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Effects of Leaf Litter on Organ Size Plasticity and Microbiome Composition in the Larval Gray Tree Frog, Hyla Versicolor

Delaney Lyons
Otterbein University, delaney.lyons@otterbein.edu

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Delaney L. Lyons

Zoo and Conservation Science & Biology

Otterbein University

Westerville, Ohio 43081

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Advisory Committee:

Sarah Bouchard, PhD.	Sarah Bouchard
Distinction Advisor	Advisor Signature
Jennifer A. Bennett, Ph.D.	Jennifer A. Bennett
Second Reader	Second Reader Signature
David G. Robertson, Ph. D.	
Distinction Representative	Distinction Representative Signature

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Abstract

Leaf litter can influence growth and development of amphibians by providing nutrients, structural support, and chemical leachates to the water. The purpose of this research was to determine the effects of Red Maple, *Acer rubrum*, and Pin Oak, *Quercus palustris*, leaf litter on organ size plasticity and microbiome composition in larval gray treefrogs, *Hyla versicolor*. Larvae were raised in outdoor mesocosms each containing 20 tadpoles. The treatments included: no leaf litter, maple litter, and oak litter. There were also treatments in which leaves were soaked in advance to remove leachates. This separated leaf structure from chemical make-up. Each treatment was replicated four times. To standardize developmental stage, tadpoles were selected based on size. Four size-matched larvae were sampled from each tank for organ analysis and three were sampled for microbiome assessment. Guts, livers, pancreas, fat bodies, and brains were weighed and guts were measured. Guts were also preserved in the -20 °C freezer for analysis of their microbiome. Data were analyzed with linear mixed effects models. There was no effect of leaf litter on growth rate of tadpoles in oak or maple treatments. Analyses indicated that leaf litter has a significant effect on organ size plasticity. The livers of tadpoles reared with oak leaf litter were significantly larger than those reared with maple in both unsoaked ($p=0.002$) and soaked treatments ($p=0.004$). Pancreases were larger in soaked oak treatments compared to soaked maple ($p=0.01926$). Fat bodies in soaked oak were over twice as large as fat bodies found in soaked maple treatments ($p=0.0035$). Brain mass from soaked maple treatments were larger than those from unsoaked maple treatments ($p= 0.0213$). There was no difference in brain mass between soaked oak and unsoaked oak treatments. We can conclude that physical and chemical characteristics of leaf litter had an effect on the organ size plasticity of larval gray treefrogs. For instance, soak leaf litter contains more lignin and cellulose, while maple leaf litter decays at a

faster rate and contains a higher amount of phenolic acids. Microbiome analyses will be completed when The Genomics Core at Michigan State University reopens post-coronavirus shutdown.

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Introduction:

Leaf litter can have profound effects on aquatic ecosystems based on different leaf litter input (Earl et al., 2012). The forest canopy plays a crucial role in the inputs of light and leaf litter on ponds and has the potential to influence aquatic communities (Rowland et al., 2016). Leaf litter shifts the physicochemical properties of water by lowering oxygen levels, increasing dissolved organic carbon levels, and lowering water pH (Rubbo, 2008). The effects of these physicochemical properties have been studied in many aquatic species.

The structural component of leaf litter is an important tool in the survival of aquatic species as it aids in predation evasion. Bell and Westby (1986) found increased leaf litter density has a positive effect on common fish and decapods. Juvenile *Cytophaga columnaris* rely on seagrass beds for protection against predators and tend to choose leaf beds that are higher in density. Similarly, anuran species adapt their habitat choice to swim into the substrate to avoid predators (Peterson et al., 1992).

Survivorship of aquatic species is influenced by the tannin and flavonoids found in litter species, which are water soluble compounds that leak during decomposition. Multiple studies suggest that high tannin concentration from the leaf litter leads to reduced tadpole developmental rate (Martin and Blossey, 2013; Cohen, 2014) and reduces the oxygen available to consumers (Earl et al., 2012). Increasing phenolic acid concentrations can lead to negative physiological effects on development of amphibians (Stoler and Relyea, 2013). On the other hand, some studies suggest the chemical properties of leaf litter are beneficial to aquatic species. For example, Zhao et al. (1997) found tannins to be essential to antibacterial activity in some species of fish, and Rubbo (2008) found high inputs of leaf litter can lead to higher levels of survival and faster development, but lower tadpole mass. Others have found litter species by itself was not

significant on tadpole development (Cohen, 2012). These contradicting findings suggest more research is needed in order to have a better understanding of the impact of leaf litter.

Anuran larvae have been shown to have very plastic responses to their environment, often in the form of altered morphology. High leaf litter inputs have been associated with wider heads and longer forelimbs in froglets, and smaller intestinal lengths and larger mouths in tadpoles (Stoler & Relyea, 2013). Leaf litter species with high nitrogen content such as red maple are associated with increased hindlimb dimensions and gut mass (Stoler & Relyea, 2013). Litter with low carbon to nitrogen ratios and high leaching rates can lead to smaller gut mass, increased time to metamorphosis, and smaller hind gut dimensions.

Leaf litter may also affect the microbial community of the habitat which could also affect tadpole gut microbiome. The gut microbiome of a species plays an important role in homeostasis of its host species. The invertebrate digestive system is inhabited by a diverse community of organisms that aid in processes like digestion and energy acquisition, immunomodulation, vitamin synthesis, and pathological defense (Bletz et al 2016). The microbial community in the gut can show a mutualistic, commensalistic, symbiotic, or pathogenic relationship with the host species. Identifying the gut microbiome of aquatic species allows us to understand and improve the health maintenance of species, disease mitigation, captive breeding and reintroduction, and introduced species management (Jimenez & Sommer, 2016).

We are only beginning to understand how variation in the environment influences the gut microbiome. The gut microbiome of species can have the potential to tell us about an individual. Yet, it is not clear how varying external environmental conditions, like leaf litter, will affect the gut microbiome of a tadpole. Multiple factors influence the gut microbiome of species, such as developmental stage, temperature, habitat, and diet; however, much is still unknown. When

wood frog larvae were inoculated with bull frog gut microbiota, tadpoles experienced accelerated growth rates (Warne et al, 2019). The study suggested establishment of the amphibian gut microbiome occurs in the critical window of hatching and shapes larval development and performance (Warne et al., 2019). Not only are these microbial communities important to the host individual, but they are also important to the wellbeing of the aquatic ecosystem. The microbiome of the gut can vary between amphibian species, despite living in the same habitat and experiencing the same environmental condition (Jimenez & Sommer, 2016).

Microbiome research has developed into a promising tool to aid in the conservation of amphibians. However, we are lacking the depth of knowledge that is needed to understand the ecological dynamics of microbiota in order to aid in conservation efforts. Specifically, microbiome research will aid in conservation efforts by maintaining host health, mitigating disease, improving captivity conditions and success rate of reintroduction, and managing invasive species. Jimenez & Somer (2016) identified major lines of research needed to meet these four goals: (1) investigate the intrinsic and extrinsic effects on amphibian gut microbiome, (2) determine the microbiome dynamics of captive breeding, (3) identify the mechanism of transmission, selection, and presence of microbes in species, (4) examine the casual effects of stress on amphibians, and (5) give a detailed explanation on the biogeography of gut microbes from amphibian species from different geographic regions and climate zones. Researchers believe if these important avenues for future research are met, then that will help to fill the gaps of our knowledge on the gut microbiome of amphibians. These actions will aid in conservation efforts.

The purpose of this research was to determine the effects red maple, *Acer rubrum*, and Pin Oak, *Quercus palustris*, leaf litter have on organ size plasticity and microbiome composition

in larval gray treefrogs, *Hyla versicolor*. We reared larvae in tanks containing one of the following: a control with no leaf litter, oak leaf litter, or maple leaf litter. For the oak and maple treatments, we also included tanks that contained previously soaked leaf litter (Figure 1). Soaking the leaf litter allowed us to significantly reduce the chemical and nutrients leaching from the litter. By separating these treatments into soaked and unsoaked, we separated the chemical and structural effects that the leaf litter provides to the tadpoles. We reared tadpoles in these tanks until they reach 1.5 centimeters in body length. We also dissected the guts for microbiome analyses in unsoaked oak, unsoaked maple, and no leaf litter tanks.

Methods:

***Sample collection*:** Research was conducted during the months of May, June, and July 2019 at Austin E. Knowlton Center for Equine Science. *Hyla versicolor* mating pairs were collected from a nearby pond on June 16, 2019 at 10pm. The conditions were wet and humid that night. Mating pairs were located in the trees by the pond and the foliage surrounding the pond (Figure 2). Mating pairs were located by following the mating call and caught using large nets. Mating pairs were placed into plastic containers with 10 centimeters of pond water. Lids with fifteen, three-centimeter holes were placed on top of the clear plastic container. The following morning, eggs from nine clutches were collected and the corresponding mating pairs were released (Figure 3). Clutches were taken back to Otterbein University's Science Center where they were incubated at room temperature until they hatched. Fresh tap water was added to each clutch twice a day to maintain a healthy environment for the tadpoles. Once tadpoles reached stage 26 (Gosner 1960), the tadpoles from all clutches were combined into a large bucket. Twenty tadpoles were haphazardly selected for each of the 40 tanks in the experiment. Each

tadpole group was photographed before transport to mesocosms at the Otterbein University Equestrian Center.

Tank description and field location: Tadpoles were reared in 416-L mesocosms and placed in an open, sunny area at the Otterbein University Equestrian Center. We lined up forty mesocosms outside in two rows of twenty each (Figure 4). Each tank was filled with tap water and covered in a mesh screen to restrict mosquitos from entering and laying eggs in the tanks. To secure the mesh, bungee cord and string was used around the edges of each tank.

Treatments: Treatments were randomly assigned to each tank. The five treatments in this experiment included: no leaf litter, soaked pin oak leaf litter, soaked red maple leaf litter, pin oak leaf litter, and red maple leaf litter (Figure 1). There were four replicates of each treatment. One month prior to this experiment, *Acer saccharum* (red maple) and *Quercus palustris* (pin oak) were collected from the edge of ponds and parks near semi-permanent bodies of water. We sorted the leaves and left them out to dry in the lab. We then prepared our soaked litter treatments by placing 120 g of litter into a mesh bag and soaking it in tap water for approximately one week. We changed the water every other day to remove the leachates and neutralize any pH changes (Hossie & Murry 2010). Both oak and maple were soaked separately until the water appeared clear (Figure 5).

Tadpoles were placed in tanks on, June 24th, 2019. Every 3 days tadpoles were given 0.25 grams of Sera Micron. We measured growth rate seven days after initial launch. Tadpoles were collected by tank using dip nets and placed into a large container with two centimeters of water and photographed. These photos were later analyzed with ImageJ software. The total length of each tadpole was measured to determine growth rate (Figure 6). Tadpoles were released back into their respective mesocosms. Based on growth rate measurements, we

predicted when the tadpoles' head-body length would reach 1.5 centimeters and develop in to Gosner stage 35-38. Once each tank reached the standard size, we sampled four tadpoles from each tank. The sampled tadpoles were euthanized using Tricaine methanesulfonate (MS222) and preserved in 10% formalin. In addition, three additional tadpoles were euthanized from each of the maple, oak, and control treatments for gut microbiome analysis.

Dissection and procedure for gut microbiome*:* Tadpoles used to examine organ size plasticity were dissected, so that the mass of the gut, brain, pancreas, fat body, and liver could be weighed. Guts were uncoiled, lined up next to a ruler for scale and photographed for analysis with ImageJ software. Gut dissections for the microbiome analysis were performed in a sterile environment to limit contamination. After each gut was removed, we used nitrogen to snap freeze the guts. The guts were stored in a -20C freezer until the DNA kit was obtained (Figure 7). Using the QIAMP Powerfecal kit (QIAGEN), DNA was extracted from unsoaked oak, unsoaked maple, and no leaf litter treatment guts (Figure 8). DNA was quantified using both the NanoDrop microvolume spectrophotometer and Qubit fluorimeter. The DNA was then used as a template in PCR test reactions using primers specific for the 16S rRNA gene. 16S V4 reverse 806r and 16S V4 forward 515f were used to amplify the DNA. The amplified sequence was then run on a gel electrophoresis. (Figure 9). Molarity of samples were then diluted to be within range provided by Michigan State University. The DNA library was then sent off to Michigan State University where it is currently in the queue for NextGen sequencing, using the Illumina HiSeq. The facility is currently closed due to the COVID-19 pandemic, but once it is reopened, the data will become available to identify the specific microbes found in each treatment. Treatments will be analyzed and compared.

Metamorphosis procedure: The remaining tadpoles in each mesocosm were raised to metamorphosis. Every morning mesocosms were checked for frogs that climbed the sides. Frogs were counted and captured in clear cups with perforated lids. These cups had tank number located on the side, as well as the date of the completion of metamorphosis. Frogs were transported back to the lab until tails were totally absorbed which took approximately one day. Frogs snout-vent length was then measured when froglets were in resting state. They were then transported back to the pond in which they were found and rereleased.

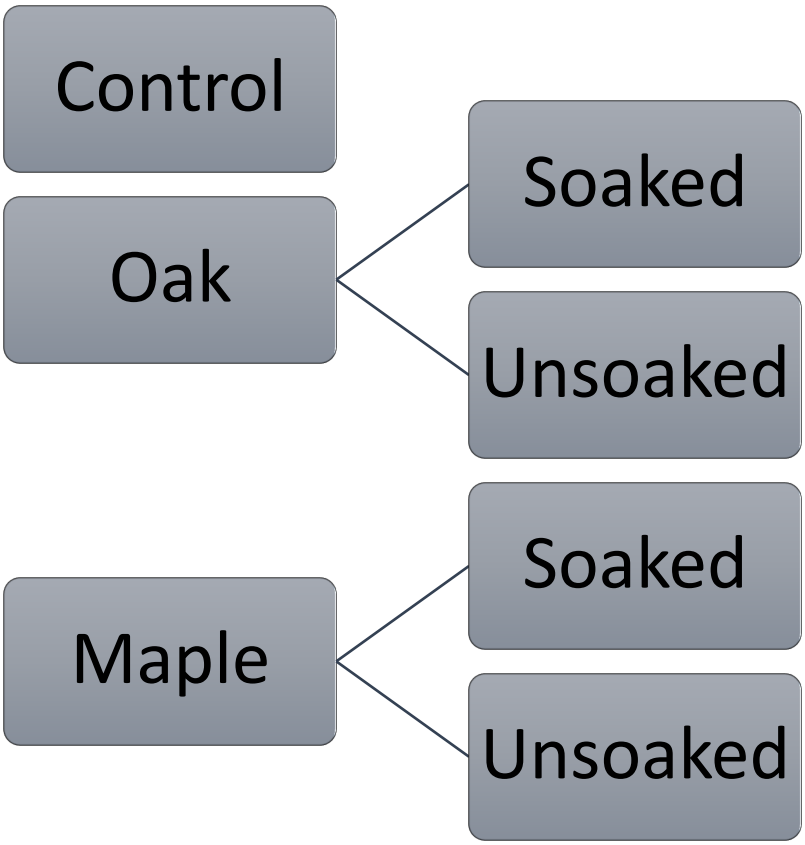

Figure 1. *Treatment description*

Figure 2. *Mating pairs.* Gray treefrog pairs that were found in the trees by the pond and the foliage surrounding the pond.

Figure 3. *One clutch of collected eggs found the morning after capturing mating pairs.*

Figure 4. *416-L mesocosms placed in an open sunny area at the Otterbein University Equestrian Center*. Mesh was used to cover the 416-liter mesocosm tanks. Each tank was filled with tap water and covered in a mesh screen to restrict mosquitos from entering and laying eggs in the tanks. To secure the mesh, bungee cord and string was used around the edges of each tank.

Figure 5. *A subset of the leaf litter was soaked in tap water for approximately one week.* One hundred and twenty grams of dry leaf litter was added to mesh bags with a rubber band secured at the end. Oak and maple were soaked separately until the water appeared clear. The water was changed every other day to remove the leachates and neutralize the solution.

Figure 6. *Growth analysis photo of tadpoles reared in no leaf litter treatment*. Photo was taken on July 1st when tadpoles were collected by dipnetting. Image was then analyzed using ImageJ software.

Figure 7. *Guts immediately after being taken out of -20C freezer.*

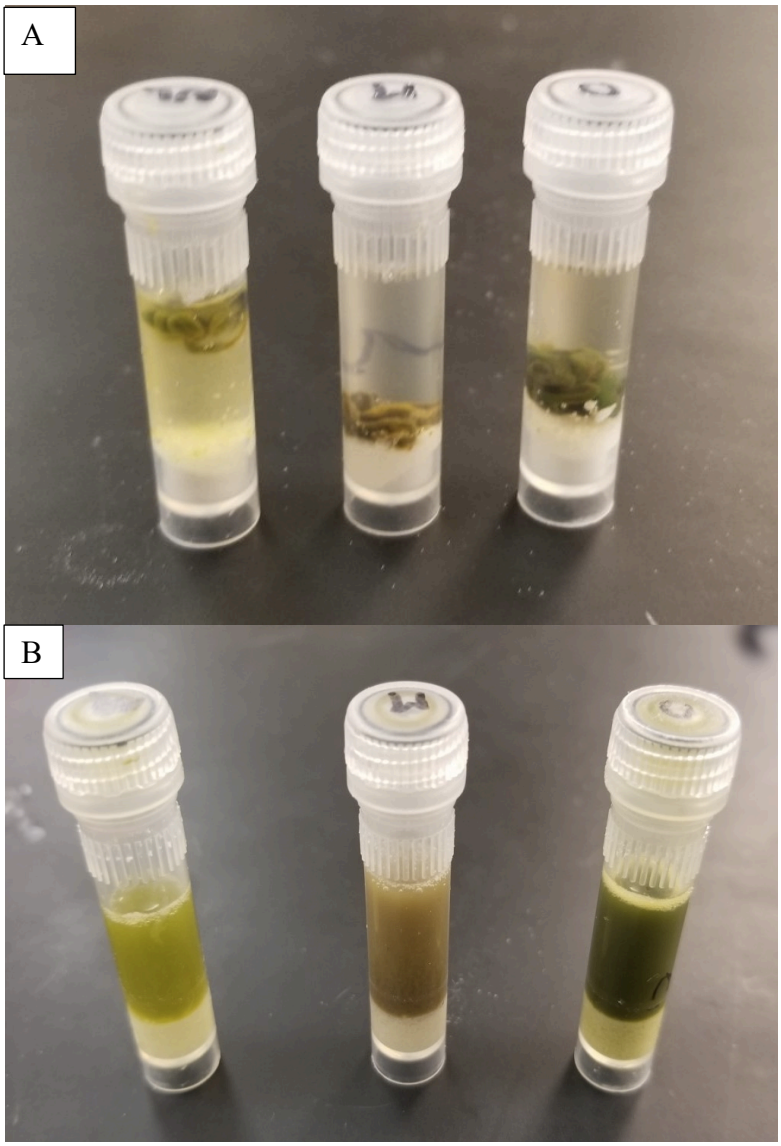

Figure 8. *Preparation of guts for microbiome analysis.* From left to right in image A and B shows the sampled gut of no leaf litter, maple, and oak treatments. Image A shows guts being prepared for DNA extraction. Image B is after subjecting the guts to bead disruption to break them down using Disruptor Gene.

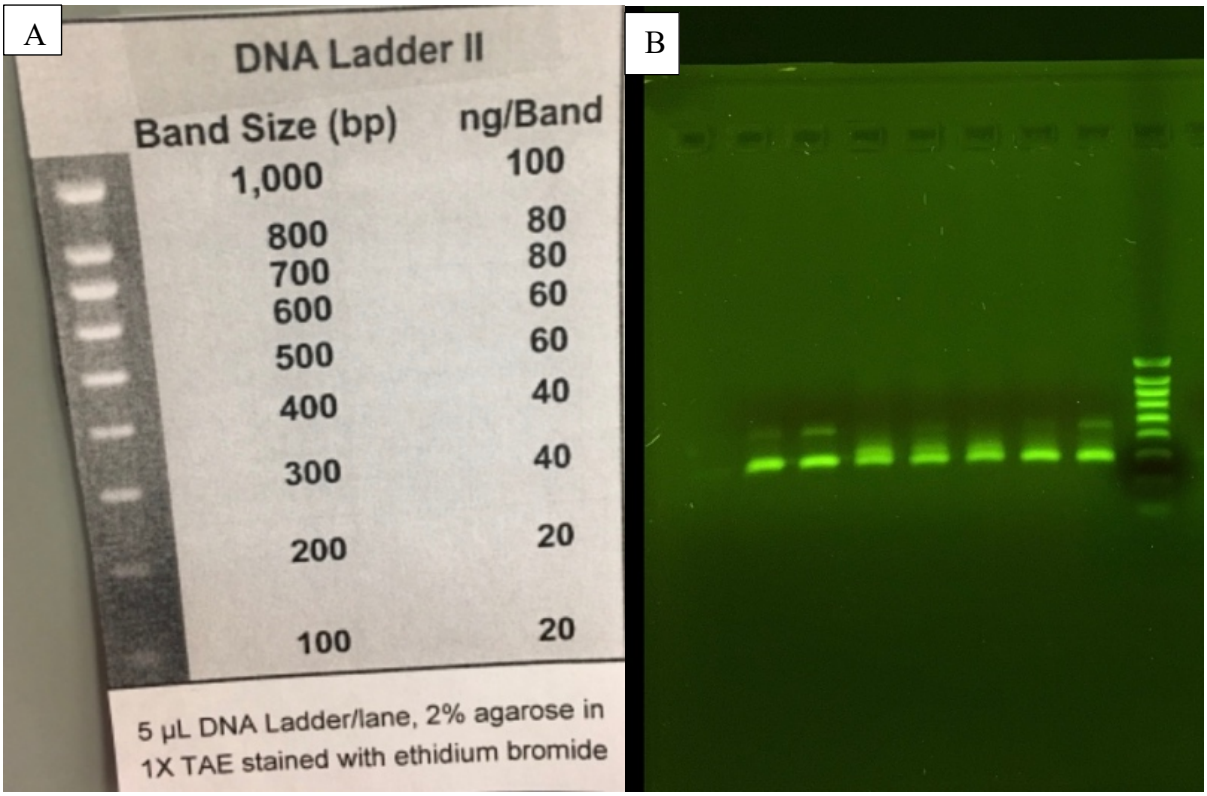

Figure 9. *Gel electrophoresis of sample DNA.* Photo A represents the DNA ladder that was run. B is a photo of the gel that was run with the ladder in the lane farthest to the right. The rest of the lanes contain the PCR reactions. This shows that the PCR product is the size we expected and that the DNA extraction worked.

Results:

Liver: Livers of tadpoles reared in oak were significantly larger than those reared in maple ($X^2 = 15.164$, $p = 0.002$) (Figure 10). The livers of tadpoles reared in soaked oak were larger than those reared in soaked maple ($X^2 = 8.462$, $p=0.004$). When comparing unsoaked maple and soaked maple we found that there was no significant difference between liver mass($X^2 = 1.341$, $p = 0.2468$). In addition, when comparing unsoaked and soaked oak treatments we found that there was no significant difference in liver mass between these two treatments ($X^2 = 0.2281$, $p = 0.633$).

Pancreas: The pancreases of tadpoles reared with oak and maple leaves were not significantly different ($X^2 = 0.169$, $p = 0.9824$) (Figure 11). Pancreas mass of tadpoles reared in soaked oak were significantly larger than those reared in soaked maple ($X^2 = 5.477$, $p = 0.019$). Pancreas mass did not vary between tadpoles reared in unsoaked maple and soaked maple treatments ($X^2 = 0.901$, $p = 0.3425$). Pancreas mass did not vary between tadpoles reared in soaked and unsoaked oak treatments ($X^2 = 0.001$, $p = 0.973$).

Fat body: Fat body mass of tadpoles reared in maple and oak treatments showed no significant difference in mass ($X^2 = 0.2534$, $p = 0.9685$) (Figure 12). Tadpoles reared in soaked oak treatments had fat bodies that were twice as large as tadpoles reared in soaked maple treatments ($X^2 = 8.495$, $p = 0.004$). The fat bodies of tadpoles reared in soaked and unsoaked maple treatments did not differ ($X^2 = 0.709$, $p = 0.400$). Fat bodies collected from soaked and unsoaked oak treatments showed no significant difference ($X^2 = 0.012$, $p = 0.912$).

Brain: The brain mass of tadpoles reared in maple and oak treatments did not differ ($X^2 = 5.780$, $p = 0.123$) (Figure 13). The brain mass of tadpoles reared in soaked maple and soaked oak treatments showed no significant difference between brain size in tadpoles ($X^2 = 0.106$, $p = 0.745$). The brain mass of tadpoles reared in soaked maple treatments were significantly larger

than those reared in unsoaked maple treatments (X^2 =5.302, p=0.021). There was no significant difference between brain mass in soaked and unsoaked oak treatments (X^2 = 0.0096, p = 0.922).

Gut: The gut mass of tadpoles reared in oak and maple treatments did not differ (X^2 = 1.2089, p = 0.7509) (Figure 14). The gut mass of tadpoles reared in soaked and unsoaked oak treatments showed no significant difference (X^2 = 2.122, p = 0.1452). The gut mass of tadpoles reared in soaked and unsoaked maple treatment did not differ (X^2 = 0.9012, p = 0.3425). The gut mass of tadpoles reared in soaked and unsoaked oak treatments showed no significant effect on gut size on tadpoles (X^2 = 0.2632, p = 0.6079).

The gut length of tadpoles reared in maple and oak treatments showed no significant difference (X^2 = 0.774, p = 0.856) (Figure 15). The gut length of tadpoles reared in soaked maple and oak treatments showed no significant difference (X^2 = 0.196, p = 0.659). The gut length of tadpoles reared in soaked and unsoaked maple treatments showed no significant difference (X^2 = 0.082, p = 0.775). The gut length of tadpoles reared in soaked and unsoaked oak treatments did not differ (X^2 = 0.035, p = 0.851).

Microbiome analyses will be completed when The Genomics Core at Michigan State University reopens post-coronavirus shutdown.

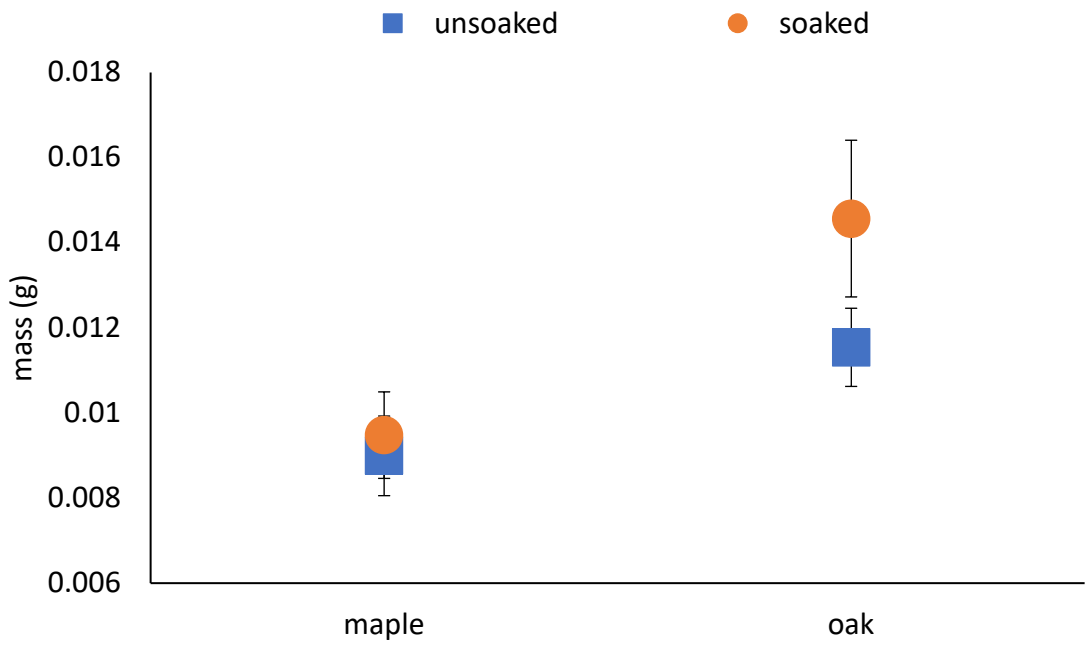

Figure 10. *Effects of leaf litter on liver mass.* The livers of tadpoles reared in oak were significantly larger than those reared in maple ($X^2 = 15.164$, $p = 0.002$). Tadpoles reared in soaked oak had significantly larger livers compared to those reared in soaked maple ($X^2 = 8.462$, $p=0.004$). Data are means +/- standard error. A line connects the significantly different data points.

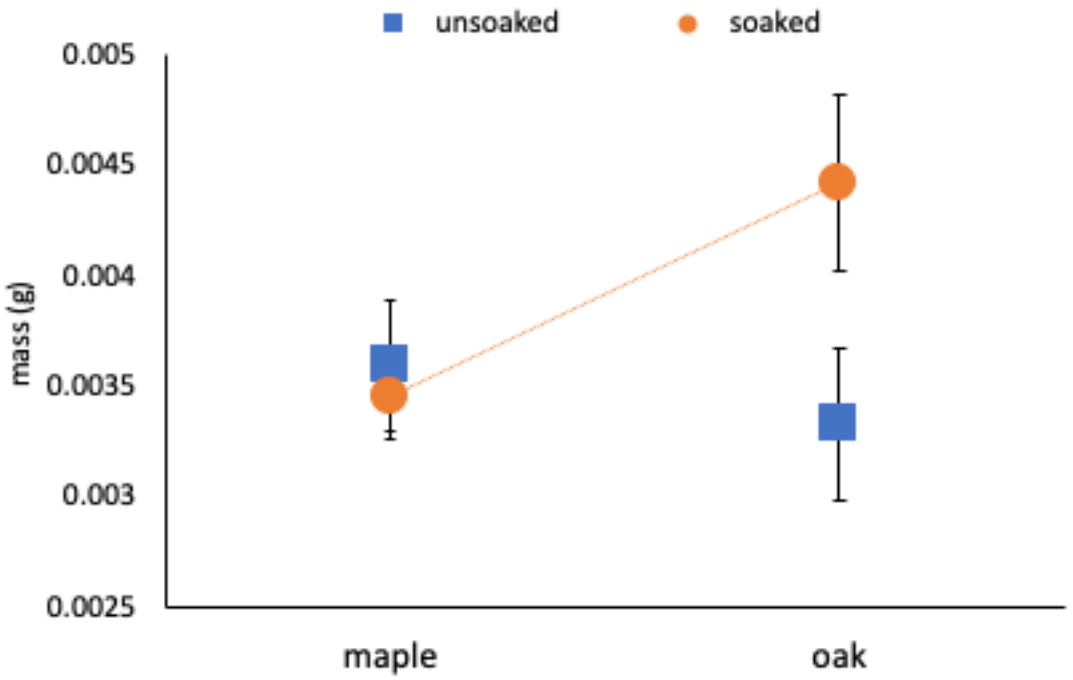

Figure 11. *Effects of leaf litter on pancreas mass.* Pancreas mass of tadpoles reared in soaked oak were significantly larger than those reared in soaked maple ($X^2 = 5.477$, $p = 0.019$). Data are means +/- standard error. A line connects the significantly different data points.

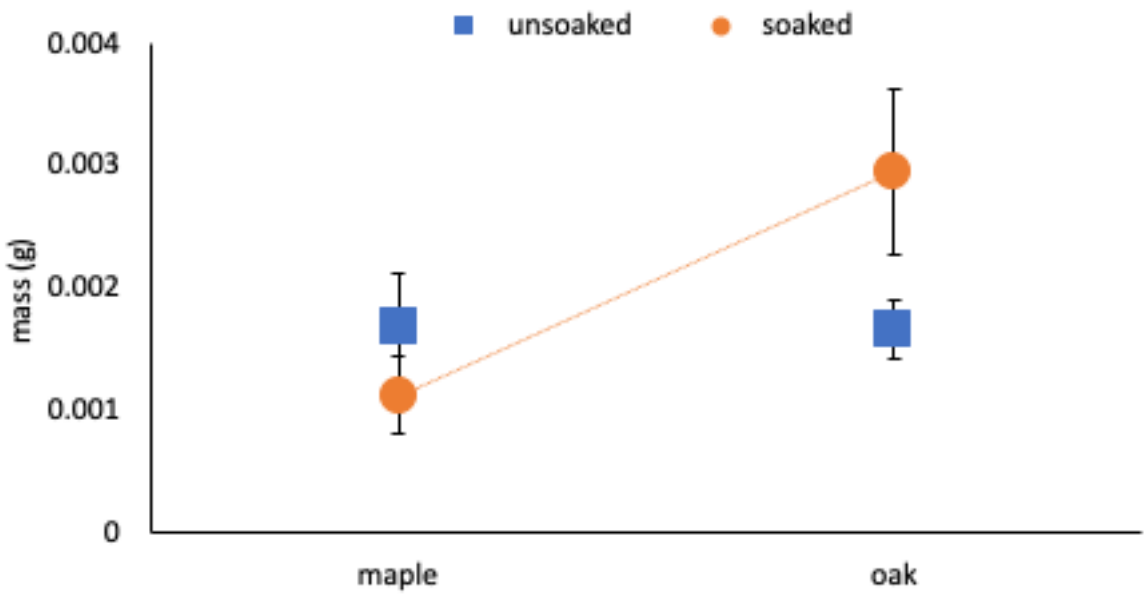

Figure 12. *Effects of leaf litter on fat body mass.* The fat bodies of tadpoles reared in soaked oak treatments were twice as large as those reared in soaked maple treatments ($X^2 = 8.495$, $p = 0.004$). Data are means +/- standard error. A line connects the significantly different data points.

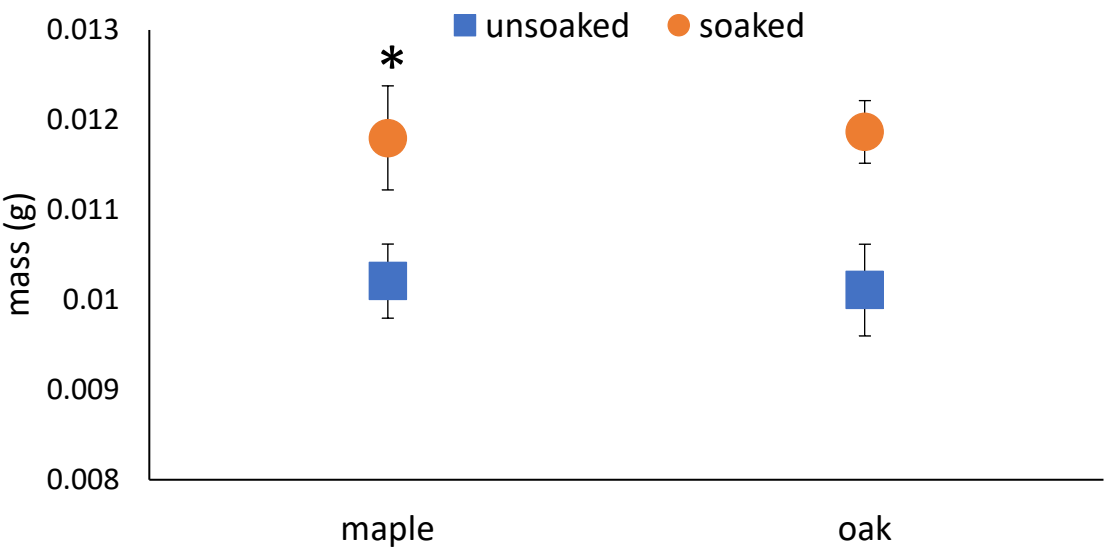

Figure 13. *Effects of leaf litter on brain mass.* The brain mass of tadpoles reared in soaked maple treatments were significantly larger than those reared in unsoaked maple treatments ($X^2 = 5.302$, $p = 0.021$). Data are means +/- standard error. * indicates significant difference.

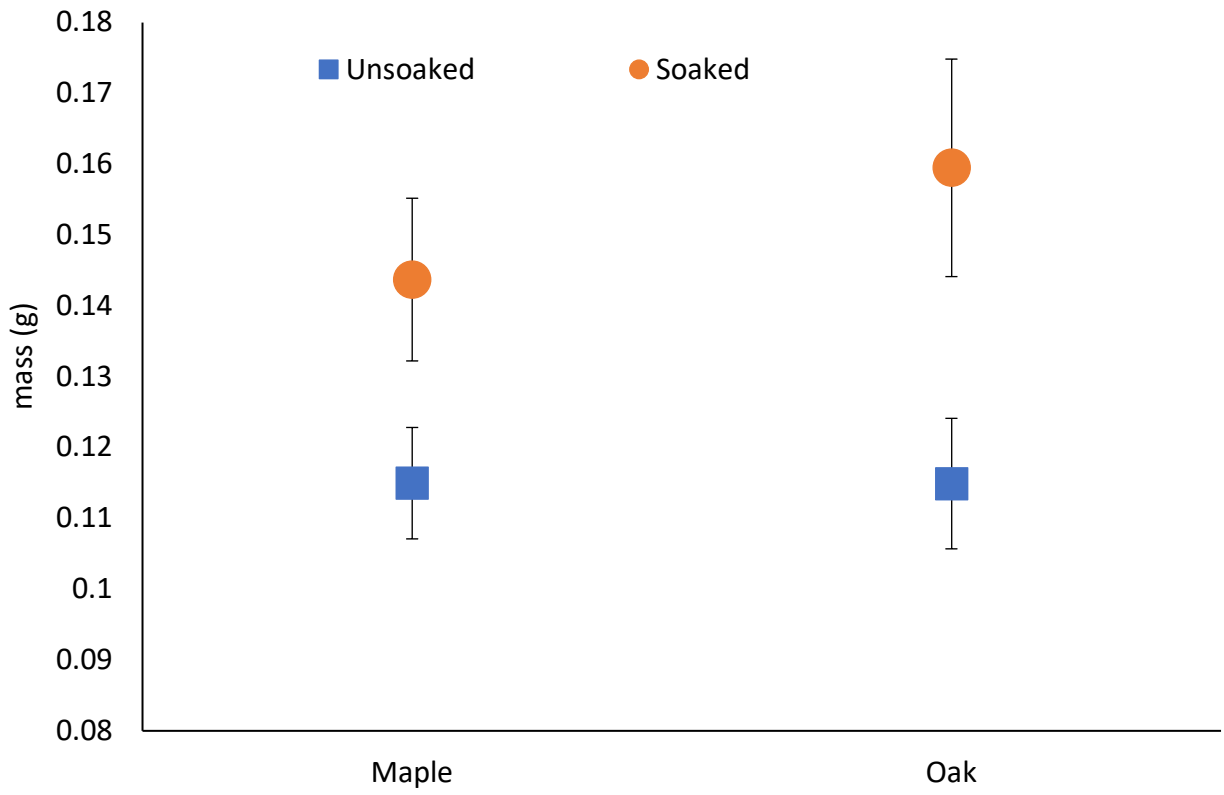

Figure 14. *Effects of leaf litter on gut mass.* The gut mass of tadpoles reared in different treatments did not differ. Data are means +/- standard error.

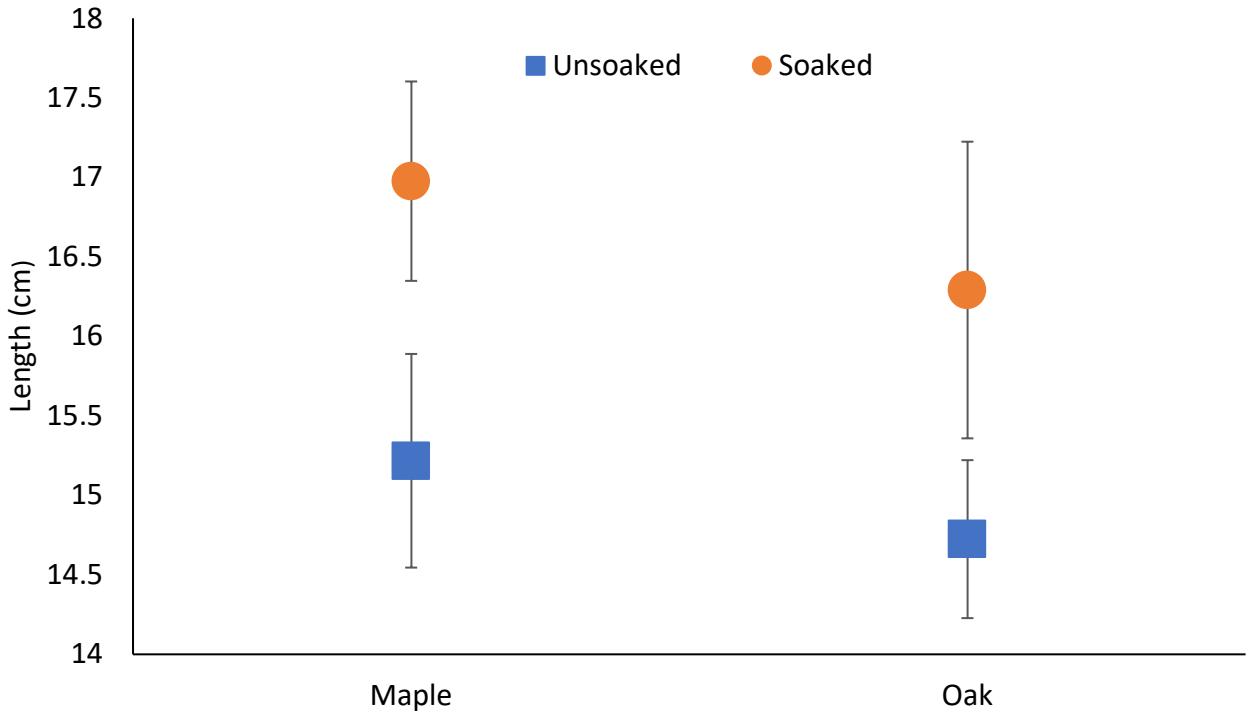

Figure 15. *Effects of leaf litter on gut length.* Gut length was not significantly affected by treatment type. Data are means +/- standard error.

Discussion

Gut length and mass did not differ in oak and maple treatment. Gut length and mass also did not differ in soaked and unsoaked treatments. Other studies have found that competition induces larger guts, and one study specifically found that litter species with greater nitrogen content, which positively correlated with phosphorus content, induced shorter intestines (Stoler & Relyea, 2013). Longer guts allow for tadpoles to be digestively efficient. This means that species with longer and bigger guts take more time to process the food that they intake presumably digesting it better. Wood frog tadpoles allocate energy towards growth and development this would explain why litter species with higher nitrogen content would have shorter guts in this experiment. Based on these findings, we predicted that in our study soaked maple and oak would have longer guts than unsoaked maple leaf litter species, however, this was not the case.

The liver is responsible for filtering blood, secreting bile, and detoxifying chemicals. In this study we found that tadpoles reared in both soaked and unsoaked oak leaf litter had significantly larger livers. There has been no previous research on this topic. This would also suggest that oak leaf litter releases a chemical when it is decomposing that influences the mechanism that causes larger livers. One study suggests that treefrogs may favor smaller livers because they can reduce the cost of transport by limiting body mass and allowing a more elongated shape (Withers & Hillman 2001).

In a tadpole fat bodies are used fat for food is unavailable. In previous studies researchers found that that the mass of fat bodies found in five different larval Southern Indian anurans tadpoles are correlated with body mass, snout vent length, and metamorphosis (Gramapurohit et al., 1998). In our study we found that the fat bodies of tadpoles reared in soaked oak were larger

than those of soaked maple. Therefore, in future studies I would be interested in comparing fat body mass to snout vent length and metamorphosis of gray treefrogs.

The brain mass of tadpoles reared in soaked maple treatments were significantly larger than those reared in unsoaked maple treatments. These results were surprising to us because initially we thought that excess nutrients in the water would have a positive correlation with brain mass. However, we speculate that soaking leaf litter allows leaf litter to decompose, which allows different nutrients and chemicals to be released into the mesocosms. These unique chemicals released by soaking maple treatments would influence a larger brain size in tadpoles.

The pancreas is an organ that is responsible for producing digestive enzymes and insulin. Insulin controls blood glucose levels. In this study we found that pancreas mass of tadpoles reared in soaked oak were significantly larger than those reared in soaked maple. We believe that this could be tied to larger fat reserves found in the individual.

Microbiome analyses will be completed when The Genomics Core at Michigan State University reopens post-coronavirus shutdown. Once results are back, treatments will be analyzed and compared. The control treatment will tell us what microbes are found in the guts of tadpoles in a no leaf litter treatment. This will allow us to standardize the treatments. I am interested in seeing the effects of leaf litter on the gut microbiome of an amphibians.

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