Routes of transmission of gut bacteria in *R. imitator* tadpoles in an experimental context

By

Natalia Belduque Correa

July 2024

Director of thesis: Kyle Summers

Major Department: Biology

The gut microbiota provides important capacities for the host, and the relationship between host-microbiome could affect, bidirectionally, different traits of the history life. Therefore, it is important to know how the microenvironment of living beings is shaped and what factors influence the community and composition of bacteria. The mother–to–offspring microbiota transmission has been extensively studied in vertebrates, focusing on human primates. In amphibians, the understanding of diversity and community of bacteria has been biased to skin microbiome. In this research, we study whether *R. imitator* mothers, a neotropical poison frog from Peru that display prolonged parental care, are transferring microbes to tadpoles by feeding them with trophic eggs. We used 16S rRNA amplicon-sequencing of the gastrointestinal tract of captive *R. imitator* mothers and their tadpoles. The analyses showed that composition of microbes is different between parents and offspring. Also, the composition of gut bacteria of tadpoles included different bacterial phyla usually found in gastrointestinal tract of vertebrates. Although, phyla Bacteroidetes and Proteobacteria were also found in gut of fish food experiment, it was seen that the proportion of Proteobacteria are higher in tadpoles from switching and control experiments, which can suggest that the proportion of microbes that comes from the maternal diet is similar than the proportion

that comes from the environment. This supports the assumption of mother's role in parental care is not limited, exclusively, to provide nutrient resources.

Routes of transmission of gut bacteria in *R. imitator* tadpoles in an experimental context

A thesis

Presented to the faculty of Biology Department Ariane Peralta and Michael Brewer

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biology

By

Natalia Belduque Correa

July 2024

Director of thesis: Kyle Summer, PhD

Thesis Committee members:
Ariane Peralta, PhD
Michael Brewer, PhD

Acknowledgments

I would like to thank my thesis advisor, Kyle Summers, for letting me be part of his lab, I would also like to thank my committee members, Dr. Ariane Peralta and Dr. Michael Brewer for their feedback and support throughout my time at ECU. In Addition, I would like to express my deepest gratitude to my family, especially Yulieth Correa, Camila Belduque and Camilo Gelvez Gonzales who always have believed in me and my dreams.

Table of Contents

List of Figures

Introduction

The association between a vertebrate host and its gut microbiota can be considered a bidirectional relationship because the gut microflora can alter the capacity of the host to adapt to its environment (Xu, et al., 2019). Moreover, the gut microbiota may play a role in several physiological processes of the hosts, such as: nutrient absorption, energy balance, and immune responses (Xia et al., 2022). In turn, the gut microbiota may be influenced by various aspects of the host's life history such as: habitat (Barelli et al., 2020), trophic level (Wong and Rawls, 2012), behavior (Weng et al., 2016), and diet (Grond, et al., 2018). Indeed, a recent study revealed that the feces of mice delivered by females exposed to a high-fat diet during pregnancy (MHFD), compared to the feces of offspring of mothers on a regular diet (MRD), had a lower diversity of microbiota and showed deficiencies in social behavior that decreased their ability to socialize in novel situations, suggesting that the dysbiosis of MHFD offspring occurred due to the absence of essential bacteria vital for displaying normal social behavior (Buffington et al., 2016).

Furthermore, the routes of transmission of gut microbiota can also determine the composition of gut microbial communities in an individual. For instance, the fecal-oral route is considered the main pathway through which the gut microbiota is transmitted to the host in insects and mammals (Bo et al., 2020; Powell et al., 2014). In humans and mammals, commensal intestinal bacteria can also be transmitted by the vagina-oral route at birth and breast milk in early life (Browne et al., 2017). Murphy et al. (2017), found identical profiles of both *Bifidobacterium breve* and *Lactobacillus plantarum* in both the infant's fecal samples and in breast milk, providing evidence of vertical transmission of symbiotic microbes from mothers to infants. Consequently,

the mother–to-offspring microbiota transmission confirms that the maternal supplements to their young positively impact the microbiota during development. Similarly, the intrauterine environment influences the establishment of intestinal microbiota in babies before delivery by transferring microorganisms from the mother to the fetus when it swallows the amniotic fluid in preterm babies (Ardissone et al., 2014; Yang et al.,2021).

The maternal transmission of symbiotic microbes throughout early life has been studied in both human and non-human animals. For example, Chen et al. (2020) reported that zebra finches can acquire gut microbiota by maternal transmission when parents provide regurgitated food. Nevertheless, the transmission of gut microbes was seen only in zebra finch chicks that obtained food from their own mothers. Similarly, Korpela et al. (2018), demonstrated that the maternal transmission of symbiont gut microbes to offspring in humans is a selective process that starts after initial inoculation by maternal fecal microbes, and can provide protection, limiting the transmission of pathogens.

Although the transmission of gut microorganisms has been studied extensively in mammals (Clayton et al., 2018; Amato et al., 2014; Amato et al., 2015; Hooper et al., 2002; Kohl et al., 2014), and birds (Berlow et al., 2022; Phillips et al., 2018; Videvall et al., 2018; Teyssier et al., 2020), it is important to study it in other vertebrates because other clades could have markedly distinct communities that could be shaped by different transmission pathways (Browne et al., 2017; Colston et al., 2016). For example, amphibians with a biphasic cycle of life have been less intensively studied with respect to which routes of transmission play a primary role in the development of microbial composition during early life. In some groups of amphibians, it has been

observed that females may transfer symbiotic bacteria to their offspring when they move through communal nests, increasing embryonic survival in nests that had a signal of the presence of pathogenic fungi. Additionally, comparisons of the skin bacteria between mothers and embryos demonstrated that the bacterial community composition was more similar, relative to bacteria in the surrounding soil (Banning et al., 2008).

Whereas in Neotropical poison frogs maternal care is associated with compensation of low nutrient availability through provision of trophic eggs to tadpoles in breeding pools, tadpole feeding can occur as a facultative behavior, and tadpoles without access to trophic eggs may grow and survive if detritus and insect larvae are available, albeit at lower growth rates (Summers et al., 2014; Brown et al., 2010; Brown et al 2008), which indicates tadpoles may acquire symbionts from their parents or the environment during development. For instance, Weinfurther et al. (2021) found symbiotic gut microbiota in tadpoles of *R. imitator*, but their main source of this microorganisms is unclear due to the facultative ability of tadpoles to utilize multiple sources of food (e.g., trophic eggs or external food sources). In addition, in other poison frogs, it was found that trophic eggs delivered to tadpoles provided not only essential nutrients for development, but also alkaloids that were sequestered and used for defense (Saporito et al., 2019; Brooks et al., 2023). However, it is not known whether trophic eggs provide additional benefits such as the transfer of symbiont microbes to the gut microbiota.

Although the mechanisms and strategies of parental care in poison frogs have been studied extensively, many of these studies have been focused on male's role (i.e., Pašukonis et al., 2016; Pašukonis et al., 2017), indicating the important of investigating additional benefits provided by

females when they provide parental care. Additionally, multiple abiotic and biotic factors impact these organisms during development (e.g., across the biphasic life cycle), and these could affect the assembly of different microbial communities, offering a valuable opportunity to determine how bacterial communities in tadpoles are affected when they are exposed to a restricted niche without access to food (e.g. breeding pools) (Brown et al., 2008; Beck et al., 2017).

Ranitomeya imitator lays arboreal clutches in laboratory conditions and both the female and the male play key roles during parental care. Male frogs attend egg clutches and transport individual tadpoles to breeding pools, and female frogs provide tadpoles with trophic eggs. Females usually lay 1-2 trophic eggs and tadpoles are fed every 6-10 days. Given the facultative behavior of tadpoles of *R. imitator*, identifying the main route of transmission of gut bacteria during development is a critical step toward understanding the additional benefits of parental care associated with the mother's role. I hypothesize that the composition of the gut bacteria of *R. imitator* tadpoles is associated with their parent's microbiome in tadpoles that were fed by trophic eggs delivered by females during development, i.e., that this diet is the main route of transmission that drives gut bacteria composition in this species. An alternative hypothesis is that the composition of gut bacteria of *R. imitator* tadpoles is influenced by environmental acquisition. Finally, it is possible that the bacterial community of the gut is influenced by both acquisition from trophic eggs and from the pool environment, resulting in a mixture.

Methods

Animal husbandry and sample collection

We selected 12 tanks with a male and a female *Ranitomeya imitator*. All frogs came from a captive breeding colony of *R. imitator*; a poison dart frog found in north-central Peru. Each tank contained plants for shelter and a 50ml falcon centrifuge tube breeding container filled with 25ml water for tadpole deposition. Tanks were misted by hand each day, and a 12/12 hr light/dark cycle was maintained. All the frogs from the captive breeding colony had the same type of diet, provided 4 times per week, that consisted of fruit flies mixed with a mineral supplement formulated for dart frogs. To identify if mothers are transferring bacteria to tadpoles by trophic eggs, we randomly assigned 6 tanks to the Probiotic diet treatment (PDT) where pairs were fed ad libitum with fruit flies mixed with a mineral supplement formulated for dart frogs, and a probiotic four times per week. The probiotic was added to act as a biological agent to modify the composition of the intestinal microbiota and maintain a healthy intestine in the frogs. Then, we assigned 6 tanks to the No Probiotic diet treatment (NPDT) where pairs were fed only with fruit flies mixed with the same mineral supplement formulated for dart frogs four times per week.

For each diet treatment, we checked each tank daily and recorded the presence of new eggs and/or tadpoles in the breeding container. Once we found a new tadpole in the breeding pool, we assigned that new individual to either the fish food experiment (FFE), control experiment (CE) or switching experiment (SE) (figure 1); tadpoles classified in FFE were re-localized in plastic containers with 25 ml of water and fed by hand with fish food. Individuals in CT were kept with their parents and once they ate between 2-3 trophic eggs, deposited by the mother, they were

collected. To identify if females are transferring microbes to progeny, a switching experiment was set up. This experiment consisted of switching tadpoles between parents from Probiotic Diet Treatment (PDT) and No Probiotic Diet Treatment (NPDT). When tadpoles were assigned to SE and before the female started to deliver trophic eggs to feed it, if the tadpole came from NPDT, we switched the breeding pool to another tank that belonged to PDT (and vice-versa) (figure 1). Also, the probiotic was used to alter, healthily, the microbiome of gastrointestinal tract of females that fed a foster tadpole. Consequently, we expected that tadpoles fed by a foster mother would have dissimilarities in the microbiome composition compared to the biological mother; If there was a dissimilarity between biological mothers and tadpoles, vertical transmission of bacteria from females to offspring could be assessed. After the tadpoles ate 1-3 trophic eggs, they were euthanized and collected. Moreover, we collected two different samples from each diet treatment: the digestive tract of 1 to 3 tadpoles per SE, CE or FFE (after euthanasia), and feces sample from the females collected at the end of the experiments where was an accumulation of feces over several weeks. Decision of collecting tadpoles after females provided 1-3 trophic eggs was established according to *R. imitator* time average feeding behavior. This species deposits trophic eggs every 7.3 days with some pairs delivering more than one trophic egg per day (Brown et al., 2008). To assure tadpoles from different pairs would obtain at least one trophic egg per week and at least two for the duration of the whole experiment (i.e. some pairs were slower breeders), we collected tadpoles three weeks after hatching. This standard time also allowed to collect the tadpoles at development stages between 26-30 (i.e. larval stage; Tumulty et al., 2014; Gosner 1969).

Additionally, the diet described above was implemented until all digestive tracts and feces were collected. Finally, all samples collected were stored at -20 °C until DNA extractions were performed. All animal procedures for this study were approved by the Institutional Animal Care and Use Committee at East Carolina University (AUP D382).

1

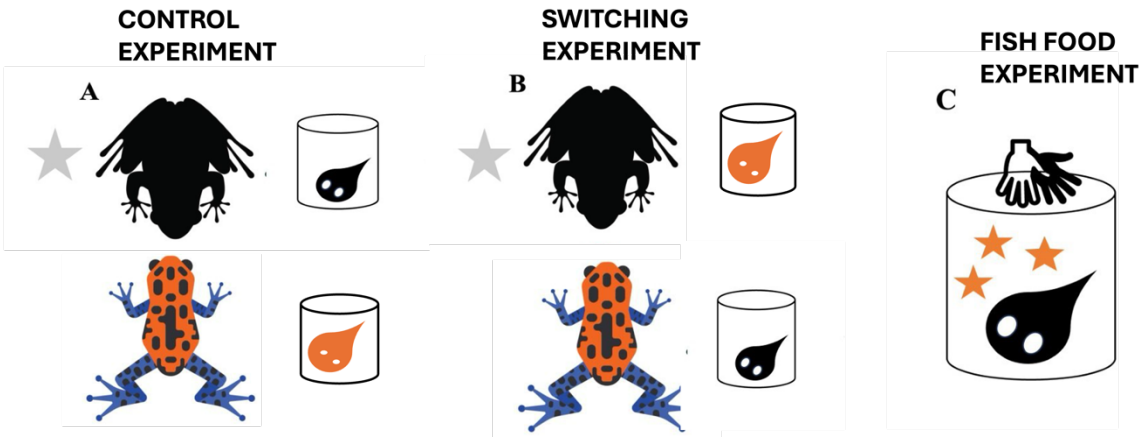


Figure 1. Experimental design to obtain microbial communities of Ranitomeya imitator. Mothers and tadpoles with the same color represent biological mother and offspring. Starts next to mothers represents individuals from Probiotic Treatment. (A) represents individuals in control experiment. (B) represents mother and tadpoles assigned to switching experiments, where mothers feed a foster tadpole. (C) represents tadpoles in fish food experiment.

Microbiome analysis:

We first determine differences in the gut bacterial community composition in tadpoles based on females exposed to two types of diet: probiotic and not probiotic. Gut bacterial communities in each sample, including probiotic, were characterized with Illumina sequencing of the highly conserved 16S rRNA gene (Yilmaz et al., 2014). After collecting the digestive tract of the tadpoles and feces sample from females, we extracted the DNA from each sample using the PowerLyzer Power Soil DNA protocol. Additionally, the DNA extracted was used as a template for PCR reactions, where barcode primers (515FB-806RB) were used to target V4-V4 region of the 16S rRNA gene (Yilmaz et al., 2014). We quantified DNA concentration by an Agarose gel electrophoresis, and a Nanodrop spectrophotometer was used to verify the amplification of DNA. To increase the quality of the sequences, the AMPure xp Mg+bead protocol was followed to purify and remove contaminants from PCR products. After cleaning up the DNA, DNA concentrations (ng/ml) from each sample were quantified using the Qubit High Sensitivity (HS) setting and converting to the final DNA concentration (ng/ul). Then, all PCR products were diluted to the same concentration. PCR products were pooled in equimolar concentrations and sequenced using the paired-end Illumina MiSeq platform at Duke University.

The raw 16S rRNA gene sequences data were processed using the Mothur standard operating procedure (SOP) analysis pipeline (v1.48.1) (Kozich et al., 2013). The sequences were aligned using the SILVA rRNA gene database and classified into operational taxonomic units (OTUs) using a 97% sequence similarity threshold. Additionally, the VSEARCH algorithm (Rognes et al., 2016) was used to remove chimeric sequences. All sequences identified as anything other than bacteria were removed

Diversity and composition analysis

Using the lmer function from the lmerTest package (Kuznesova et al., 2017), Species richness, Shannon diversity (H'), and Pielou's evenness were analyzed by a linear regression model with interaction for evaluating the effects of 'Treatment' and 'Diet' on diversity metrics. Following diversity metric analysis, analysis of composition of bacterial communities was performed using permutational multivariate analysis of variance (PERMANOVA) and Principal Coordinates Analyses (PCoA) were completed. PCoA was performed based on the Bray-Curtis dissimilarity of bacterial community composition. Then, a PERMANOVA analysis was conducted to identify significant differences in bacterial communities based on experiment and type of diet. PERMANOVA was performed using the adonis function from the vegan package (Oksanen et al., 2017). A statistical significance threshold of $p<0.05$ was used for diversity metrics and PCoA analysis. Because we found significant differences in bacterial communities based on the experimental treatment, we used a Kruskal Wallis and Dunn post hoc test to determine if relative abundances of OTUs between tadpoles from switching, Control and Fish Food experiments, feces obtained from females, and probiotic, differed from each other. Then, we executed indicator species analysis using the multipatt function from the indicspecies package (Caceres et al., 2009) to identify OTUs found more often between experiments compared to another. Finally, phylum compositions with relative abundances greater than 0.05 were plotted.

Results

Alpha diversity metrics

To evaluate whether mothers are transferring gut microbiome to offspring by feeding them with trophic eggs, we performed a microbiome analysis in the collected digestive tract of *R. imitator* tadpoles from different experiments, and feces from mothers that were classified into two types of diet; regular food mixed with probiotic and regular food without probiotic. We collected 23 tadpoles from the switching experiment (NPDT =12; PDT = 11), 14 tadpoles from the fish food experiment (NPDT = 8; PDT = 6), and 24 from the control experiment (NPDT = 10; PDT = 13), and 12 feces samples (NPDT = 6; PDT = 6). After classification, 6859 OTUs were obtained from all 74 samples. We found that the linear model analysis with interaction results suggested that the number of species was similar within experiments, indicating that our model was not affected by type of diet provided to the parents (adjusted R^2=-0.003, *P*=0.4). However, interesting trends were seen with the fish food experiment, which was the only experiment that showed a change in the number of species (t value=-2.039; Pr(>|t|=0.0457;) compared to tadpoles, with access to trophic eggs, who were similar in richness (i.e. individuals in control experiment and switching experiment) (Supplemental Table 2A). Also, parents from both treatment shared similar mean OUT richness, and it was higher that OTUs from tadpoles classified into the different experiments. Furthermore, our model could be explained mainly by the effects of the parent treatment, it showed that the bacterial community in this group was highly different from control and switching experiment (tvalue=-4.503; Pr(>|t|=2.96e-05). In addition, we observed interesting trends with these plots. Parents from the probiotic treatment shared similar mean OTU richness

with tadpoles from switching experiment compared to tadpoles who did not have access to trophic eggs (fish food experiment). On the other hand, OTU richness was higher in tadpoles who grew up with their own parents from non-probiotic treatment, in contrast to fish food tadpoles (Figure 2). In fact, the fish food experiment was less diverse compared to tadpoles from the control and switching experiment from probiotic treatment (Figure 3). Lastly, the Parent treatment group had the highest equitability (Pielou), differing significantly from all other groups (Pielou's Evenness: t value=3.329; Pr(>|t|=0.001458)

Community Composition Analysis

A multivariate ANOVA analysis test with permutations (PERMANOVA) was performed to investigate if the centroids from the sample clusters based on type of diet and three different experiments (control experiment, switching experiment and fish food experiment) were different. This analysis showed that gut bacterial composition differed significantly based only on "experiment" (P= 0.0009, R^2 = 0.26806, df = 4), but there was not an interaction between "experiment" and "type of diet" (P = 0.4155, R^2 = 0.033, df = 3). A PCoA was plotted to visualize the difference in compositional clusters between different experiments, where it was observed that there is a cluster between bacterial communities from control and switching experiment (Figure 5).

Given the striking similarities in bacterial community among tadpole's gut microbiome from three experiments and parent, after being exposed to two distinct types of diet, we next

examined whether any of the identified taxa were limited to a specific experiment, which would indicate whether mothers are transferring microbes to offspring.

We did not find any OTUs that were restricted and shared among mothers and tadpoles from control and/or switching experiments when all data were combined (Figure 6), with mother samples harboring the largest proportion of unique OTUs (300 species), and it was seen that fish food, control, and switching experiment shared one of the most abundant identified OTUs with mothers (Figure 6). Interestingly, the relative abundance of otu00009 was bigger in tadpoles from switching experiment compared to the other groups (Figure 7).

To analyze the effect of treatment in Relative Abundance between groups, we performed a Kruskal-Wallis with a Dunn test. The Kruskal-Wallis test showed that the effect of treatment on relative abundance is big (R2= 1334.1; df=4; P = 2.2e-16), but we found that the relative abundance of OTUs is highly variable between all treatments (Dunn test R^2= 1334.1; df=4; P = < 0.000). Mothers and switched tadpoles showed differences in their relative abundance, which highlighted that the route of transmission is different from maternal route (Figure 8). Finally, as significant differences in the OTUs relative abundances were identified, taxonomic analysis was performed to see how phylum community composition varied between treatments. The relative abundance between treatments was high, we plotted the relative abundance at phylum level between experiments and type of diet. Initial phylum differences were seen between tadpoles from switching and control experiments compared to fish food tadpoles. Proteobacteria, Actinobacteria and Bacteroides were the main phyla who had high abundance between experiments. Nevertheless, Deferribacteres and Verrucomicrobia were phylum who were more often in switched and control

experiment tadpoles (figure 9). Finally, Fish food experiment showed a high relative abundance of Bacteroidetes compared to parents and individual from switching and control experiments.

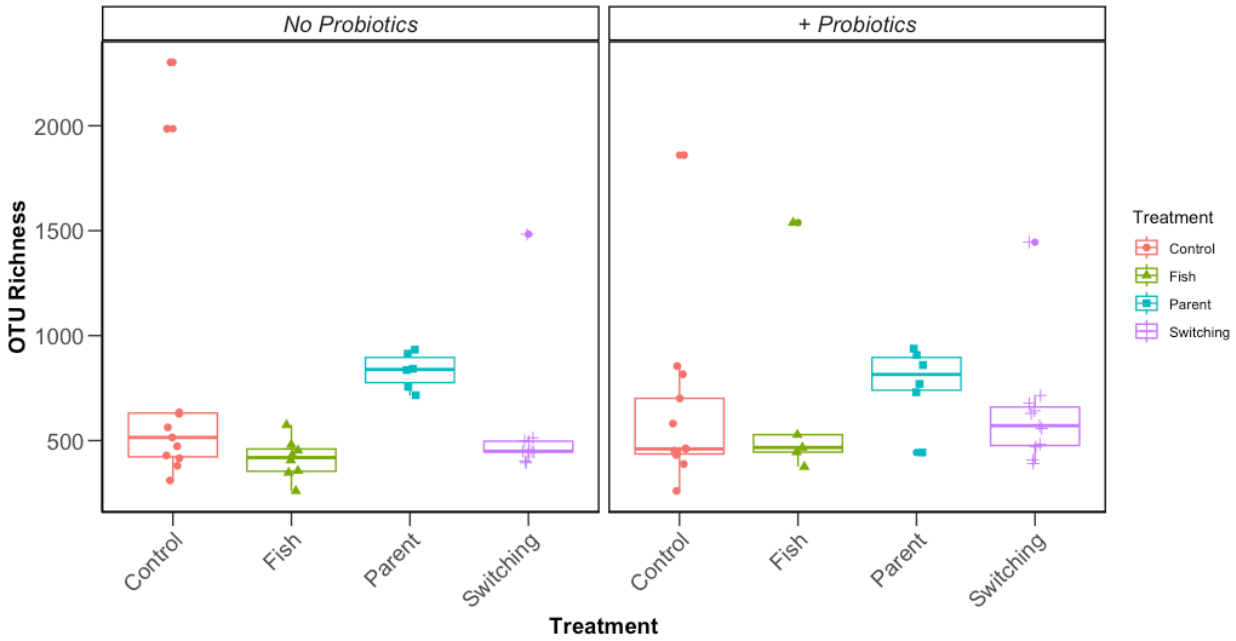


Figure 2. Boxplots represent species richness between individuals from different experiments. The boxplot also shows female feces and the sequenced probiotic. Squares are colored according to experiment (red square= control, pink square=switching, green square= fish food experiment, green square=parent (female feces), blue square=sequenced probiotic)

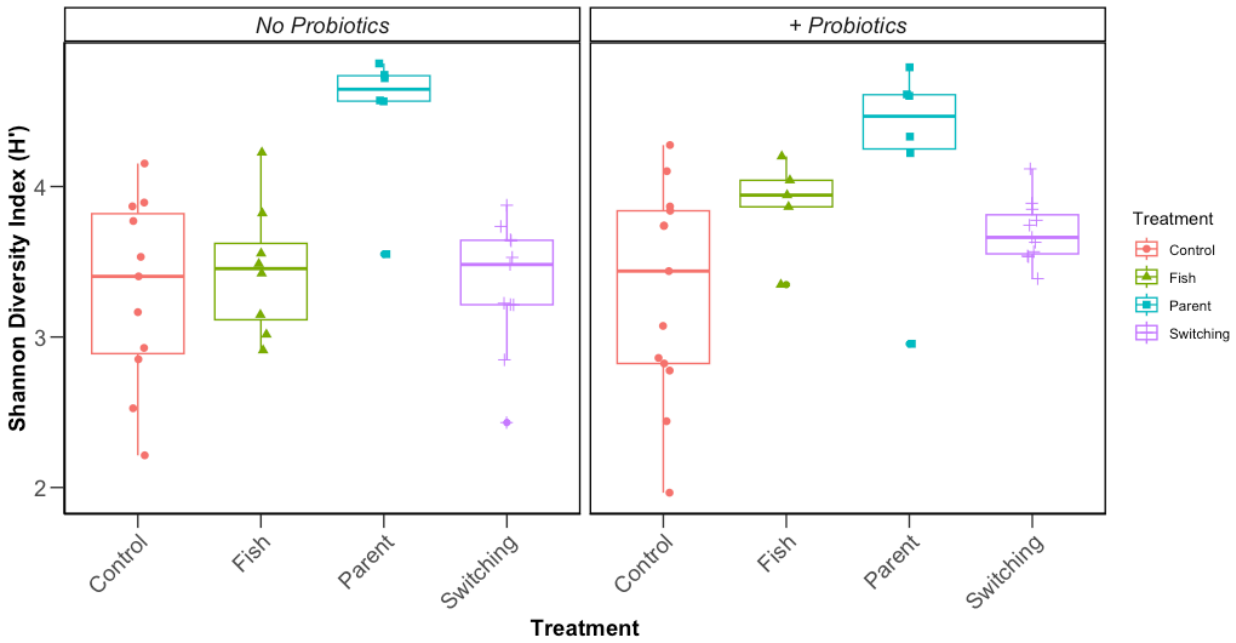


Figure 3. boxplots represent species diversity index between individuals from all treatments and experiments. squares are colored according to experiment (red square=control, pink square=switching, green square=fish food experiment, green square=parent (female feces, blue square=sequenced probiotic).

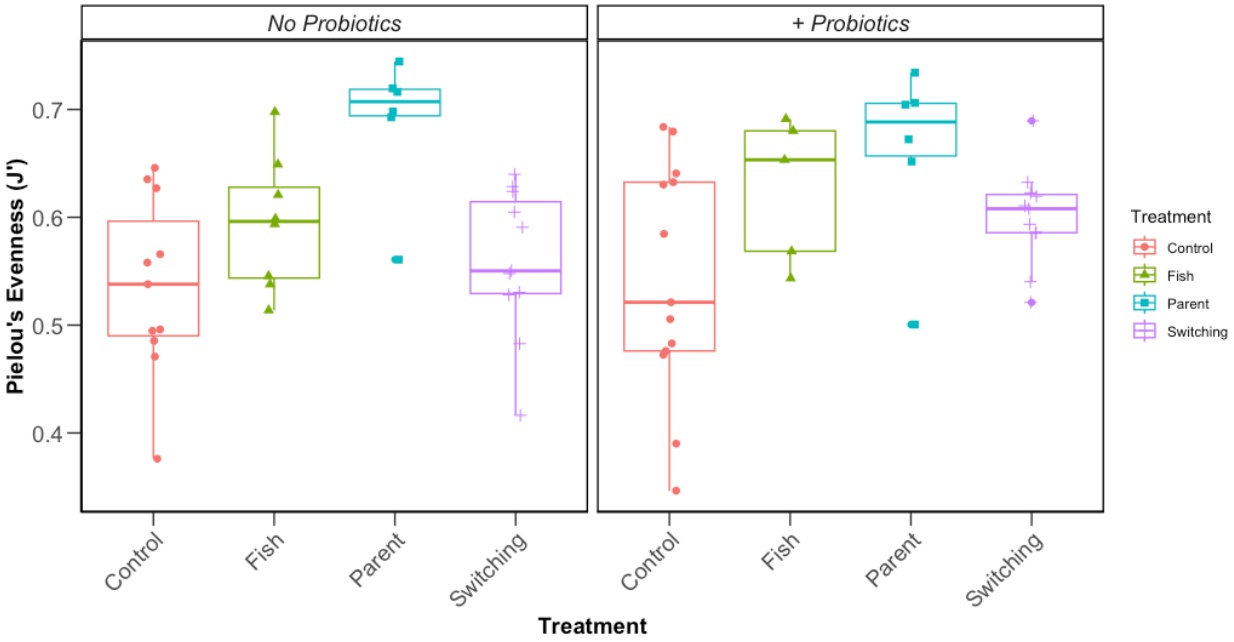


Figure 4. boxplots representing species evenness between experiment and type of diet.

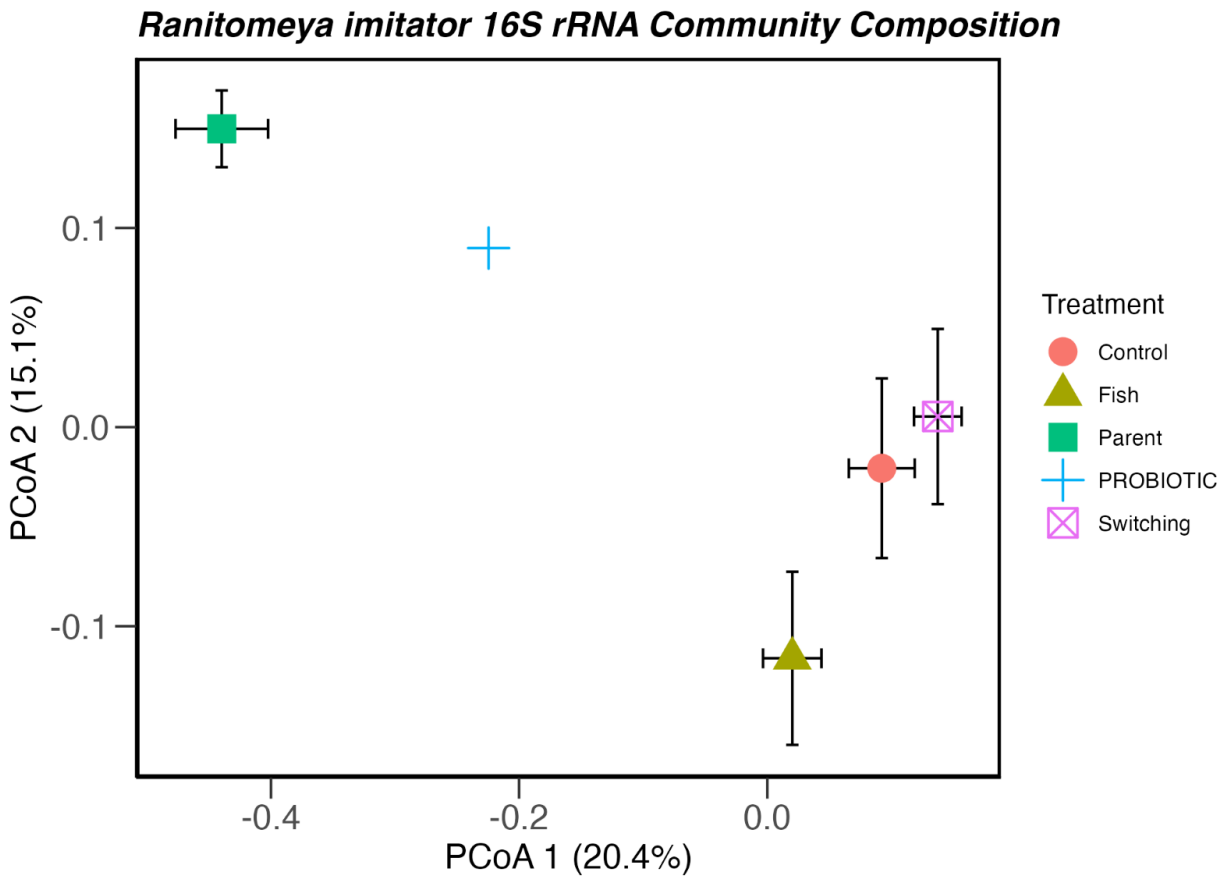


Figure 5. Ordination based on Principal Coordinate Analysis (PCoA) showing bacterial community composition based on experiments. Symbols are colored according to experiment (red circle = control, olive triangle = fish food, green square = parent (females feces), blue cross = sequenced probiotic, pink square = switching experiment).

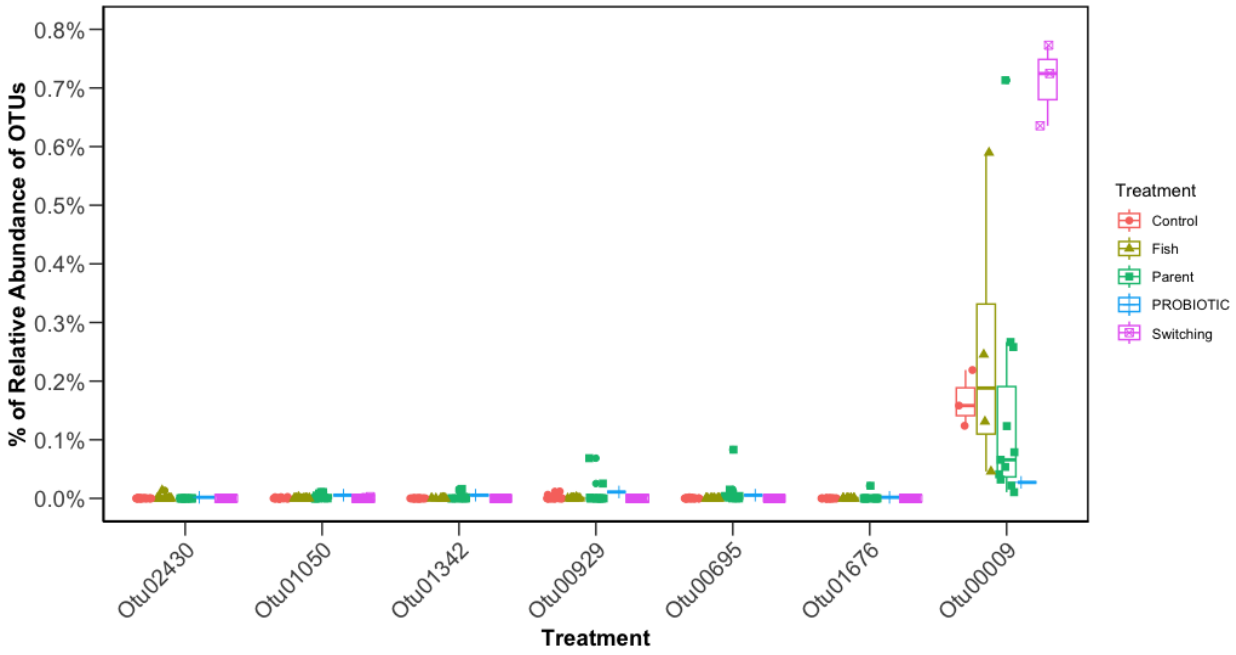


Figure 6. Boxplots represent the percentage of relative abundance of most abundant OTUs between treatments. Symbols are colored according to experiment (red circle = control, olive triangle = fish food, green square= parent (feces female), blue cross = sequenced probiotic, pink squared = switching experiment).

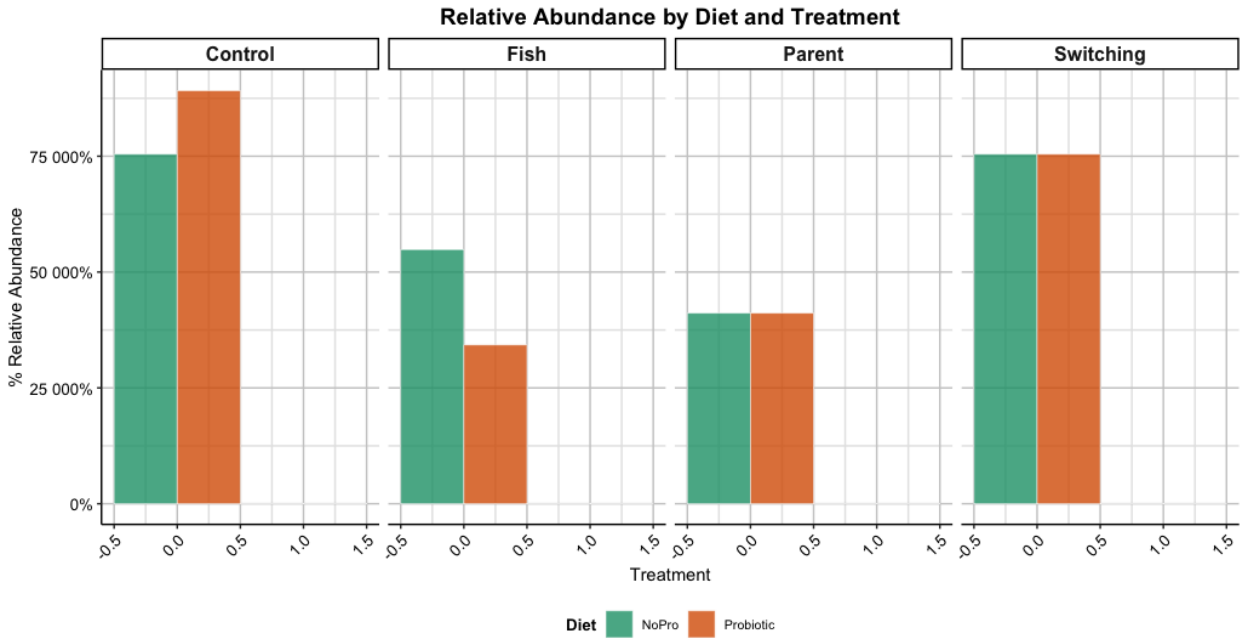


Figure 7. Relative abundance of OTUs between experiments and Diet provided to the mother. Stack bars were graph using relative abundance matrices of OTUs obtained for performing analysis of composition of bacterial communities.

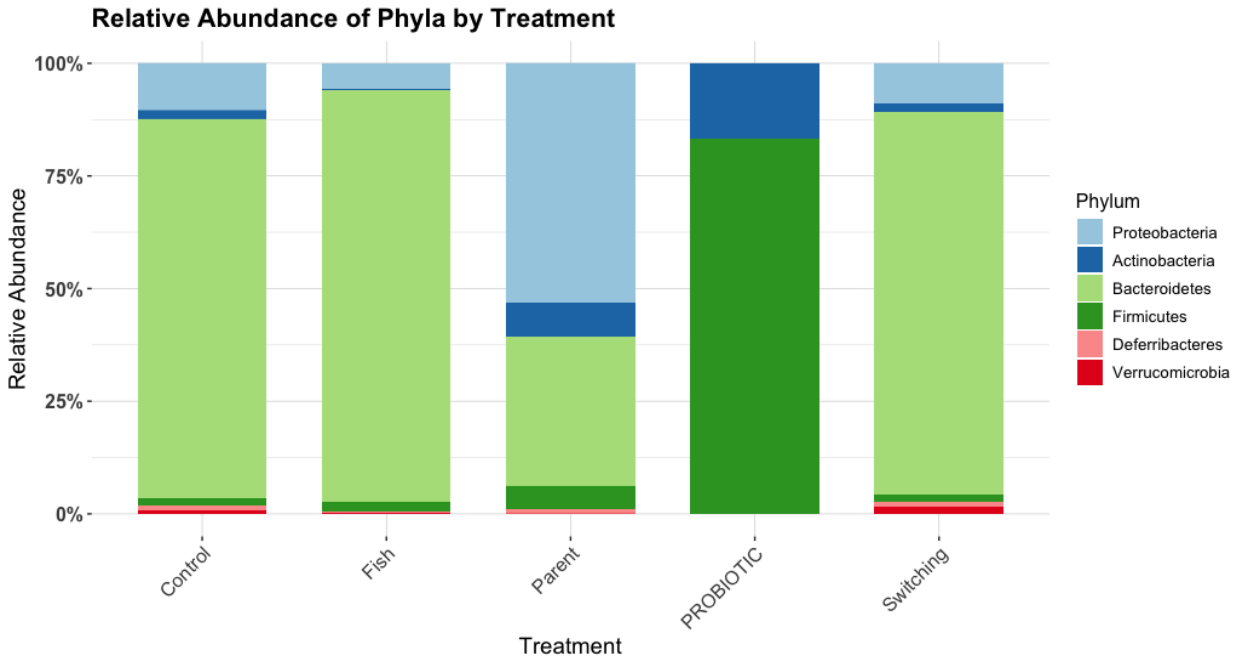


Figure 8. Stack bar analysis representing phylum level bacteria abundances in Ranitomeya imitator based on experiment. Color represents individual phyla. Sky blue = Proteobacteria; Blue = Actinobacteria; Green Apple = Bacteroidetes; Green = Firmicutes; Pink = Deferribacteres; Red = Verrucomicrobia.

Discussion

The goal of this research was to determine the main routes of transmission of gut bacteria during the development of *Ranitomeya imitator* tadpoles. To address this aim, I hypothesized diet is the main route of transmission that drives gut bacteria composition in this species. Results suggest that diet and environment play a crucial role in shaping the gut bacteria composition in offspring of the mimic poison frog, *R. imitator*. The experiments revealed that tadpoles who grew up with (actual or foster) parents, individuals in both the control experiment and switching experiment showed different levels of alpha diversity of gut bacteria compared to tadpoles from the fish food experiment. Although we did not see that mothers directly transfer microbes through trophic eggs to the offspring, maternal diet still impacts the acquisition of the gut microbiota in the progeny, even though the offspring have a facultative ability to utilize diverse sources of food when the female is absent. This suggests that female poison frogs who participate in brood care, is not limited, exclusively, to the provision of nutrients.

Moreover, the results suggested that the bacterial community of the gut is influenced by both acquisition from trophic eggs and from the pool environment, it seems the composition of the gut bacteria that comes from environment is greater than the proportion that comes from the maternal diet in *R. imitator* (figure 8). The composition of the gut bacterial community (microbiome) is very similar between the control and switched treatment, and both are different from the parental microbiome (Figure 5 and 8).

The alteration of the relationship between gut bacteria and their hosts can generate important consequences. For example, it can change the relationship between the hosts and its central nervous system (Wood, 2007). In frogs, a disruption of this association, for example, could affect the ability of the tadpoles to identify glycinamide in the environment, which is a chemical

compound that works to recognize other species of tadpoles and it is found in all species **(**Schulte et al., 2011**).** The males of poison frogs during the adulthood use the presence of glycinamide, that in the combination with heterospecific compounds could provide information of the tadpole's species present in the phytotelma, to avoid depositing tadpoles in pools with competitors, and/or predators during tadpole transportation (Schulte et al., 2015; Summers et al., 1999).

As with other vertebrates, the population of the gut bacteria of adults and tadpoles *R. imitator* consists of Bacteroidetes, Proteobacteria, Actinobacteria and Firmicutes (Ley et al., 2008). However, the dominant bacterial taxa in the gut of tadpoles from fish food treatment did not include Deferribacteres **(Figure 8)**, a phylum that is not normally found in animal's guts (Newsome et al., 2020). Proteobacteria and Actinobacteria were found mainly in tadpoles who grew up under a mother's provision of food. Furthermore, the presence of these phyla in the switching and control experiment may indicate that the tadpoles from these experiments are healthier, in contrast to tadpoles from fish food treatment because species from these groups are key in the production of vitamins for the host, inhibition of Gram-negative pathogens, and to aid in maintaining hosts homeostasis (Colston et al., 2016).

In amphibians, the ability to maintain a balanced homeostasis during early development is crucial to adapt to unexpected environmental situations that can affect physiological function and behavior in later life (Crespi et al., 2013). Also, the difference between the relative abundance of Proteobacteria and Actinobacteria between tadpoles from switching and control experiment compared to fish food tadpoles suggests that the prolonged provision of female care in *R. imitator* not only can have positive repercussions on the fitness offspring as in other Anura species (Valencia et al., 2016), but can also influence their overall health.

In Addition, parental environment during the development can have powerful transgenerational effects on life-history on animals. For example, when parents are raised having access to good quality food, this can improve the immune reactivity of the offspring (Triggs et al., 2012). Finally, one of the most abundant OTUs between offspring and parents *R. imitator* was the OTU 00009. This OTU is associated with the relatively new genus, *Parabacteroides,* which is important for energy utilization in the gut of vertebrates (Ezeji et al., 2021).

There are several studies that have described external (skin: Kueneman et al., 2014; Jimenez et al., 2019; Rebollar et al., 2020; Walke et al., 2016) and internal (gut: Warne et al., 2017; Brunetti et al., 2023; Fontaine et al., 2018; Bletz et al., 2016) microbiomes in anurans. Nevertheless, how parental care has a role in shaping the gut microbiome of offspring is less well known. Our results suggests that, even the absence of horizontal transmission of gut microbiome from mother to offspring in *R. imitator*, frequent maternal diet delivery is likely a source of both microbiome and nutrition provision in offspring, similar to that found in other vertebrate taxa such as birds (Diez-Mendez et al., 2023; Chen et al., 2020) and mammals (Ge et al., 2021; Fagan et al., 2024.

In our experimental design, we assumed that the fish food treatment is considered an "environmental situation" where mothers are not providing parental care. However, the proportion of relative abundance of bacteria in fish food treatment is higher than in adults for No Probiotic treatment (Figure 8). This result can be explained by the composition of fish food that usually includes squid meal, shrimp meal, earthworms, spirulina, and vitamins and minerals. Such provisioning of fish food would result in high presence of Bacteroidetes, which is one of the most abundant bacteria phyla found in vertebrate gastrointestinal tract and are important for both herbivorous and carnivorous diet, it would be interesting analyzing how the relative abundance of

Bacteroidetes can be affected when tadpoles grow in wild conditions with a poor maternal care, and where phytotelma chosen by *R. imitator* males are smaller in volume and are limited in availability of nutrients (Brown et al., 2008).

Conclusions

While it was initially predicted that mothers are transferring microbes to offspring by delivering trophic eggs, we did not identify vertical transmission of microbes as a major factor, as has been described for other groups of amphibians (Kouete et al., 2023). Nonetheless, our results indicate that parental care by *R. imitator* can have a specific maternal effect in progeny in shaping some aspects of the bacteria of gastrointestinal track by providing trophic eggs, and the diet and environment are main factors that may contribute to the microbiome composition in *R. imitator*. Future field research may focus on understanding mechanisms of microbiome transfer from the mother's gut to the offspring's gut in poison frogs that feed their young, and to understanding whether is important to produce a good microenvironment to be able to recognize chemical cues in the environment that are used later in life.

Bibliography

- Amato, K. R., Leigh, S. R., Kent, A., Mackie, R. I., Yeoman, C. J., Stumpf, R. M., ... & Garber, P. A. (2014). The role of gut microbes in satisfying the nutritional demands of adult and juvenile wild, black howler monkeys (*Alouatta pigra*). *American journal of physical anthropology*, *155*(4), 652-664.

- Amato, K. R., & Righini, N. (2015). The howler monkey as a model for exploring host-gut microbiota interactions in primates. *Howler monkeys: adaptive radiation, systematics, and morphology*, 229-258.

- Ardissone, A. N., de la Cruz, D. M., Davis-Richardson, A. G., Rechcigl, K. T., Li, N., Drew, J. C., ... & Neu, J. (2014). Meconium microbiome analysis identifies bacteria correlated with premature birth. *PloS one*, *9*(3), e90784.

- Bailey, M. T., & Coe, C. L. (1999). Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Developmental Psychobiology: The Journal of the International Society for Developmental Psychobiology*, *35*(2), 146-155.

- Banning, J. L., Weddle, A. L., Wahl Iii, G. W., Simon, M. A., Lauer, A., Walters, R. L., & Harris, R. N. (2008). Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, Hemidactylium scutatum. *Oecologia*, *156*, 423-429.

- Barelli, C., Albanese, D., Stumpf, R. M., Asangba, A., Donati, C., Rovero, F., & Hauffe, H. C. (2020). The gut microbiota communities of wild arboreal and ground-feeding tropical primates are affected differently by habitat disturbance. *MSystems*, *5*(3), 10-1128.

- Beck, K. B., Loretto, M. C., Ringler, M., Hödl, W., & Pašukonis, A. (2017). Relying on known or exploring for new? Movement patterns and reproductive resource use in a tadpole-transporting frog. *PeerJ*, *5*, e3745.

- Berlow, M., Wada, H., & Derryberry, E. P. (2022). Experimental exposure to noise alters gut microbiota in a captive songbird. *Microbial ecology*, *84*(4), 1264-1277.

- Bletz, M. C., Goedbloed, D. J., Sanchez, E., Reinhardt, T., Tebbe, C. C., Bhuju, S., ... & Steinfartz, S. (2016). Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nature communications*, *7*(1), 13699.

- Brooks, O. L., James, J. J., & Saporito, R. A. (2023). Maternal chemical defenses predict offspring defenses in a dendrobatid poison frog. *Oecologia*, *201*(2), 385-396.

- Brown, J. L., Morales, V., & Summers, K. (2008). Divergence in parental care, habitat selection and larval life history between two species of Peruvian poison frogs: an experimental analysis. *Journal of Evolutionary Biology*, *21*(6), 1534-1543

- Brown, J. L., Morales, V., & Summers, K. (2010). A key ecological trait drove the evolution of biparental care and monogamy in an amphibian. *The american naturalist*, *175*(4), 436-446.

- Browne, H. P., Neville, B. A., Forster, S. C., & Lawley, T. D. (2017). Transmission of the gut microbiota: spreading of health. *Nature Reviews Microbiology*, *15*(9), 531-543.

- Brunetti, A. E., Lyra, M. L., Monteiro, J. P., Zurano, J. P., Baldo, D., Haddad, C. F., & Moeller, A. H. (2023). Convergence of gut microbiota in myrmecophagous amphibians. *Proceedings of the Royal Society B*, *290*(2011), 20232223.

- Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell*, *165*(7), 1762-1775.

- Cáceres M, Legendre P (2009). "Associations between species and groups of sites: indices and statistical inference." Ecology, 90, 3566-3574. doi:10.1890/08-1823.1.

- Colston, T. J., & Jackson, C. R. (2016). Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. *Molecular ecology*, *25*(16), 3776-3800.

- Clayton, J. B., Gomez, A., Amato, K., Knights, D., Travis, D. A., Blekhman, R., ... & Johnson, T. J. (2018). The gut microbiome of nonhuman primates: lessons in ecology and evolution. *American journal of primatology*, *80*(6), e22867.

- Chen, C. Y., Chen, C. K., Chen, Y. Y., Fang, A., Shaw, G. T. W., Hung, C. M., & Wang, D. (2020). Maternal gut microbes shape the early-life assembly of gut microbiota in passerine chicks via nests. *Microbiome*, *8*, 1-13.

- Crespi, E. J., & Warne, R. W. (2013). Environmental conditions experienced during the tadpole stage alter post-metamorphic glucocorticoid response to stress in an amphibian.

- Diez-Méndez, D., Bodawatta, K. H., Freiberga, I., Klečková, I., Jønsson, K. A., Poulsen, M., & Sam, K. (2023). Indirect maternal effects via nest microbiome composition drive gut colonization in altricial chicks. *Molecular Ecology*, *32*(13), 3657-3671.

- Ezeji, J. C., Sarikonda, D. K., Hopperton, A., Erkkila, H. L., Cohen, D. E., Martinez, S. P., ... & Rodriguez-Palacios, A. (2021). Parabacteroides distasonis: intriguing aerotolerant gut anaerobe with emerging antimicrobial resistance and pathogenic and probiotic roles in human health. *Gut microbes*, *13*(1), 1922241.

- Fagan, B. T., Constable, G. W., & Law, R. (2024). Maternal transmission as a microbial symbiont sieve, and the absence of lactation in male mammals. *Nature Communications*, *15*(1), 5341.

- Fontaine, S. S., Novarro, A. J., & Kohl, K. D. (2018). Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *Journal of Experimental Biology*, *221*(20), jeb187559.

- Ge, Y., Zhu, W., Chen, L., Li, D., Li, Q., & Jie, H. (2021). The maternal milk microbiome in mammals of different types and its potential role in the neonatal gut microbiota composition. *Animals*, *11*(12), 3349.

- Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica, 16(3), 183-190.

- Grond, K., Sandercock, B. K., Jumpponen, A., & Zeglin, L. H. (2018). The avian gut microbiota: community, physiology, and function in wild birds. *Journal of Avian Biology*, *49*(11), e01788.

- Hooper, L. V., Midtvedt, T., & Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual review of nutrition*, *22*(1), 283-307.

- Kohl, K. D., Weiss, R. B., Cox, J., Dale, C., & Denise Dearing, M. (2014). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology letters*, *17*(10), 1238-1246.

- Korpela, K., Costea, P., Coelho, L. P., Kandels-Lewis, S., Willemsen, G., Boomsma, D. I., ... & Bork, P. (2018). Selective maternal seeding and environment shape the human gut microbiome. *Genome research*, *28*(4), 561-568.

- Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology*, *79*(17), 5112-5120. Accessed on June 15.

- Kouete, M. T., Bletz, M. C., LaBumbard, B. C., Woodhams, D. C., & Blackburn, D. C. (2023). Parental care contributes to vertical transmission of microbes in a skin-feeding and direct-developing caecilian. *Animal Microbiome*, *5*(1), 28.

- Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., & McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular ecology*, *23*(6), 1238-1250.

- Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest package: tests in linear mixed effects models. *Journal of statistical software*, *82*(13).

- Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., & Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology*, *6*(10), 776-788.

- Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C. A., Dempsey, E. M., O'Toole, P. W., ... & Stanton, C. (2017). The composition of human milk and infant faecal microbiota over the first three months of life: a pilot study. *Scientific reports*, *7*(1), 40597.

- Newsome, S. D., Feeser, K. L., Bradley, C. J., Wolf, C., Takacs-Vesbach, C., & Fogel, M. L. (2020). Isotopic and genetic methods reveal the role of the gut microbiome in mammalian host essential amino acid metabolism. *Proceedings of the Royal Society B*, *287*(1922), 20192995.

- Pašukonis, Andrius, Katharina Trenkwalder, Max Ringler, Eva Ringler, Rosanna Mangione, Jolanda Steininger, Ian Warrington, and Walter Hödl. "The significance of spatial memory for water finding in a tadpole-transporting frog." *Animal behaviour* 116 (2016): 89-98.

- Pašukonis, A., Beck, K. B., Fischer, M. T., Weinlein, S., Stückler, S., & Ringler, E. (2017). Induced parental care in a poison frog: a tadpole cross-fostering experiment. *Journal of Experimental Biology*, *220*(21), 3949-3954.

- Phillips, J. N., Berlow, M., & Derryberry, E. P. (2018). The effects of landscape urbanization on the gut microbiome: an exploration into the gut of urban and rural white-crowned sparrows. *Frontiers in Ecology and Evolution*, *6*, 148.

- Rebollar, E. A., Martínez-Ugalde, E., & Orta, A. H. (2020). The amphibian skin microbiome and its protective role against chytridiomycosis. *Herpetologica*, *76*(2), 167-177.

- Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open-source tool for metagenomics. *PeerJ*, *4*, e2584.

- Saporito, R. A., Russell, M. W., Richards-Zawacki, C. L., & Dugas, M. B. (2019). Experimental evidence for maternal provisioning of alkaloid defenses in a dendrobatid frog. *Toxicon*, *161*, 40-43.

- Summers, K. (1999). The effects of cannibalism on Amazonian poison frog egg and tadpole deposition and survivorship in Heliconia axil pools. *Oecologia*, *119*, 557-564.

- Summers, K., & Tumulty, J. (2014). Parental care, sexual selection, and mating systems in neotropical poison frogs. In *Sexual selection* (pp. 289-320). Academic Press.

- Schulte, L. M., Yeager, J., Schulte, R., Veith, M., Werner, P., Beck, L. A., & Lötters, S. (2011). The smell of success: choice of larval rearing sites by means of chemical cues in a Peruvian poison frog. *Animal Behaviour*, *81*(6), 1147-1154.

- Schulte, L. M., Krauss, M., Lötters, S., Schulze, T., & Brack, W. (2015). Decoding and discrimination of chemical cues and signals: Avoidance of predation and competition during parental care behavior in sympatric poison frogs. *PLoS One*, *10*(7), e0129929.

- Teyssier, A., Matthysen, E., Hudin, N. S., De Neve, L., White, J., & Lens, L. (2020). Diet contributes to urban-induced alterations in gut microbiota: experimental evidence from a wild passerine. *Proceedings of the Royal Society B*, *287*(1920), 20192182

- Triggs, A. M., & Knell, R. J. (2012). Parental diet has strong transgenerational effects on offspring immunity. *Functional Ecology*, *26*(6), 1409-1417.

- Tumulty, J., Morales, V., & Summers, K. (2014). The biparental care hypothesis for the evolution of monogamy: experimental evidence in an amphibian. Behavioral Ecology, 25(2), 262-270.

- Valencia, L. B., & Delia, J. (2016). Maternal care in a glassfrog: care function and commitment to offspring in Ikakogi tayrona. *Behavioral ecology and sociobiology*, *70*, 41-48.

- Videvall, E., Strandh, M., Engelbrecht, A., Cloete, S., & Cornwallis, C. K. (2018). Measuring the gut microbiome in birds: comparison of faecal and cloacal sampling. *Molecular ecology resources*, *18*(3), 424-434.

- Walke, J. B., & Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS pathogens*, *12*(9), e1005796.

- Warne, R. W., Kirschman, L., & Zeglin, L. (2017). Manipulation of gut microbiota reveals shifting community structure shaped by host developmental windows in amphibian larvae. *Integrative and comparative biology*, *57*(4), 786-794.

- Weinfurther, K. D., Stuckert, A. M. M., Muscarella, M. E., Peralta, A. L., & Summers, K. (2023). Evidence for a parabasalian gut symbiote in egg-feeding poison frog tadpoles in Peru. *Evolutionary Biology*, *50*(2), 239-248.

- Weng, F. C. H., Yang, Y. J., & Wang, D. (2016). Functional analysis for gut microbes of the brown tree frog (Polypedates megacephalus) in artificial hibernation. *BMC genomics*, *17*, 31-42.

- Wong, S., & Rawls, J. F. (2012). Intestinal microbiota composition in fishes is influenced by host ecology and environment.

- Wood, J. D. (2007). Neuropathophysiology of functional gastrointestinal disorders. *World journal of gastroenterology: WJG*, *13*(9), 1313.

- Xu, L. L., Chen, H., Zhang, M., Zhu, W., Chang, Q., Lu, G., ... & Zhu, L. (2020). Changes in the community structure of the symbiotic microbes of wild amphibians from the eastern edge of the Tibetan Plateau. *MicrobiologyOpen*, *9*(4), e1004.

- Yang, L., Sakandar, H. A., Sun, Z., & Zhang, H. (2021). Recent advances of intestinal microbiota transmission from mother to infant. *Journal of functional foods*, *87*, 104719.

- Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., ... & Glöckner, F. O. (2014). The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. *Nucleic acids research*, *42*(D1), D643-D648.

Appendix A: Supplemental tables

Supplemental Table 1. Statistical output of linear model with type of diet (probiotic and not probiotic) and treatment (Fish food, Control, Switching, and feces) as categorical predictors.

	NumDF	R^2	Adjusted R^2	F-test	p-value
Richness	63	0.10	-0.00	0.96	0.47
Diversity	63	0.49	0.43	7.85	2.94e-07
Evenness	63	0.28	0.19	3.18	0.00

Supplemental Table 2. Summary of linear model Table comparing bacteria diversity metric (OTU richness, Shannon Diversity Index (H'), and Simpson's Evenness) associated with type of diet (probiotic and not probiotic) and treatment (Fish food, Control, Switching, and feces).

A. OTU Richness

	Estimate	Srt. Error	t-value	Pr(>t)
Fish	-371.59	182.24	-2.03	0.04*
Parent (Feces)	47.74	199.05	0.24	0.81
Probiotic	-328.85	407.01	-0.80	0.42
Switching	-236.73	167.24	-1.41	0.16
Diet-Probiotic	-158.24	160.68	-0.98	0.32
Fish:Diet	415.34	275.34	1.50	0.13
Parent:Diet	100.24	277.64	0.36	0.71
Switching:Diet	245.15	231.92	1.05	0.29

B. Shannon's Diversity (H')

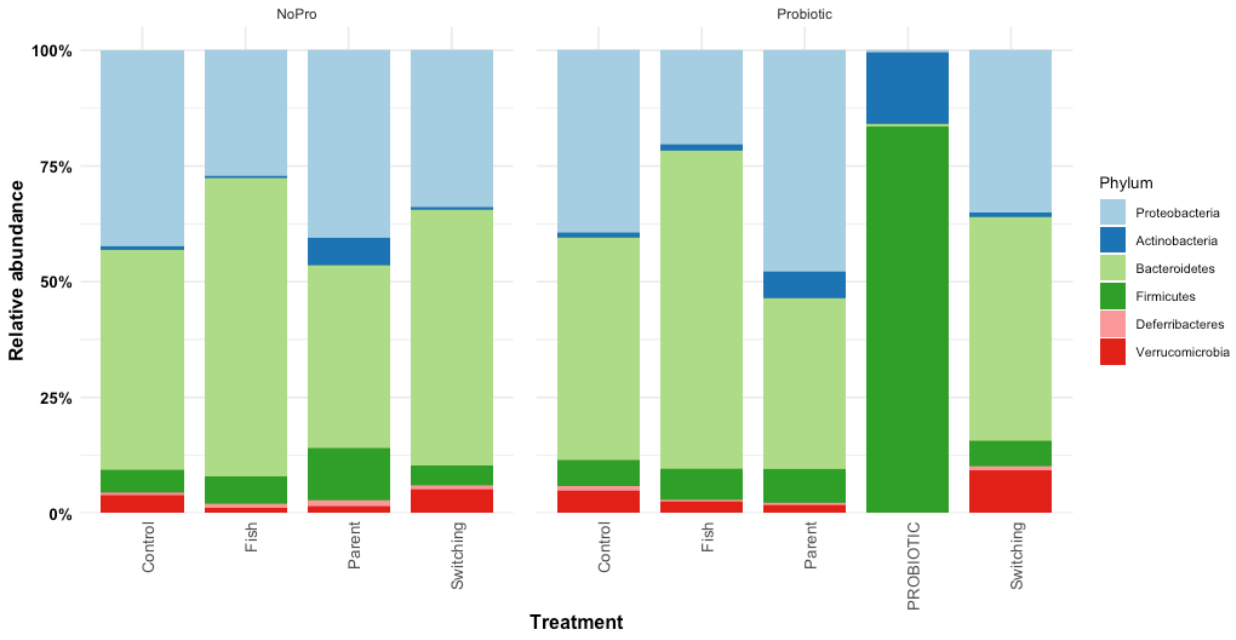
	Estimate	Srt. Error	T-value	Pr(>t)
Fish	0.14	0.24	0.60	0.54
Parent (Feces)	1.19	0.26	4.50	0.00 ***
Probiotic	-2.20	0.54	-4.06	0.00 ***
Switching	0.04	0.22	0.20	0.84
Diet-Probiotic	-0.00	0.21	-0.00	0.99
Fish:Diet	0.43	0.36	1.17	0.24
Parent:Diet	-0.23	0.37	-0.64	0.52
Switching:Diet	0.35	0.30	1.14	0.25

C. Pielou's Evenness

	Estimate	Srt. Error	t-value	Pr(>t)
Fish	1.59e-02	9.89e-03	1.61	0.11
Parent (Feces)	3.55e-02	1.08e-02	3.28	0.00**
Probiotic	-2.01e-02	2.20e-02	-0.91	0.36
Switching	4.97e-03	9.07e-03	0.54	0.58
Diet-Probiotic	5.23e-03	8.72e-03	0.60	0.55
Fish:Diet	9.73e-03	1.49e-02	0.65	0.51

Parent:Diet	-6.87e-03	1.50e-02	-0.45	0.65
Switching:Diet	-7.99e-05	1.25e-02	-0.00	0.99

Supplemental figure 8. Stack bar analysis representing phylum level bacteria abundances in *Ranitomeya imitator* based on experiment and diet provided to the female (mother). OTUs were classified according to phylum to calculate the relative abundances using the mean frequency at phylum level by treatment. Color represents individual phyla. Sky blue = Proteobacteria; Blue = Actinobacteria; Green Apple = Bacteroidetes; Green = Firmicutes; Pink = Deferribacteres; Red = Verrucomicrobia.



Appendix B: ECU IACUC Approval letter

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 – 4354
252-744-2436 office | 252-744-2355 fax

January 13, 2023

Kyle Summers, Ph.D.
Department of Biology, ECU

Dear Dr. Summers:

Your Animal Use Protocol entitled, "Maternal provisioning of symbiont microbes by egg feeding in the Peruvian mimic poison frog, Ranitomeya imitator" (AUP#D382) was reviewed by this institution's Animal Care and Use Committee on 01/13/2023. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice-Chair Animal Care and Use Committee

JD/GD

enclosure