Influence of Alternative Feeding Modes on Gene Expression and Microbiome Composition in Poison Frog Tadpoles

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Dendrobatid frogs have evolved a variety of unique behaviors related to parental care of tadpoles. However, few studies have investigated physiological adaptations and responses of tadpoles associated with different behaviors. The genus *Ranitomeya* provides a unique opportunity for comparative study as it includes two species that exhibit vastly different modes of tadpole feeding strategies: *R. imitator* tadpoles rely on infertile eggs provided by their parents, while *R. variabilis* tadpoles feed mainly on detritus. Despite these differences, tadpoles of both species can survive on alternative diets. We developed an experimental field study to compare responses to alternative feeding strategies and natural diets. To this end, we analyzed gut transcriptomes with accompanying microbiomes to investigate changes in bacterial composition and within the gut itself. Preliminary microbiome analyses revealed gut bacteria previously unknown from *Ranitomeya* poison frog tadpoles. Transcriptomic analyses uncovered 17 differentially expressed transcripts in *R. imitator* treatments, and 2,451 in *R. variabilis*. Critically, genes from a known group of symbiotic protists were highly expressed in egg-fed *R. imitator* tadpoles compared to those fed detritus. These results provide initial evidence for gut symbionts in these tadpoles, indicating the possibility that this symbiosis coevolved with egg-

feeding in this species and facilitated the expansion of *R. imitator* into previously uninhabitable breeding pools.

INFLUENCE OF ALTERNATIVE FEEDING MODES ON GENE EXPRESSION AND MICROBIOME COMPOSITION IN POISON FROG TADPOLES

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INTRODUCTION

The interactions between an organism and its microbiome—defined as associated bacterial, protozoal, bacteriophagal, viral, and fungal communities (Amon & Sanderson 2017; Dethlefsen et al. 2006)—have increasingly been considered an important factor in ecological adaptation (Alberdi, Aizpurua, Bohmann, Zepeda-Mendoza, & Gilbert 2016; Zilber-Rosenberg & Rosenberg 2016). Microbial communities play major roles in the morphology, physiology and development of their hosts (Alberdi et al. 2016; Bletz et al. 2016; Zilber-Rosenberg & Rosenberg 2016), though the full extent of their influence is still under investigation. Such an integrative relationship introduces the possibility of a "hologenome" mechanism of evolution (Zilber-Rosenberg & Rosenberg 2016). This hypothesis considers the holobiont—the host organism and its associated microorganisms—along with their corresponding genomes, as a possible unit of selection in evolution (Zilber-Rosenberg & Rosenberg 2016). While support for integrated roles has been linked to well-known microbial influences such as those of *Wolbachia* on arthropod reproductive isolation (Bordenstein 2003; Bordenstein, O'Hara & Werren 2001) and wood-digesting gut microbiota on termites (Brune & Dietrich 2015;Hongoh 2011; Ohkuma & Brune 2010), the restrictions necessary for this relationship—partner fidelity, acquisition via parent (vertical) or otherwise (horizontal), and equal levels of selection—has led some to question whether the concept applies on a broader scale (Douglas & Werren, 2016).

Alternatively, Douglas and Werren (2016) suggest that the host and microbiome can be considered as an ecological community. As a community—a group of interacting species co-occurring—units of selection could differ or act on the combined unit. Under specific conditions, changes in community function for the organism could therefore result in a gradual shift of community structure.

In the digestive system, changes in microbial communities and their effects have been documented extensively, particularly in humans (Broderik, Buchon & Lemaitre 2014; Gilbert et al. 2015). The vertebrate digestive system is inhabited by a wide array of complex microbial communities that can differ greatly between species. These communities have been shown to influence the immune system, cooperate in food breakdown, induce specific gene expression in intestinal cells (Bosch & McFall-Ngai 2011; Cash et al. 2006; Hooper et al. 2002), and participate in the structural buildup of blood vessels (Stappenbeck et al. 2002) and fat accumulation (Bäckhed et al. 2004). Microbial variation may result from phage infection, temperature change, or nutrient availability (among other factors), mediated by genetic drift or selection (Dethlefsen et al. 2006). Variation has also been shown to be affected negatively (reduced) by parasitic infection during specific developmental stages (Knutie, Wilkinson, Kohl, & Rohr 2017).

External ecological factors strongly influence microbiome composition. Available food resources, temperature, microbes in the soil, water, and air, and the microbiomes of sympatric plant and animal species can alter the makeup of different microbial communities, leading to adaptive changes (Bletz et al. 2016). For example, selection could favor changes in diet based on the functional ability of gut microbes to degrade specific molecules in novel food sources (Kohl, Amaya, Passement, Dearing, & Mccue 2014; Brune & Dietrich 2015; Kohl, Stengel & Dearing 2016). Kohl, Weiss, Cox, Dale, & Dearing (2014) showed that animals feeding on tannin-rich plants contained specific tannin-degrading bacteria in their gut, allowing the consumption of an otherwise toxic food source. Thus, diet-associated microbes selected based on their tannin-degrading abilities represent gut colonists; when introduced to the now tannin-rich environment in the gut, these microbes provided a selective advantage to their hosts in the form of a new

dietary niche. Gut colonists provide novel opportunities to influence the physical and biochemical reactions as well as the genetic composition of the gut itself, potentially manifesting as changes in patterns of gene expression. Given specific compositions, gut microbes can influence both energy intake and storage (Backhed et al. 2004; Sommer et al. 2016), greatly influencing host fitness and adaptive abilities. Therefore, studying both microbial composition and gene expression can provide unique insights into the interactions influencing biological adaptation.

Given these unique possible insights made by exploring changes in the microbial communities, the microbiome is an excellent system for comparative studies examining factors critical to evolutionary adaptations. Intra- and interspecific comparisons of microbial composition and diversity can be particularly useful in amphibians, which have independently evolved a wide array of novel adaptations (Summers et al. 2006). For example, species of neotropical poison frogs (Dendrobatidae) utilize a variety of reproductive strategies and parental care including trophic egg-feeding, a strategy in which females deposit unfertilized eggs for their tadpoles to feed on (Tumulty, Morales, & Summers 2014). However, little research has been done to understand the physiological adaptations in tadpoles that are associated with this behavior, or how these adaptations have influenced and were influenced by the host microbiome. Within dendrobatids, the genus *Ranitomeya* provides a unique opportunity for a comparative study as it includes two closely related species, *Ranitomeya imitator* and *Ranitomeya variabilis*, with dramatically different modes of parental care and associated tadpole feeding strategies, despite sharing similar habitats. *Ranitomeya imitator* breed in tiny bodies of water inside terrestrial plants called phytotelmata, and regularly feed their tadpoles protein-rich unfertilized eggs, as other nutritional sources are lacking in such small pools (Brown, Twomey, Morales, &

Summers 2008). *Ranitomeya variabilis* breed in pools of water within comparatively larger phytotelmata and do not feed their offspring; instead their tadpoles consume mosquito larvae and other less protein-rich nutrient sources such as available detritus and algae inside these pools (Brown, Twomey, Morales, & Summers 2008). A key characteristic of this study system is that tadpoles of both species are able to survive on other foods if available (although detritus is often minimal in the small pools used by *R. imitator*), suggesting that ancestral populations likely had to periodically subsist on alternative sources of nutrition (Brown, Twomey, Morales, & Summers 2008). Based on feeding habits of other closely related species (Brown, Twomey, Morales, & Summers 2008), the ancestral feeding mode is represented by *R. variabilis*: tadpoles were deposited in larger pools with more food available, but with higher levels of competition. The behavior of egg feeding is hypothesized to be an independently derived adaptation, and the shift in feeding ability potentially allowed this species to expand its range, rearing tadpoles in smaller pools in different phytotelma, where external food sources that otherwise would be necessary for growth and survival (as well as competition from other congenerics) were otherwise lacking (Yeager & Amorós 2020; Brown et al. 2008).

Taking advantage of each species' facultative ability to utilize multiple sources of food, we developed an experimental design for a comparative analysis to answer the following question: do changes in diet induce molecular (differences in gene expression) or microbial (differences in gut microbiome composition and diversity) changes in the gut of *Ranitomeya* tadpoles? If molecular, we would expect to see upregulation of genes related to the breakdown of detritus and/or egg protein in the respective diets of both species. Similarly, if microbial, we would expect to find a microbial community associated with the breakdown of plant or egg material. Additionally, we might see evidence of both predictions supported by different subsets

of the data (i.e. a dietary treatment may result in subsequent horizontal transmission of microbes, as well as the up- or down-regulation of genes in the gut necessary for the breakdown of specific material). If feeding strategy does not influence gene expression or microbial composition, we expect to see no differences between feeding groups of different species regardless of diet. If this is the case, feeding strategies may instead be mediated by comparable changes in the parents rather than tadpoles, in changes to tadpole gene expression or microbiota composition in areas other than the gut, or through another mechanism such as hormonal or other physiological response.

Based on our original question, we propose the following predictions: 1) Specific microbial communities will consistently characterize individuals based on feeding behavior, and patterns of gene expression will change with the colonization of new microbes in the gut of the tadpole. 2) Microbial communities will be consistent across species, but will change functionally based on feeding behavior, also resulting in gene expression changes based on food type. 3) A core microbiome will remain constant across intraspecific feeding treatments, and interspecific differences in certain gene expression patterns will be found that represent long-term adaptation between the different lineages. In short, altering tadpole feeding strategies will have noticeable effects on gut microbe communities and the expression of genes associated with the breakdown of different materials in both species tested, though these changes may not necessarily be exhibited in the exact same way in each species.

METHODS

Field Work

Field work was conducted at four field sites around Tarapoto, Peru from May through August 2017. After identifying field sites, we identified breeding pairs of both *R. imitator* and *R. variabilis* and monitored their breeding behavior following the methods of Tumulty and Summers (2014). After deposition and before the first egg feeding occurred in *R. imitator*, each tadpole was removed from their pool, then weighed and measured with a scale and calipers. Tadpoles in the control treatments were returned to their original pools. Tadpoles in the crossed treatment were placed in the opposite pool type: *R. imitator* tadpoles in large artificial pools to mimic those used by *R. variabilis*, and *R. variabilis* tadpoles in small, artificial pools to mimic those used by *R. imitator*. *Ranitomeya imitator* tadpoles in the larger pools fed on algae, detritus and mosquito larvae. *Ranitomeya variabilis* tadpoles in the artificial pools were fed eggs collected from the field every 3 days. Each pool was monitored on a weekly basis for development at Gosner stage 30 (Gosner 1960), in order to avoid any changes in the gut brought on by metamorphosis. After the designated stage was reached, we collected each tadpole in a sterile falcon tube.

Gut Transcriptome Analysis

After collection, tadpoles were weighed and measured, anesthetized using 250g/l tricaine methanesulfonate (MS-222) buffered by sodium bicarbonate to maintain neutral pH, then euthanized by pithing. We then rinsed the bodies with 25 mL of sterile water to remove the anesthetizing agent and to prevent other sources of microbial contamination, and dissected out the stomach and intestines (collectively, the gut). Before storing, the gut samples were rinsed again with 25 mL of sterile water. Samples were stored in RNA later for preservation until

extraction. RNA was extracted from the half of the tissue samples using a standardized Trizol protocol, cleaned with DNAse and RNAsin, and purified using Qiagen RNEasy mini kit. Libraries were prepared using standard poly-A tail purification with Illumina primers, and barcoded using a New England Biolabs Ultra Directional kit as per manufacturers protocol. Samples were pooled and sequenced using paired end reads at Novogene on the Illumina platform. We used the Oyster River Protocol v2.2.7 (MacManes 2018) to assemble the dataset. Error correction was done using RCorrector 1.01 (Song & Florea 2015), followed by adapter removal and quality trimming by trimmomatic v0.36 at a Phred score of ≤ 3 (Bolger et al. 2014). We constructed assemblies using Trinity 2.4.0 (Grabherr et al. 2011) SPAdes assembler v3.11 using 55 and 75 kmers (Bankevich et al. 2012), and Shannon version 0.0.2 (Kannan et al. 2016). We merged assemblies using Orthofuser (MacManes 2018). Using BUSCO version 3.0.1 (Simão et al. 2015) and TransRate 1.0.3 (Smith-Unna et al. 2016). We used Diamond version 0.9.10 (Buchfink et al. 2015) to annotate the transcriptome with peptide databases for *Xenopus tropicalis*. We then pseudo-quantified alignments for each library and technical replicate using Kallisto version 0.43.0 (Bray et al. 2016) and tested for differential gene expression in R version 3.4.2 (R Development Core Team 2017) using Sleuth version 0.29.0 (Pimentel et al. 2017). Figure 1 shows the design of the comparisons. Differentially expressed genes were searched against known sequences using a translated nucleotide database (tblastx). Lastly, we conducted gene ontology analyses using GOrilla (Eden et al. 2009).

Gut Microbiome Analysis

We characterized tadpole gut microbiome composition via amplicon sequencing of the 16S rRNA gene. To this end, we extracted genomic DNA from the remaining half of the tissue samples using the DNeasy PowerLyzer Powersoil Kit (Qiagen), and standardized DNA concentrations to a maximum of 10 ng/µL prior to PCR. We used primers 515F and 806RB barcoded primer set designed by the Earth Microbiome Project to amplify the V4-V5 region of the 16S subunit of the ribosomal RNA gene in bacteria archaea (Caporaso et al. 2012). For each sample, we prepared libraries by combining 38.35 µL molecular grade water, 5 µL Amplitaq Gold 360 10x buffer, 2.4 µL MgCL2 (25mM), 1 µL dNTPs (40mM total, 10mM individual), 0.25 µL Amplitaq Gold 360 polymerase, 1 µL forward barcoded primer (10M), 1µL 806 reverse primer (10M), and 1 µL DNA template (10 ng/µL). Thermocycler conditions for reactions were as follows: initial denaturation (94˚C, 3 minutes); 30 cycles of denaturing at 94˚C for 45 seconds, annealing at 50˚C for 30 seconds, and extending at 72˚C for 90 seconds; final elongation (72˚C, 10 minutes). For each sample, triplicate PCR products were combined then cleaned using the Agencourt AMPure XP magnetic bead cleanup kit (Axygen) and quantified using Quant-iT dsDNA BR (broad-range) assay (Thermo Scientific, Waltham, MA, USA). PCR products were mixed in equimolar concentrations and 250bp paired-end sequenced on an Illumina MiSeq platform (Illumina Reagent Kit v2, 500 reaction kit) at Indiana University's Center for Genomics and Bioinformatics. Sequences were assembled and analyzed using a standard mothur pipeline (v1.40.1) (Schloss et al. 2009, Kozich et al. 2013). Briefly, we assembled contigs from paired end reads, trimmed low quality bases, aligned sequences to the Silva Database (Quast et al. 2013; SSURef v132), and removed chimeric sequences using the VSEARCH algorithm (Rognes et al. 2016). Operational taxonomic units (OTUs) were binned at 97% sequence identity, and

taxonomic classifications were carried out for the resulting bacterial gene sequences using the Silva database.

We ran all statistical analyses in the R Environment (R v3.6.3, R Core Development Core Team 2020). Intraspecific comparisons were made between egg-fed and detritus-fed samples of both *R. imitator* and *R. variabilis*. To visualize patterns of microbial community composition among the two treatments and species, we used principal coordinate analysis of the bacterial community composition based on the Bray-Curtis dissimilarity coefficient. The adonis function in the vegan package (Oksanen 2015) was used to run permuted analysis of variance (PERMANOVA) to test for clustering significance. We rarefied sequences prior to calculating bacterial richness, evenness, and diversity metrics. We conducted indicator species analysis to identify taxa representative of each diet for each species using the labdsv package (Roberts 2016).

RESULTS

Gene Expression

Figure 1 shows the experimental design of the differential expression experiments analyzed for this thesis, including the number of genes found to be differentially expressed in each comparison. Controlling for multiple comparisons, between egg-fed, natural pool and detritus-fed, large pool treatments of *R. imitator*, 17 transcripts were significantly differentially expressed. While half of these transcripts did not yield any significant hits using BLAST searches of the GenBank databases, the majority of those that did return significant hits were protein digesting enzymes (e.g. cysteine peptidases) most closely related to peptidases from a group of protists known as parabasalians (Table 1). For example, transcript 69809 (Table 1) is most closely related to a cathepsin L-like cysteine peptidase from the parabasalian protist *Tritichomonas foetus*. This transcript was highly expressed in the egg-fed treatment, but not in the detritus-fed treatment (Fig 8).

Four other transcripts (108710, 25604, 70345, 68184) also matched proteases (peptidases) closely related to proteases previously identified in parabasalian protists. The protein-digesting function of these enzymes is of obvious significance given the high protein content of an egg-based diet (see discussion). We also found increased expression of common gene products (actin, elongation factor 1-alpha 2) in the egg-fed treatment that also are most similar to sequences of parabasalian protist proteins. Taken together, these results suggest a symbiotic relationship wherein related protists inhabit the guts of *R. imitator* tadpoles, assisting in the breakdown of the unfertilized eggs and reaching higher population densities in the egg-fed treatment.

Another gene differentially expressed between *R. imitator* treatments is keratin 8 (Fig 9). Keratin 8 is a filament protein that stabilizes and protects intestinal tissues and has been implicated in lipid metabolism (see discussion). The results of our BLAST search reveal that the differentially expressed *R. imitator* transcript identified in this study is closely related to the protein from *Rana catesbiana*, implying that it is likely produced by *Ranitomeya imitator* itself, rather than by a symbiont.

Conversely, comparison of egg-fed and detritus-fed treatments of *R. variabilis* tadpoles yielded a substantial number of differentially expressed genes. Of the 2,451 differentially expressed transcripts, a number of those up-regulated in the egg-fed treatment closely match (in BLAST searches) the sequences of genes are associated with lipid processing. These include apolipoprotein A1, a major component of high-density lipoproteins intimately involved in cholesterol metabolism and well-known in the context of human cardiovascular disease. Another exemplar gene is CYP51A1, a member of the cytochrome P450 group of enzymes. These enzymes are also heavily involved in the metabolism of cholesterol, steroids and other lipids. Up-regulation of these genes in the guts of the *R. variabilis* tadpoles fed on an egg-diet appears to be a response to the high lipid levels associated with that diet.

Of all differentially expressed genes across the four treatments, only one was shared by both *R. variabilis* and *R. imitator*. This gene was a hydrolase, and may have been upregulated in response to the need to process plant cell wall components associated with a detritus diet. We did not see differential expression of any of the parabasalian genes seen for the *R. imitator* comparisons. These results suggest the perceived novel symbiotic relationship between *R. imitator* tadpoles and an unknown parabasalian likely does not extend to *R. variabilis*, representing a novel, derived “trait” in *R. imitator*.

Gene ontology analyses of the differentially expressed gene in *R. variabilis* revealed several categories of genes associated with lipid processing were upregulated, including sterol metabolic processes, steroid metabolic processes, and lipid transport (Fig. 2). It is noteworthy that *R. imitator* tadpoles did not up-regulate lipid processing genes similar to *R. variabilis* tadpoles. One possible explanation is that some or all of these genes are constitutively up-regulated in *R. imitator*, given that eggs are the normal diet for tadpoles in this species. Tadpoles of *R. variabilis*, conversely, would have access to eggs less frequently (although egg cannibalism by tadpoles can occur in this species). Hence, the up-regulation of lipid processing enzymes in these tadpoles might occur through a facultative physiological response, rather than being a constitutive property of the species.

Microbiome

Indicator species analysis identified one Operational Taxonomic Unit (OTU) from *R. variabilis* treatments (Table 3) in the family Rikenellaceae (detritus fed), and three in *R. imitator* treatments (Table 2), from one unclassified bacteria (egg-fed), one Bacteroidaceae (detritus-fed), and one Desulfovibrionaceae (egg-fed). Results for diversity showed no significant differences, but some patterns were seen in the data. Bacterial diversity measured using Shannon Diversity Index (*H*) trended towards being higher in the egg-fed vs detritus-fed treatment in *R. imitator* ($p < 0.167$), but the opposite relationship was observed between *R. variabilis* ($p<0.079$) detritus-fed and egg-fed treatment tadpoles. Bacterial species richness trended toward higher variability in the egg-fed *R. imitator* ($p < 0.767$) treatment, but lower than the detritus-fed treatment, a pattern similarly seen in *R. variabilis* ($p<0.100$). Simpson’s Evenness trended higher in the egg-fed *R.imitator* ($p<0.175$), but lower in egg-fed *R. variabilis* ($p<0.364$)(Fig 7).

DISCUSSION

Due to the major role of microbial communities in ecological adaptations, we developed a study to look at changes in microbial composition, as well as associated influences on physiological and behavioral adaptations in poison frog tadpoles fed on ancestral and derived diets. By investigating these influences on molecular and microbial composition in poison frog tadpoles, we uncovered what could be an important factor in the ecological adaptation of egg-feeding in *Ranitomeya*.

Analyses of microbial community composition of *R. variabilis* and *R. imitator* uncovered bacteria aiding in digestion commonly found in the gut microbiome of many animals (Rikenellaceae in *R. variabilis,* Bacteroidaceae in *R. imitator*). The single family found in *R. variabilis* was identified to genus level (*Mucinivorans*), which is known from one isolation from the digestive tract of a leech (Nelson, Bomar, Maltz, & Graf 2015), and could be associated with organisms digested by the tadpoles. Desulfovibrionaceae, found in egg-fed *R. imitator*, are composed of sulfate-reducing bacteria commonly found in aquatic environments often with high amounts of organic material. Some bacteria from this group have also been isolated from animal and human intestines, although their role in digestion is unknown. It is unclear why Desulfovibrionaceae were more abundant in the microbiome of *R. imitator*, where tadpoles are found in pools with very little organic material to feed on, rather than *R. variabilis* tadpoles which are exposed to larger pools where a variety of organic material can be found. It is possible that these sulfate-reducing bacteria play a role in the digestion of eggs specifically, which would account for their absence in *R. variabilis*. Bacterial diversity metrics showed no significant differences between diet treatments, which was likely a result of low sample sizes. Although sample sizes were low, overall the natural diet of *R. imitator* trended toward more stability than

that of the natural detritus diet of *R. variabilis*. This is most likely due to the variable nature of food composition/availability in pools where *R. variabilis* tadpoles are found.

Our transcriptomic analyses of the guts of *R. imitator* tadpoles that were fed a natural diet of eggs (a derived diet shared with only one closely related species, *R. vanzolinii*), or detritus, algae and insect larvae (the ancestral diet for this genus) identified differences in expression in genes from a group of single-celled eukaryotes known as the Parabasalia. Parabasalians are anaerobic flagellate protists, most of which are symbionts found in the intestinal tracts of many vertebrate and insect hosts (Cepicka et al. 2017). They are perhaps most well known as gut mutualists of termites (Kirby 1931), which contribute to the digestion of wood as part of the termite gut microbiome. The most well-studied parabasalians are those found in humans, such as *Trichomonas vaginalis* (a urogenitotract parasite), or in domesticated animals, such as *Tritrichomonas foetus* (a venereal parasite of cattle, but a harmless commensal in pigs) (BonDurant & Honigberg 1994). The prevalence of studies on these two taxa (and subsequent availability of sequence data) likely explains why these species provided the closest matches to the differentially expressed sequences in our BLAST searches. Parabasalians have been identified in amphibians (e.g. *Trichomitus batrachorum* (Dobell 1909)), but there is comparatively little sequence data available for these species. As the gut microbiomes of Amazonian poison frogs were virtually unstudied until now, it is likely that the sequences we identified are from an as-yet-undescribed species of symbiotic parabasalian inhabiting the guts of *R. imitator* tadpoles. Given that these sequences are from gut microfauna rather than from genes in the *R. imitator* gut transcriptome, it is likely that the differential expression observed results from different population densities of the parabasalian microfauna, suggesting that these

microorganisms are responding to the gut microenvironment as affected by the differential dietary treatments.

While some of the parabasalian genes were common genes not associated with nutrient digestion (e.g. actin), most were protein digestion enzymes (cathepsins and legumains). These genes are known to be key mediators of protein catabolism. In fact, they are key enzymes involved in the processing of high protein substrates, such as blood meals in ticks and other blood feeding animals (e.g. Alim et al. 2009; Santiago et al. 2017). Cathepsins are also involved in lipid processing (Thibeaux et al. 2017). These enzymes likely play key roles in the digestion of proteins and lipids, which would likely be useful in processing the concentrated proteins and lipids associated with an egg diet.

Our results provide initial evidence for gut symbionts in the tadpoles of *Ranitomeya imitator*, indicating the possibility that symbionts coevolved with egg-feeding in this species. These preliminary results are an important first step in understanding the evolution of this novel strategy and have opened the door for further research. Importantly, future studies examining and better characterizing these symbionts, as well as whether said symbionts are transmitted vertically from parents or acquired independently from the environment, are needed. Studies examining growth rate and other physiological responses in tadpoles to alternate feeding strategies could also provide new insight into the evolution of this group. Finally, examining gene expression and microbial composition in parental frogs, as well as adult frogs metamorphosed from tadpoles raised on alternative diets, may uncover additional mechanisms shaping the unique adaptations facilitating the diversification of these frogs.

In summary, the evidence for differential expression of parabasalian genes in the guts of *R. imitator* tadpoles feeding on an egg diet (compared to the ancestral detritus diet) implicates

these protists as gut symbionts in these tadpoles. The high expression of proteolytic enzymes associated with the breakdown of proteins and lipids in other taxa further suggests that these protists are symbionts that specifically aid in the digestion of large quantities of proteins and lipids associated with an egg-based diet. We believe this is evidence of a new form of symbiosis that provided a novel mechanism for a “key innovation” in the life history of *R. imitator*: the evolution of egg-feeding. This trait likely allowed *R. imitator* to greatly expand its geographic range into those of the northern species that it is currently sympatric with by allowing this species to use tiny pools that provided insufficient nutrients to other species, resulting in the formation of large mimicry complex (Symula et al. 2003; Twomey et al. 2013). Future research will focus on characterizing these putative symbionts (and their functional role) in more detail.

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TABLES

Table 1. Transcripts, associated proteins, and most closely related organisms from BLAST searches (based on amino acid sequence) of transcripts showing significant differential expression between egg-fed and detritus-fed treatments for *R. imitator*

Transcript	Protein	Organism
69809	Cathepsin L-like cysteine peptidase	*Tritrichomonas foetus*
108710	Cathepsin L-like cysteine proteinase precursor	*Trichomonas vaginalis*
25604	Cathepsin L-like cysteine proteinase precursor	*Trichomonas vaginalis*
68484	Actin	*Tritrichomonas foetus*
39181	Actin	*Tritichomitus batrachorum*
67755	Elongation factor 1-alpha 2	*Tritrichomonas foetus*
70345	Cysteine protease 8	*Tritrichomonas foetus*
68184	Asparaginyl endopeptidase-like cysteine peptidase	*Trichomonas vaginalis*
169742	Cell-wall associated hydrolase	*Trichuris trichiura*
22062	Keratin 8	*Rana catesbiana*

Table 2. Indicator species OTU groups for *R. imitator* diet comparisons. Cluster 1 represents detritus-fed individuals, while Cluster 2 represents egg-fed.

OTU	Cluster	IndVal	Prob	Domain	Family	Genus
Otu0008	1	0.916426	0.045	Bacteria	Bacteroidaceae	*Bacteroides*
Otu0011	2	0.94979	0.023	Bacteria	Bacteria_unclass.	Bacteria_unclass.
Otu0014	2	0.855155	0.05	Bacteria	Desulfovibrionaceae	*Desulfovibrio*

Table 3. Indicator species OTU groups for *R. variabilis* diet comparisons. Cluster 1 represents detritus-fed individuals.

OTU	Cluster	IndVal	Prob	Domain	Family	Genus
Otu0005	1	0.749395	0.009	Bacteria	Rikenellaceae	*Mucinivorans*

FIGURES

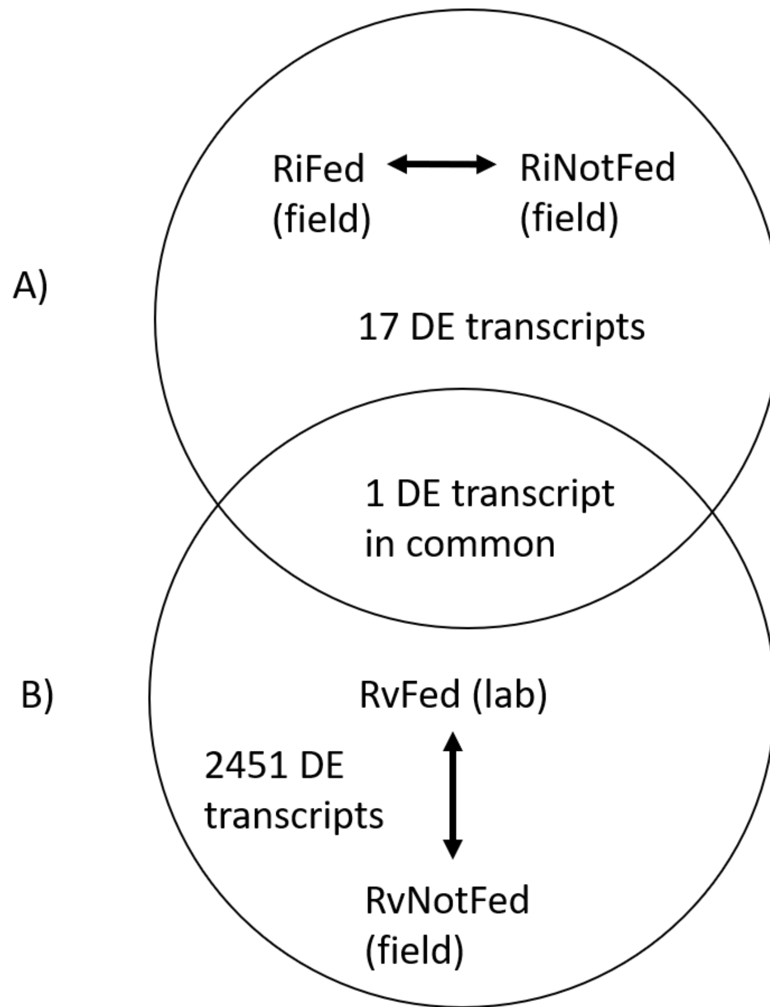

Fig. 1. This figure shows the differential gene expression comparisons made for A) *R. imitator* tadpole treatments (egg-fed versus detritus fed in the field) and for B) *R. variabilis* treatments (egg-fed in the lab versus detritus-fed in the field).

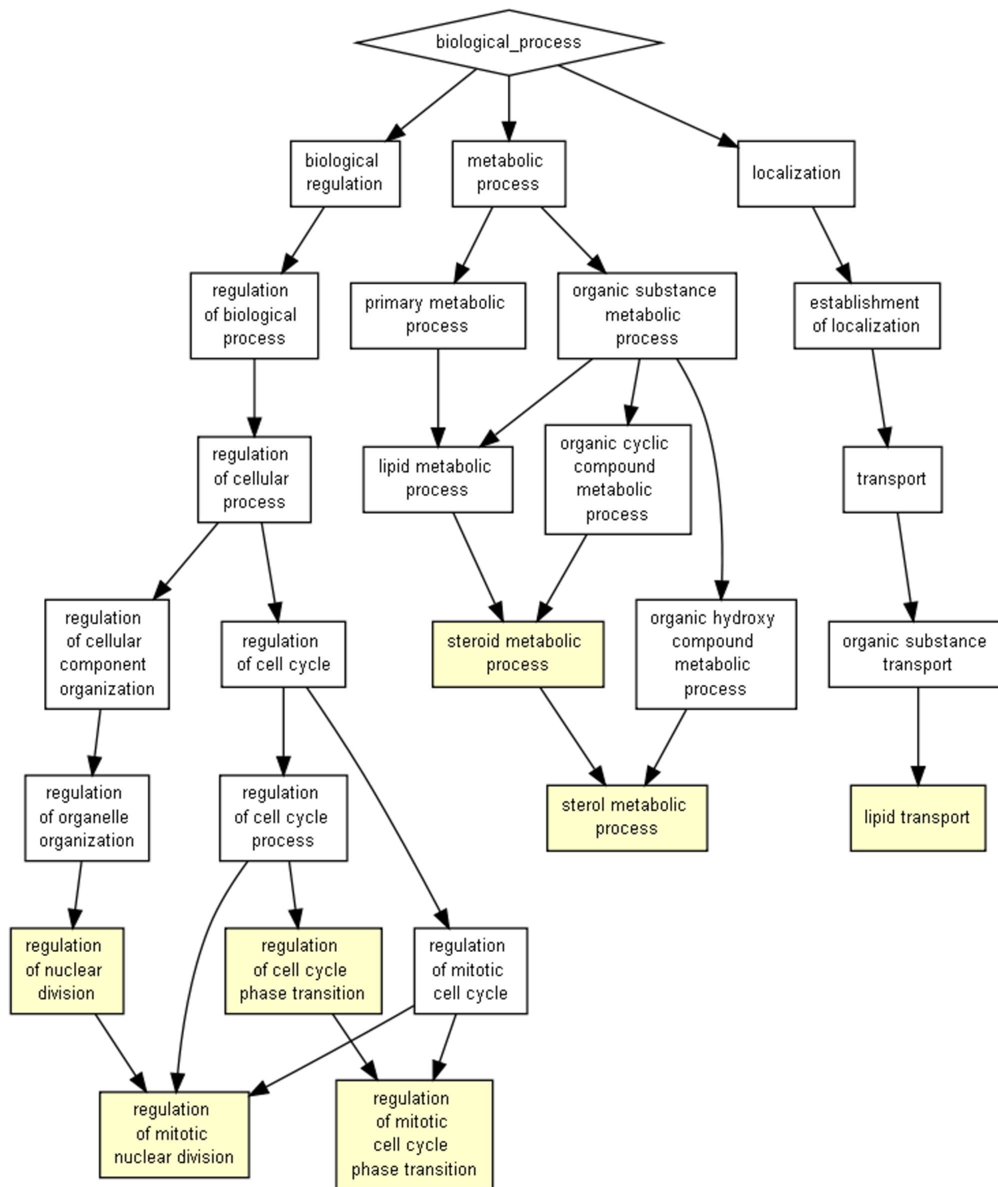

Fig. 2. Gene categories over-represented in the set of differentially expressed genes between egg-fed and detritus-fed *R. variabilis* tadpoles.

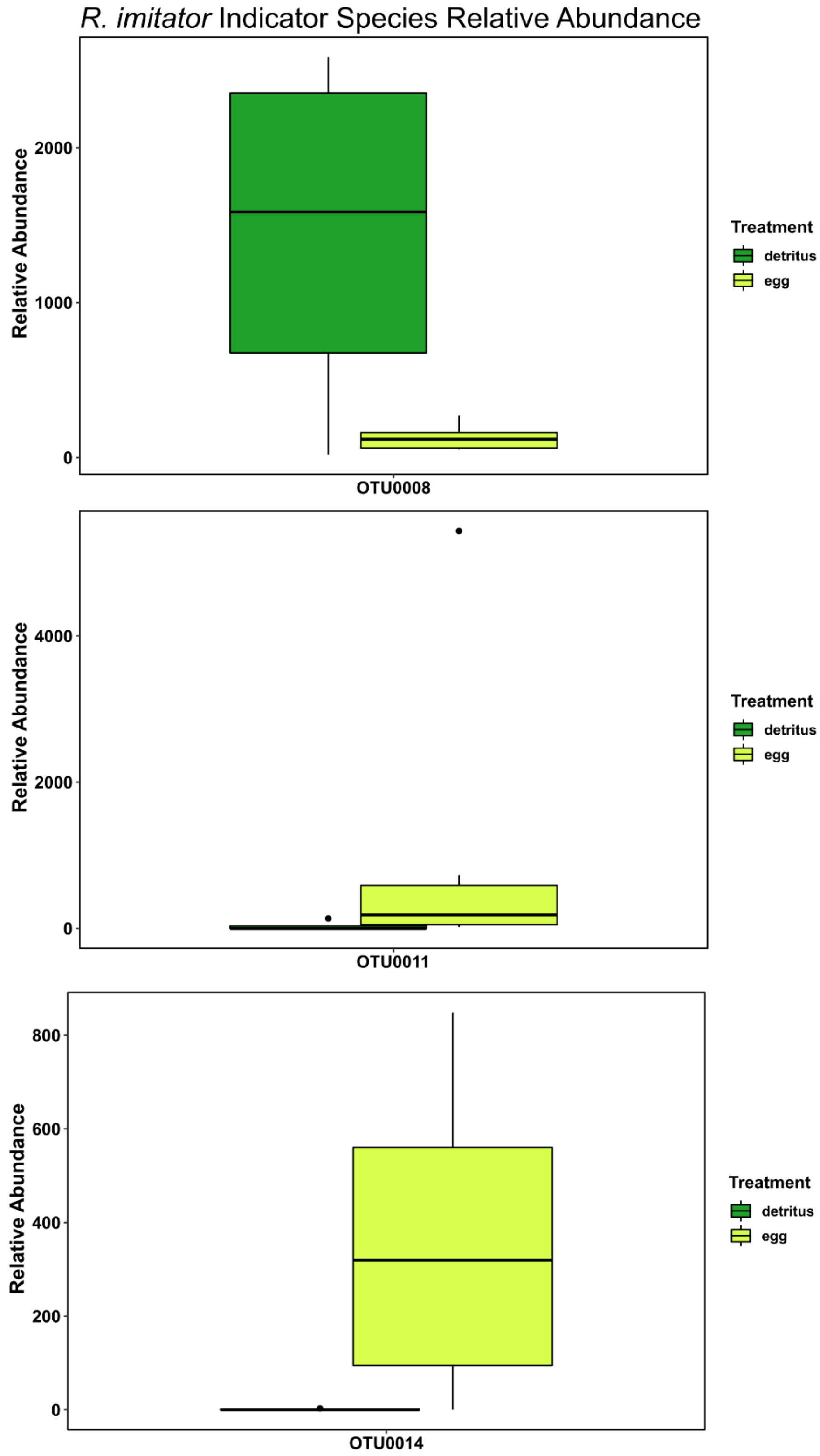

Figure 3. Relative abundance of Indicator Species OTU's in *R. imitator* treatments (green=detritus-fed, yellow=egg-fed).

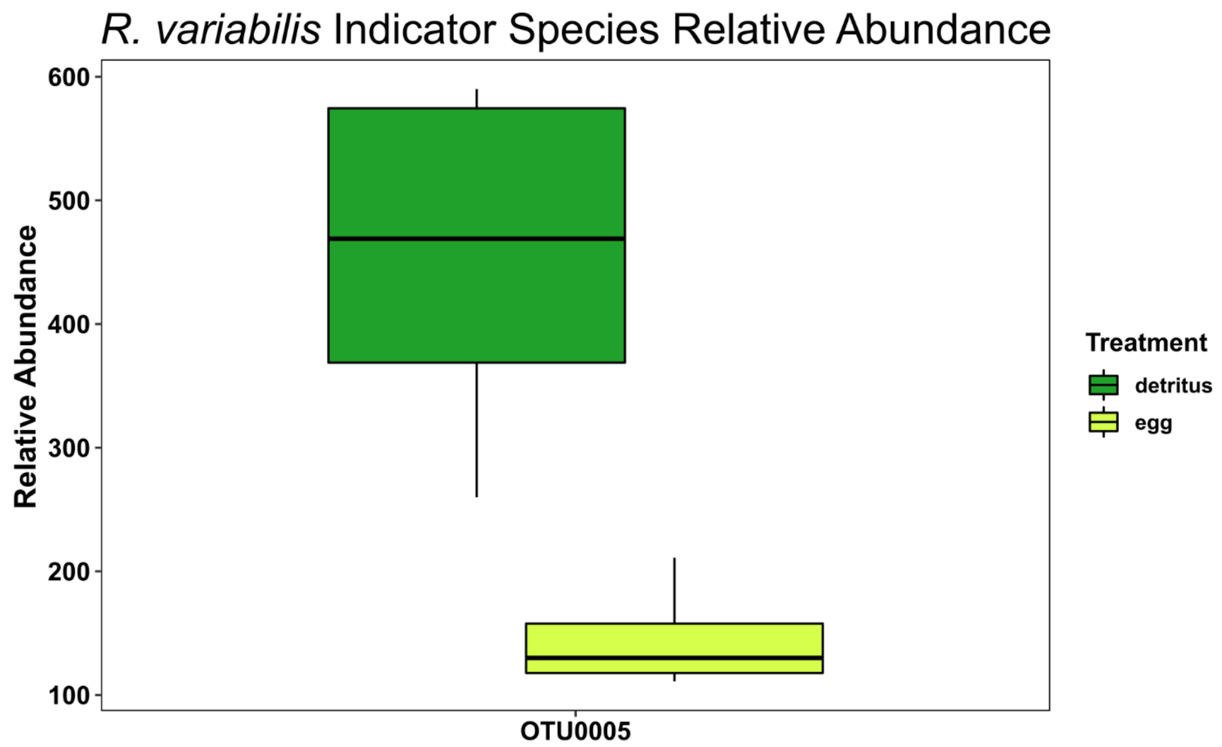

Figure 4. Relative abundance of Indicator Species OTU's in *R. variabilis* treatments (green=detritus-fed, yellow=egg-fed).

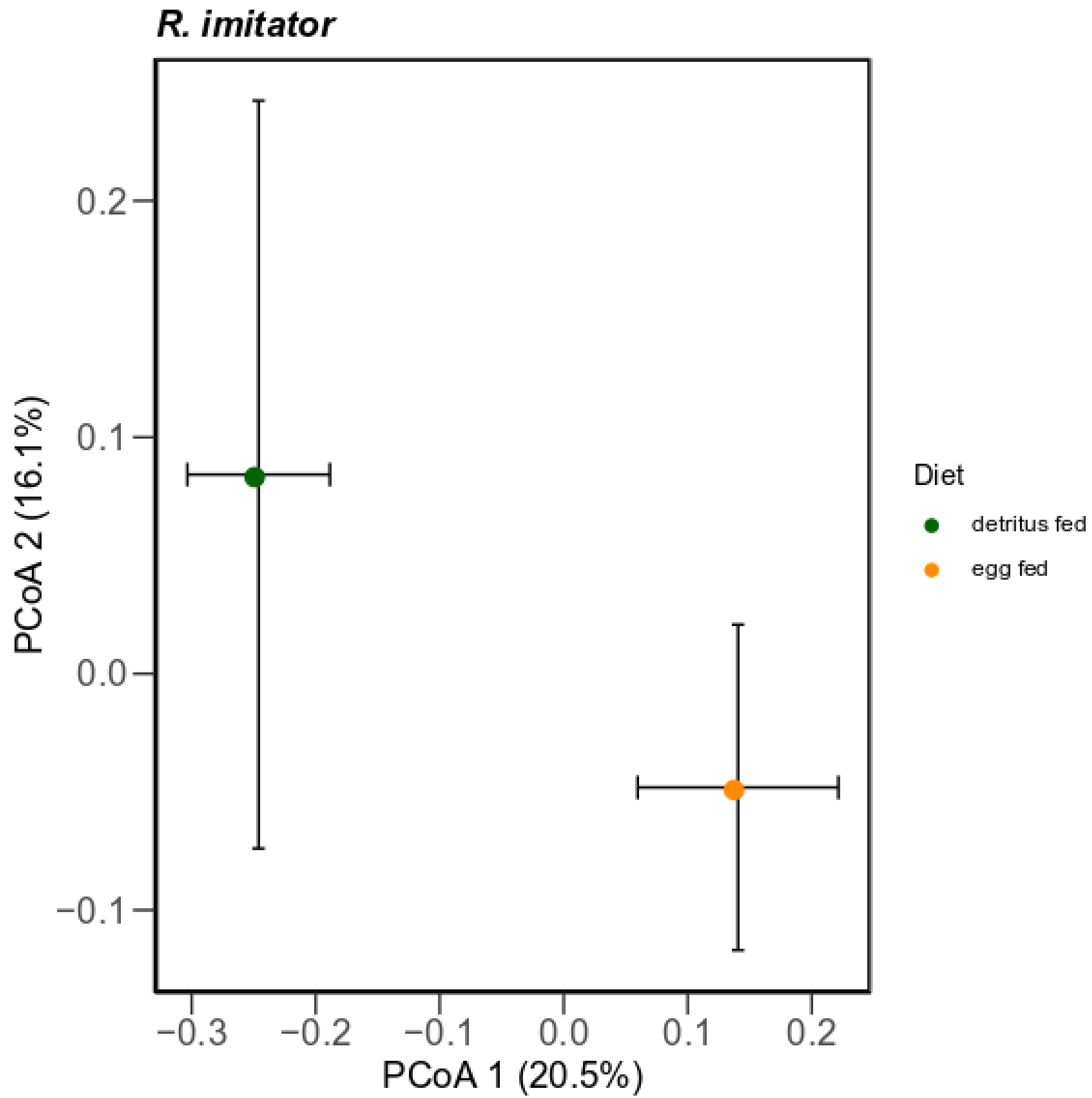

Figure 5. Ordination plot based on Principal Coordinate Analysis community composition (R^2:0.1564, $p<0.015$) for each diet for *R. imitator*.

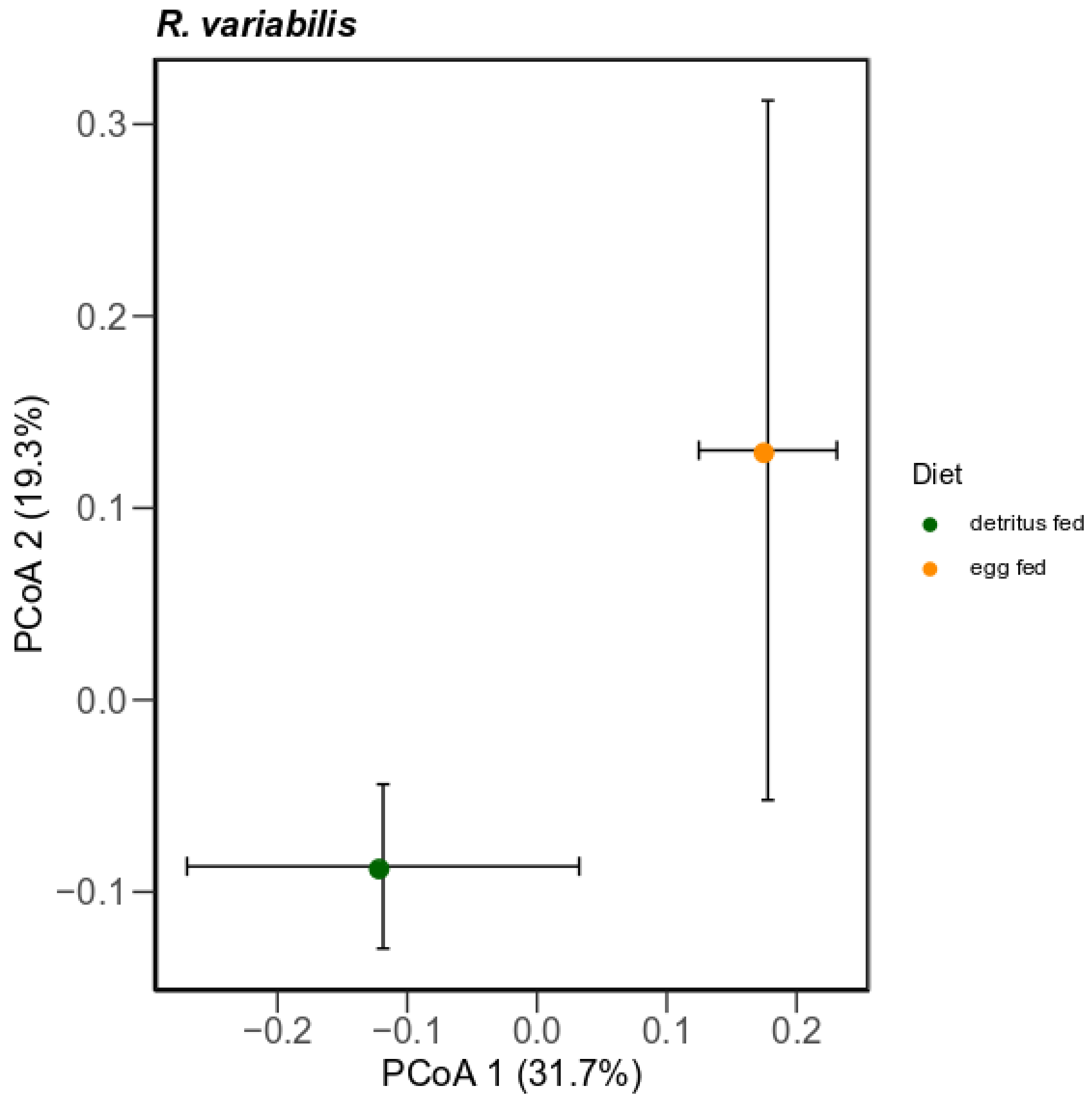

Figure 6. Ordination plot based on Principal Coordinate Analysis community composition (R^2:0.15314, $p<0.115$) for each diet for *R. variabilis.*

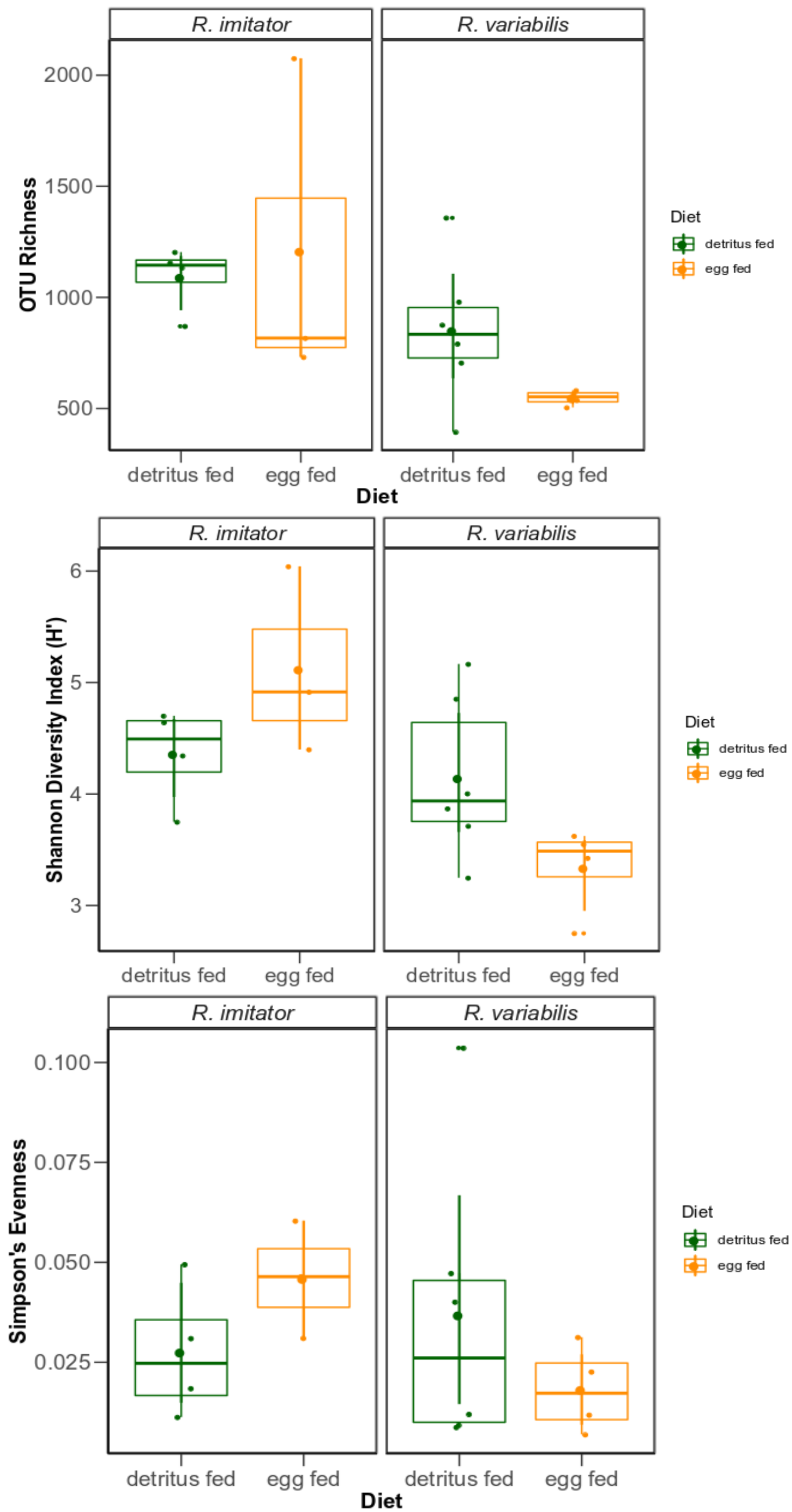

Figure 7. Box plots representing total bacterial diversity (Shannon Diversity Index *H*), richness, and evenness (Simpson's Evenness) between feeding groups in each species.

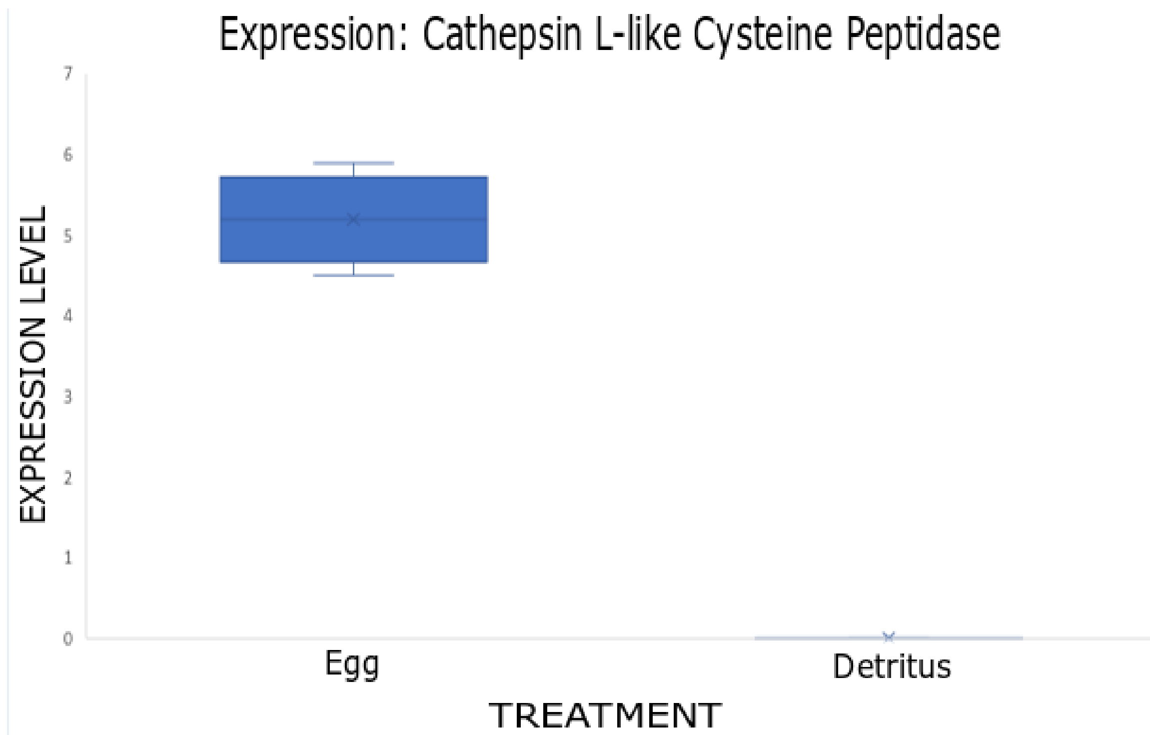

Figure 8. Differential expression of Cathepsin L-like Cysteine Peptidase in *R. imitator*.

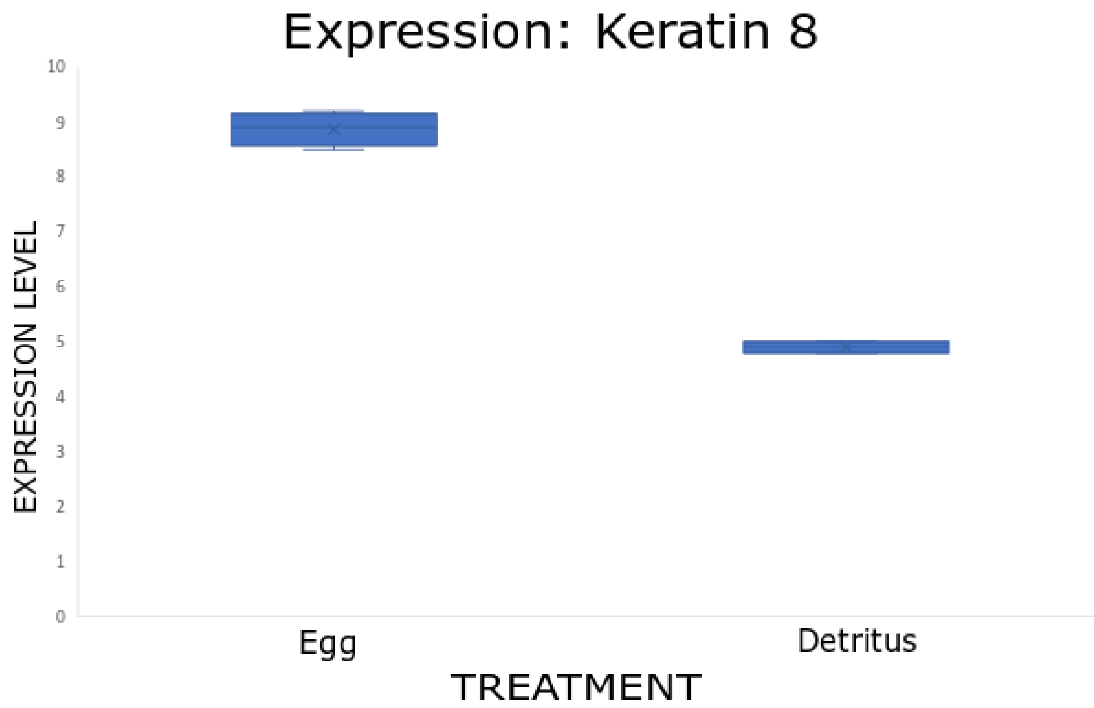

Figure 9. Differential expression of Keratin 8 in *R. imitator*.

APPENDIX A: AUP APPROVAL

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

February 16, 2017

Kyle Summers, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Summers:

Your Animal Use Protocol entitled, "Comparative Analysis of Feeding Mode, Gene Expression, and Microbiome Composition in Poison Frog Tadpoles" (AUP #D348) was reviewed by this institution's Animal Care and Use Committee on February 16, 2017. The following action was taken by the Committee:

"Approved as submitted"

A copy 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 and are familiar with its contents.**

Sincerely yours,
Eddie Johnson/jd

Eddie Johnson, MS
Vice-Chair, Animal Care and Use Committee

EJ/jd

Enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

Comparative analysis of feeding mode, gene expression and microbiome composition in poison frog tadpoles

	Principal Investigator	**Secondary Contact**
Name	Kyle Summers	Kayla Weinfurther
Dept.	Biology	Biology
Office Ph #	326-6304	Click here to enter text.
Cell Ph #	Click here to enter text.	Click here to enter text.
Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	Click here to enter text.	Click here to enter text.
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For IACUC Use Only

AUP # D348			
New/Renewal New 2/13/17			
Full Review/Date	DR/Date		
Approval Date 2/16/17			
Study Type evolution			
Pain/Distress Category D			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS
OHP Enrollment			
Mandatory Training			
Amendments Approved			

1

I. Personnel

A. Principal Investigator(s):

Kyle Summers

B. Department(s):

Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Kyle Summers, PhD	PI, oversees project	Yes	25 years of frog research, renewed IACUC online April 2013
Kayla Weinfurther, BS	Graduate student, project supervisor, project researcher	Yes	6 months of captive frog husbandry and research, IACUC completed Sept. 15 2016
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

In this study, we plan to investigate the ecological factors and genetic, physiological and behavioral adaptations associated with transitions to obligatory parental feeding of offspring in Peruvian poison frogs. This is an important issue with widespread implications, because transitions from independent offspring to offspring that are intensively fed and cared for have occurred in multiple taxa across the animal kingdom, and these transitions are associated with the evolution of complex family interactions and advanced sociality. Egg-feeding can be considered a first step towards intensive offspring provisioning by parents, providing a valuable opportunity to study adaptations associated with evolutionary shifts toward intensive parental provisioning of offspring.

The work planned under this grant proposal is to carry out comparative studies of the evolution of gene expression and microbiome composition in the guts of tadpoles of two closely related species of poison frogs (*Ranitomeya imitator* and *Ranitomeya variabilis*) that live in sympatry, yet have dramatically different reproductive strategies. *Ranitomeya* imitator breeds in tiny plant pools (phytotelmata), and regularly feed their tadpoles infertile eggs. *Ranitomeya variabilis* breed in substantially larger phytotelmata, and don't feed their offspring. We will sample intestinal tissue from both species, and compare their patterns of gene expression and microbial communities.

An important advantage of working with *R. imitator* is that egg consumption is "facultative", so tadpoles of this species can survive on other foods, if they are available (which they are not in the tiny pools they typically use). This gives us the opportunity to compare gene expression and microbiomes in tadpoles of the same species (*R. imitator*) when developing on the normal diet (eggs) and when developing on the "ancestral" diet of algae and detritus (typically consumed by *R. variabilis*). Reciprocally, we can feed eggs to *R. variabilis*, to look at gene expression and microbiome composition in the gut of a species that normally feeds on detritus (*R. variabilis* tadpoles will eat eggs). This will provide important insights into the key changes in gene expression associated with recent adaptation to a diet of trophic eggs, a transitional point in the evolution of complex reproductive strategies and intensive parental care.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No

If yes, why is it necessary? (note: teaching by definition is duplicative)

Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Tadpole euthanasia

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	2/10/17
Database(s) searched:	Scopus, Science Direct
Time period covered by the search (i.e. 1975-2013):	1945-2017
Search strategy (including scientifically relevant terminology):	Term 1: tadpole euthanasia alternatives, Term 2: tadpole collection alternatives
Other sources consulted:	Click here to enter text.

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers

to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

The method of tadpole euthanasia proposed (immersion in a solution of buffered MS222) appears to be the standard method for tadpole euthanasia. This conclusion has resulted from the refinement of euthanasia methods for aquatic organisms over the years, and has replaced previous (less efficient) methods. In terms of reduction, we are proposing relative conservative sample sizes to minimize the number of tadpoles that will be euthanized.

C. Hazardous Agents

1. **Protocol related hazards (chemical, biological, or radiological):**
   Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Click here to enter text.	Choose an item.
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Click here to enter text.	Choose an item.
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.

Cell lines, tissues or other biological products injected or implanted in animals	DCM	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. **Incidental hazards**

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

Individuals, primarily Kayla Weinfurther and Kyle Summers, will be exposed to some incidental hazards. These are unavoidable in the field. The nature of field work always poses some hazards, but individuals on this protocol are all experienced with field work and work in the tropics. Kayla has already completed nearly several months of field work in Central America. As a result, she is well aware of these hazards. These are easily mitigated with forethought and caution, however some are unavoidable (air travel, stinging insects, defended plants, etc). We will hire local field assistants in a secondary effort to reduce the risk of working in Peru.

The CDC listed a number of diseases that travelers to Peru should be take precaution against. The majority of these are general vaccines, including Hep A and B, polio, MMR, and tetanus. Most of these are requirements for the university. The CDC also recommends yellow fever and malarial medicines depending on location. These do not appear to be risks in the areas of Peru in which we work as their occurrence is quite rare. Other mosquito borne diseases such as Zika and chikungunya will be mitigated through use of appropriate clothing and/or mosquito repellant.

III. Animals and Housing

A. Species and strains:

Ranitomeya imitator and Ranitomeya variabilis

B. Weight, sex and/or age:

Larval phase (tadpoles)

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project

R. imitator: 10 treatment, 10 control R. variabilis: 10 treatment, 10 control	+50 (10 extra, 40 breeding frogs)	= 90

2. Justify the species and number (use statistical justification when possible) of animals requested:

These numbers represent a reasonable sample size for the types of analyses (gene expression and microbiome composition) we plan to perform. Determining sample sizes necessary to ensure statistical rigor (power) in these types of analyses is not possible beforehand due to the variable nature of gene expression levels and microbiome compositions between species, but these sample size are considered adequate by most current researchers in these fields.

3. Justify the number and use of any additional animals needed for this study:

We are adding ten animals to substitute for any losses due to unexpected accidents or mortality during the experiments.

a. For unforeseen outcomes/complications:

10

b. For refining techniques:

Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Ten breeding pairs of each species will be identified in fieldsites near Tarapoto, Peru, and we will monitor their breeding behavior

d. Indicate if following IACUC tail snip guidelines: Choose an item.

(if no, describe and justify)

N/A

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? No **(if yes, describe)**

Click here to enter text.

5. Are there any deviations from standard husbandry practices?

No **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**

Click here to enter text.

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study? No **(If yes, describe housing and justify the need to singly house social species):**

Click here to enter text.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No

(If yes, describe and justify the need to withhold enrichment)

Click here to enter text.

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):

Pending (research permits are not granted ahead of time – we obtain them when we go to Peru – we have been able to obtain permits for our research in the past (15 years))

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

Tarapoto, Peru

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

Ten breeding pairs of each species will be identified in fieldsites near Tarapoto, Peru, and we will monitor their breeding behavior, as in previous research. Once breeding begins, a sample of two tadpoles from each family will be removed from the pools, and weighed and measured four days after deposition (before the first egg-feeding occurs in R. imitator). Tadpoles in the "non-crossed" treatment (five tadpoles) will be returned to their original pool. Tadpoles in the "crossed" treatment will be placed in the opposite pool type: R. imitator tadpoles in large pools with detritus (separated from egg-feeding parents), and R. variabilis tadpoles switched into small pools without detritus. "Crossed" Ranitomeya imitator tadpoles in R. variabilis pools will feed on pool detritus. "Crossed" Ranitomeya variabilis tadpoles in R. imitator pools will feed on eggs that we will collect from other pools). After four weeks, we will collect and euthanize all 40 tadpoles in the experiment.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** Click here to enter text. **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**
   Click here to enter text.
   c. **Water restricted for** Click here to enter text. **hours**
   d. **Water restriction is not recommended in any species for routine pre-op prep and must be justified:**
   Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered
Pre-procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
Click here to enter text.

4. For which procedure(s):
Click here to enter text.

5. Methods for monitoring anesthetic depth:
Click here to enter text.

6. Methods of physiologic support during anesthesia and recovery:
Click here to enter text.

7. Duration of recovery:
Click here to enter text.

8. Frequency of recovering monitoring:
Click here to enter text.

9. Specifically what will be monitored?
Click here to enter text.

10. When will animals be returned to their home environment?
Click here to enter text.

11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
Click here to enter text.

C. Use of Paralytics

1. Will paralyzing drugs be used? Choose an item

2. For what purpose:
Click here to enter text.

3. Please provide scientific justification for paralytic use:

Click here to enter text.

4. Paralytic drug:

Click here to enter text.

5. Dose:

Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:

Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:**
   a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
   b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

Click here to enter text.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks.

Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. **For what procedure(s):**
Click here to enter text.
2. **Explain why non-restraint alternatives cannot be utilized:**
Click here to enter text.
3. **Restraint device(s):**
Click here to enter text.
4. **Duration of restraint:**
Click here to enter text.
5. **Frequency of observations during restraint/person responsible:**
Click here to enter text.
6. **Frequency and total number of restraints:**
Click here to enter text.
7. **Conditioning procedures:**
Click here to enter text.
8. **Steps to assure comfort and well-being:**
Click here to enter text.
9. **Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. **Describe methodology:**
Click here to enter text.
2. **Expected model and/or clinical/pathological manifestations:**
Click here to enter text.
3. **Signs of pain/discomfort:**
Click here to enter text.
4. **Frequency of observations:**
Click here to enter text.
5. **Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):
Click here to enter text.
2. Conditioning:
Click here to enter text.
3. Safeguards to protect animal:
Click here to enter text.
4. Duration:
Click here to enter text.
5. Frequency:
Click here to enter text.
6. Total number of sessions:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

1. Food Regulation

a. Amount regulated and rationale:
Click here to enter text.
b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.
c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:
Click here to enter text.
b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.
c. Frequency of observation/parameters documented (body weight, hydration status, etc.):
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

3. Dietary Manipulations

a. **Compound supplemented/deleted and amount:**
Click here to enter text.
b. **Frequency and duration (hours for short term/week or month for long term):**
Click here to enter text.
c. **Frequency of observation/parameters documented:**
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.
2. Duration of procedure:
Click here to enter text.
3. Frequency of observations during procedure:
Click here to enter text.
4. Frequency/total number of procedures:

Click here to enter text.

5. Method of transport to/from procedure area:

Click here to enter text.

6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:

Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:

Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:

Click here to enter text.

3. Will ascites be generated: Choose an item.

i. **Criteria/signs that will dictate ascites harvest:**
Click here to enter text.

ii. **Size of needle for taps:**
Click here to enter text.

iii. **Total number of taps:**
Click here to enter text.

iv. **How will animals be monitored/cared for following taps:**
Click here to enter text.

4. What will be done to minimize pain/distress:
Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):
Click here to enter text.

2. Duration:
Click here to enter text.

3. Intensity:
Click here to enter text.

4. Frequency:
Click here to enter text.

5. Frequency of observations/parameters documented:
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:
Click here to enter text.

2. Will conditioning occur? If so, describe:
Click here to enter text.

3. If aversive stimulus used, frequency, intensity and duration:

Click here to enter text.

4. Length of time in test apparatus/test situation: ***(i.e., each test is ~10 mins)***

Click here to enter text.

5. Frequency of testing and duration of study: ***(i.e., 5 tests/week for 6 months)***

Click here to enter text.

6. Frequency of observation/monitoring during test:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:

Click here to enter text.

2. Maximum time animal will be in capture device:

Click here to enter text.

3. Frequency of checking capture device:

Click here to enter text.

4. Methods to ensure well-being of animals in capture device:

Click here to enter text.

5. Methods to avoid non-target species capture:

Click here to enter text.

6. Method of transport to laboratory/field station/processing site and duration of transport:

Click here to enter text.

7. Methods to ensure animal well-being during transport:

Click here to enter text.

8. Expected mortality rates:

Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:

Click here to enter text.

2. Approximate time required for data collection per animal:

Tadpoles can be collected rapidly by inverting the small breeding pools into a plastic container. Tadpoles will be measured by pouring them into a small sieve and placing them on a small plastic sheet for measurement with calipers. Weight will be determined by weighing the plastic sheet, then the plastic sheet with tadpole, on a small portable electronic field scale. At the end of the experiments, tadpoles can then be transferred into sieves and then into MS222 for euthanasia.

3. Method of restraint for data collection:

NA

4. Methods to ensure animal well-being during processing:

We will handle all animals carefully.

5. Disposition of animals post-processing:

Tissues will be collected for genomic/microbiome analyses as described above.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

We will work quickly to ensure that euthanasia is achieved in a rapid and humane fashion.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):

Click here to enter text.

2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:

Click here to enter text.

2. Describe methods to ensure animal comfort and well-being:

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

1. Location of Surgery (Building & Room #):
Click here to enter text.

2. Type of Surgery (check all that are appropriate):
Click here to enter text.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**
☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**
☐ **Minor survival surgery**
☐ **Multiple survival surgery**
If yes, provide scientific justification for multiple survival surgical procedures:
Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**
Click here to enter text.
c. **Water restricted for** Click here to enter text. **hours**
d. **Water restriction is not recommended in any species for routine pre-op prep and be justified:**
Click here to enter text.

4. **Minimal sterile techniques will include (check all that apply):**

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

☐ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. **Describe all surgical procedures:**

a. Skin incision size and site on the animal:

Click here to enter text.

b. Describe surgery in detail (include size of implant if applicable):

Click here to enter text.

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. **Anesthetic Protocol:**

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):
Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):
Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:
Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):
Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:
Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure. ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

Tadpoles will be euthanized by immersion in a solution of 0.1% (w/v) tricaine methanesulfonate (MS222) prepared in 25 mM sodium bicarbonate. This is the standard method used in studies that sacrifice tadpoles during experiments.

2. Inhalant Method- Choose an item.
(if other, describe the agent and delivery method)
Click here to enter text.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:
Click here to enter text.

b. Dose or concentration:
Click here to enter text.

c. Route:
Click here to enter text.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Immediate dissection/Carcasses will be dissected and collected for genomic and microbiome analyses.

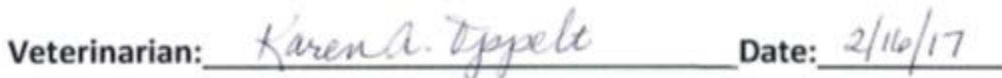
C. Describe disposition of carcass following euthanasia:

Following euthanasia, gut tissue will be dissected out, cleaned and preserved for genomic/microbiome analyses in RNAlater buffer. The rest of each tadpole carcass will be preserved in RNAlater buffer as well in case they might be valuable for future genetic analyses.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: Kyle Summers Date: 2/10/17


Veterinarian: Karen A. Tppele Date: 2/16/17

Vice-IACUC Chair: Eddie Johnson/jd Date: 2/16/17