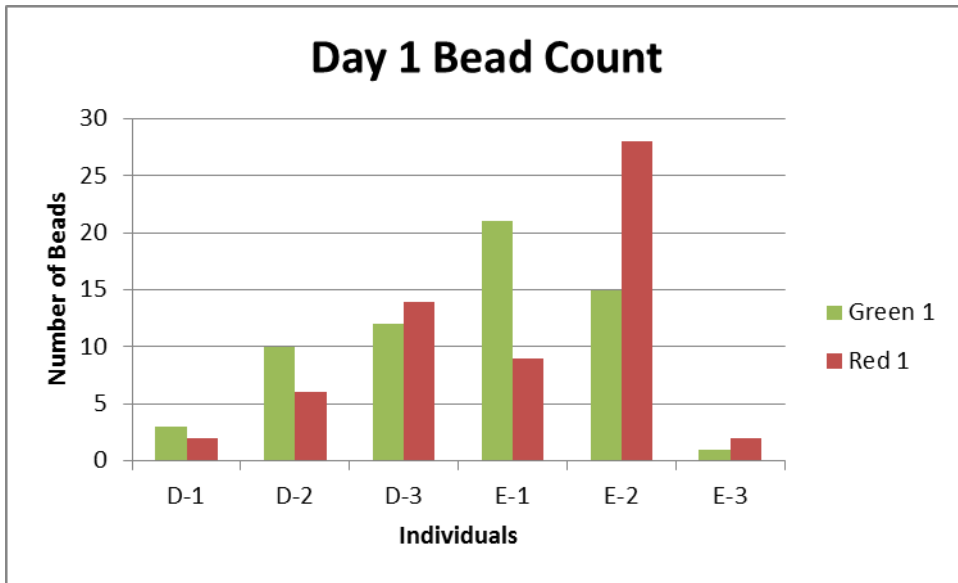
Figure 16: Diet preference of tadpoles on Day 2 of feeding trial using gelatin as the

binder.

Feed trial took place after 14 days of feeding to stabilize gut microbiota in the laboratory.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a

diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles.

Green: food containing cellulose (Timothy grass). Red: food containing chitin.

Figure 17: Diet preference of tadpoles on Day 3 of feeding trial using gelatin as the

binder.

Feed trial took place after 14 days of feeding to stabilize gut microbiota in the laboratory.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a

diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles.

Green: food containing cellulose (Timothy grass). Red: food containing chitin.
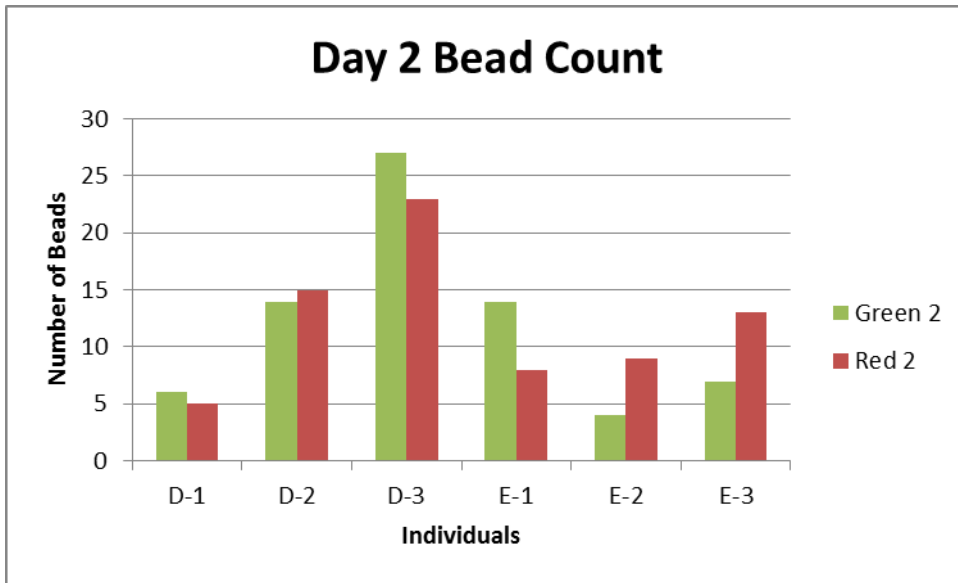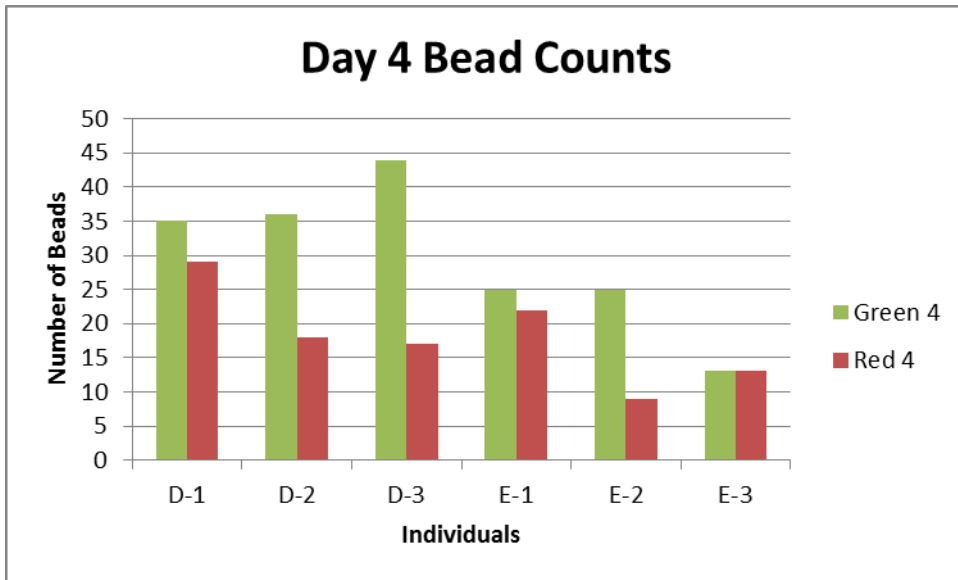
Figure 18: Diet preference of tadpoles on Day 4 of feeding trial using gelatin as the

binder.

Feed trial took place after 14 days of feeding to stabilize gut microbiota in the laboratory.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a

diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles.

Green: food containing cellulose (Timothy grass). Red: food containing chitin.
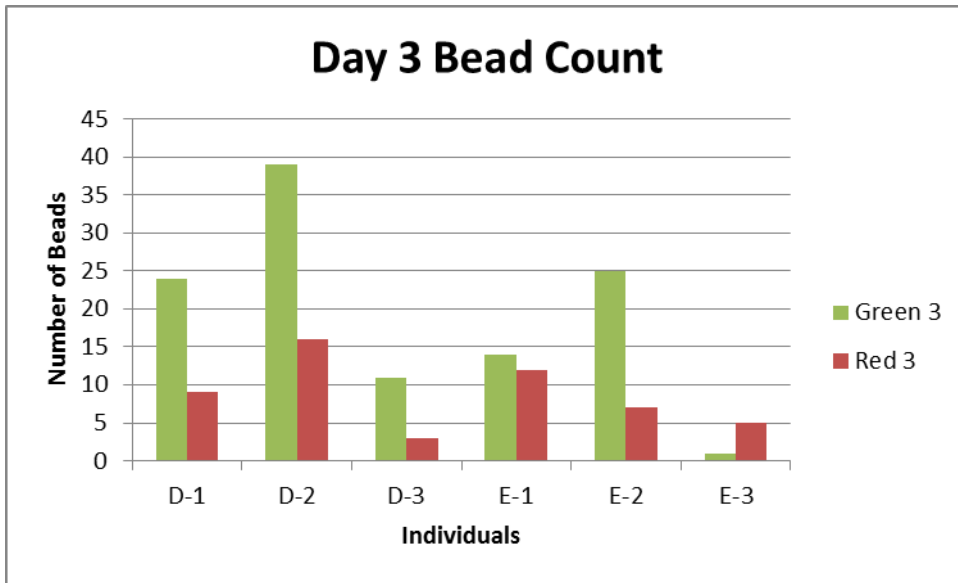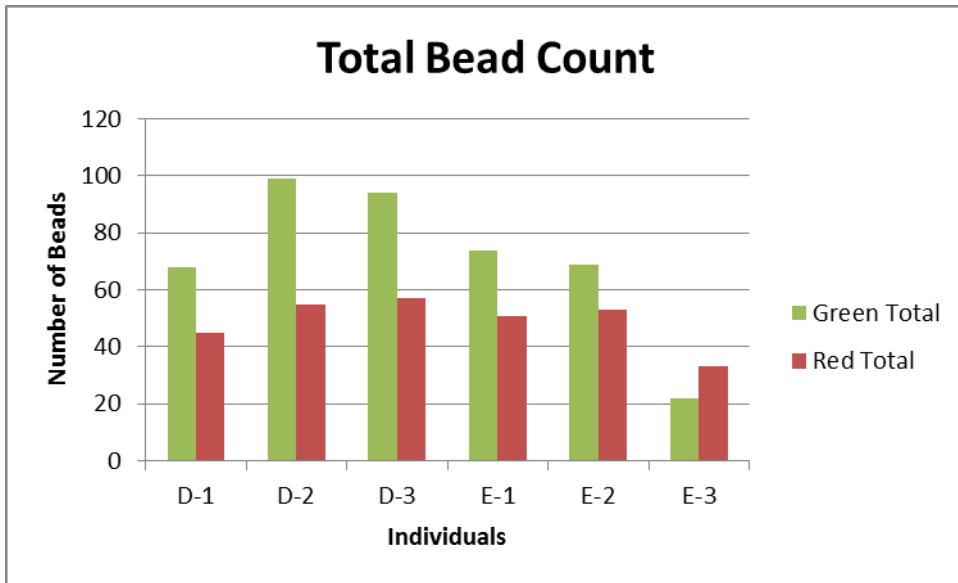
Figure 19: Cumulative bead counts over four days of testing with food containing gelatin

     as the binder.

Feed trial took place after 14 days of feeding to stabilize gut microbiota in the laboratory.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a

diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles.

Green: food containing cellulose (Timothy grass). Red: food containing chitin.

Individual	Ratio G:R	$(x-\mu)^2$	σ	z-score	p-value
D-1	1.51	0.47	1.02	0.673	0.7486
D-2	1.8	0.953	1.02	0.957	0.8289
D-3	1.65	0.682	1.02	0.81	0.791
E-1	1.45	0.392	0.512	1.22	0.8888
E-2	1.15	0.106	0.512	0.637	0.7357
E-3	0.667	0.025	0.512	-0.307	0.6443

Table 3: Z-score statistics of food preference by tadpoles.

D: tadpoles that had been fed a diet enriched with chitin; E: tadpoles that had been fed a diet enriched with cellulose (Timothy grass). Numbers represent individual tadpoles. Green: food containing cellulose (Timothy grass). Red: food containing chitin. Numbers represent individual tadpoles. Ratio G:R is the ratio of green beads (that indicated preference for food containing cellulose) to red beads (that indicated preference for food containing chitin).

The green to red bead ratio in the test diet was 0.824.

Chapter 5. Discussion/Conclusion

Tadpoles in the first diet test did not show a significant diet preference after 4 days of testing. I think that the tadpoles were unable to detect the differences in the foods due to the overwhelming amounts of agar and frog brittle that were contained in the foods that blocked the tadpole's ability to determine if the diet contained timothy grass or chitin.

In experiment number two the diets were altered to help the tadpoles distinguish between the diets. Spirulina and chlorella were chosen to allow the tadpoles to associate these different algae with the Timothy Grass and chitin. Also, the binder was changed to gelatin which has less of an odor compared to the agar. Frog brittle remained in the diets to provide the tadpoles with appropriate sources of nutrition. In this experiment the values for the percentiles in Table 3 were higher. Overall, this group showed higher values for preference, however these values are not in a significant range to determine that preference truly exists.

In Figure 7 the DGGE results show that microbiota communities in the gut of tadpoles fed Diets A, B and C are different. To determine exactly which microbial communities are present among the groups Illumina Next Generation Sequencing will be used to obtain additional information about how the diets affect the communities of bacteria that reside in the host's intestinal tract.

To further study this topic more tadpoles should be examined to gain a better statistical model. More tadpoles would allow the research more data to see if there truly is a preference among the diets that occurs. For the current results it can be concluded that tadpoles do not have a preference, but feed on whatever diet the tadpole's first bump

into. Also, the containers that were used to monitor the diet preference were very small.

Possibly increasing the size of these containers would yield better results. In Richter-Boix

(et al., 2006) experiment he concluded that environmental stressors such as overcrowding

and pond drying causes changes in diet preference. Finally, the bead ratio may not be

enough to determine preference. Possibly adding video data that monitors which food the

tadpoles congregate around the most would give additional information to determine if

preference exists. At this point in the research the hypothesis that microbiota affects diet

preference in tadpoles is not supported.

Although microbiota does not affect diet preference, the diets do change the

communities of bacteria found in the gut of tadpoles. In the HRM data the curves all

fluoresced at different temperatures between the groups. After the HRM was conducted

these fluorescent readings were recorded at 0.2°C intervals from 79°C-89°C. These were

then used to form a cluster dendrogram. This dendrogram has individuals with similar gut

microbiota grouped together. In the dendrogram most of the individuals from each group

were grouped fairly closely together.

Those individuals that were not clustered together could be from individual

differences from the wild. Not all of the tadpoles were at the same stage in development,

and Kohl observed that the stomach in tadpoles goes from a non-acidic stomach with a

small hindgut to a stomach that is acidic with an enlarged hindgut during development

(Kohl et al., 2013). With these changes to the gut, tadpoles from the same group could

have initially had different microbes in their gut. Overall, tadpoles fed on diets G and E

clustered closely together and tadpoles fed on diets D and F clustered closely. Diets G

and E both contained chlorella, but differed in Timothy Grass and chitin. Diets D and F

both contained spirulina, but differed in Timothy Grass and chitin.

This result was unexpected because spirulina and chitin are both similar in

nutrition and should not be the cause of change between gut microbiota. The algae are

digestible by the tadpoles; however the Timothy Grass and chitin are indigestible and

should need different bacteria groups to aid in the digestion. While each group contains

differences in gut microbiota, these results do not support chitin and Timothy Grass being

the cause of the difference. To determine specific microbes that are present among each

group an Illumina Next Generation Sequencing test can be used.

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Appendix A

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal 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 three year 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 (see attached) 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: **12/2013 – 9/ 2015**
PROJECT TYPE: **New**
PRINCIPAL INVESTIGATOR(S):   **Shiao Wang**
DEPARTMENT: **Biological Sciences**
FUNDING AGENCY/SPONSOR:
IACUC COMMITTEE ACTION: **Full Committee Approval**
PROTOCOL EXPIRATON DATE: **September 30, 2015**

Frank Moore, Ph.D.   Date   12-18 -2013
IACUC Chair

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