The influence of environmental variation on the microbiome during early-life stages in reptiles

by

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Abstract

My dissertation research seeks to understand how environmental variation, including maternal effects, might influence the microbiome of reptiles and how those differences translate to phenotypic variation. My research framework integrates both observational and experimental science through field and lab-based methods. Documenting environmentally mediated changes in the microbiome and their effects on hosts will provide a robust foundation for understanding the role of microbiome plasticity in shaping host phenotypes, including growth, physiology, and behavior.

For my first chapter, I sought to understand how gut homeostasis is influenced by environmental variation (in the form of aquatic pollutants like estrogen). I experimentally assigned 23 hatchling American alligators (*Alligator mississippiensis*) to three ecologically relevant treatments (control, low, and high estrogen concentrations) for ten weeks. Gut microbial samples were collected following diet treatments and microbial diversity was determined using 16S rRNA gene-sequencing. Individuals in estrogen-treatment groups had decreased microbial diversity, but a greater relative abundance of operational taxonomic units than those in the control group. This effect was dose-dependent; as individuals were exposed to more estrogen, their microbiota became less diverse, less rich, and less even. Findings from this study suggest that environmental contamination can influence wildlife populations at the internal, microbial level, which may lead to future deleterious health effects.

For my second chapter, I sought to effectively sample and manipulate the microbiome of eggshells. Although most vertebrates are oviparous, little is known about microorganisms on the surface of eggshells and their functions, particularly on eggs of non-avian reptiles. I developed a

novel method to effectively sample (i.e., whole-egg sonication) and manipulate the eggshell microbiome of non-avian reptiles while minimizing contamination from external sources. Overall, my results provide useful guidelines for future manipulative studies that examine the source and function of the eggshell microbiome.

For my third chapter, I experimentally manipulated the maternal gut microbiome using antibiotics and evaluate consequences on offspring phenotype in the brown anole lizard (*Anolis sagrei*). DNA was extracted from maternal gut tissue and cloacal samples and sequenced at the 16S rRNA gene. Eggs were incubated and embryo/hatchling phenotypes were recorded (e.g., survival, hatchling morphology). I found that treatment mothers had reduced gut microflora diversity and produced larger eggs/hatchlings than control mothers. Findings from this study provide new insight into the role of maternal gut microbiota and its potential functional significance on offspring.

For my concluding chapter, I conducted a systematic review on vertical transmission of microbiota in non-human animals. I found that many studies examining vertical transmission of microbiomes fail to collect whole microbiome samples from both maternal and offspring sources, particularly for oviparous vertebrates. An ideal microbiome study incorporates host factors, microbe-microbe interactions, and environmental factors.

Together, results from my dissertation suggest that the gut microbiome is highly influenced by environmental variation, including maternal effects, in ways that may affect offspring fitness. As evolutionary biologists continue to merge microbiome science and ecology, examining microbiomes in oviparous taxa may provide insight into how microbiota shape host phenotypes.

Acknowledgements

My seventh birthday party was Steve Irwin themed. I had a crocodile piñata, a cake with a plastic "Crocodile Hunter" wrestling a croc in the chocolate frosting, and party favors with little toy reptiles in them. On this day, I received a package with a letter inside addressed from the "Crocodile Hunter." He told me how proud he was of my interest in wildlife and encouraged me to continue on that path. That letter hung in my bedroom for years. I promised myself that I would make Steve Irwin proud. It wasn't until I was in high school that I found out that Steve Irwin did not write me that letter- my mother did. I reflect on that letter now. If it had not been for the enthusiastic support and dedication to my dream that my parents showed me at such an early age, I might not be writing this acknowledgements section in my dissertation today. Thank you mom and dad (and my two bonus parents, Cory and Annie) for always believing that I could be a biologist, a scientist, and an academic. I set out to make Steve Irwin proud, but I know that you were always my biggest fans.

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Background Information

Most surfaces, including the internal and external membranes of many vertebrates, are colonized by microorganisms that form interactive and fluctuating communities, commonly referred to as the "microbiome." The microbiome is a network of bacteria, archaea, fungi, algae, and protists as well as associated structural elements (e.g., DNA/RNA) and metabolite products that create a habitat with distinct bio-physio-chemical properties (Berg et al. 2020). Microorganisms are important to the health and function of organ systems in many species (Nicholson, Holmes, and Wilson 2005), and often exhibit symbiotic relationships with their host (Chow et al. 2010). Thus, documenting the microbiome over space and time is critical for understanding how microbiota may influence host phenotypes.

Variation in environmental conditions allows for phenotypic plasticity, or the ability for an organism with a single genotype to express multiple phenotypes in different environments. A growing area of study involves the influence of microbiome plasticity, or the variability in structure and composition of microbiota measured through ß-diversity metrics (e.g., Bray Curtis or Unifrac), on phenotypes of organisms (Grembi et al. 2020). A classic example of this is the interaction between host immune systems and resident gut microbiota; immune systems must maintain mutualistic homeostasis with microbial communities, while at the same time, the gut microbiome shapes host immunity (Hooper, Littman, and Macpherson 2012). Another example is gut microbiota dysbiosis following toxin exposure that can lead to changes in physiological processes important for survival in wildlife (Adamovsky et al. 2018). Environmental variation drives microbiome composition in many taxa and across life stages (Grembi et al. 2020; Trevelline, MacLeod, et al. 2019; Kohl et al. 2017; Jacob et al. 2015) and host phenotypic associations result

from change in microbiota community structure. However, the origin and initial colonization of the microbiome can also influence host phenotypes.

It is well documented that neonates obtain microbial symbionts/microbiota via vertical transmission from mothers in humans (Mueller et al. 2015) and other viviparous species (mammals: Law et al. 2021; Zhu et al. 2021; reptiles: Kohl et al. 2017). This vertical transmission has been shown to aid in the development of neonates including stimulation of metabolic processes (Mueller et al. 2015) and immune system priming. This transfer may serve to initiate immune function of offspring (Mueller et al. 2015), or influence acquisition of critical bacterial groups that can affect survival (Korpela et al. 2018). Importantly, maternal effects (a type of developmental plasticity transmitted through the mother) arise when a mother's environment or phenotype, rather than her genes, can influence the phenotypes of her offspring (Wade 1998). Thus, sampling at this early stage is critical to understand how microbial communities may be passed on from mother to offspring or necessary to determine how the microbial community changes throughout growth.

Oviparous reptiles serve as excellent models to study the influence of environmental variation on the microbiome and whether microorganisms can affect phenotypic traits of individuals. As in other oviparous species, embryos are subject to environmental conditions that mediate development (Joanen and Mcnease 1989), growth (Lance 2003), and homeostasis (Seebacher, Elsey, and Trosclair III 2003). Reptiles represent 17% of the world's vertebrate biodiversity, however, little remains known about the reptilian microbiome and the influence of varying environmental conditions on microbial symbionts (Norris and Lopez 2010). Still, a large portion of studies seek to characterize the internal and external microbiome of different species. Documenting environmentally mediated microbiome changes and their effects on hosts will

provide a robust foundation for understanding the role of microbiome plasticity in shaping host phenotypes, including growth, physiology, and behavior.

My dissertation questions seek to understand how environmental variation, including maternal environments, might influence the microbiome of reptiles and how that variation translates to phenotypic variability. My research framework integrates ecology, physiology, microbiology, and evolution through both field and lab-based methods. To address questions on this topic, I use two reptilian species that differ in trophic position and reproductive strategy. My research will contribute to this framework by 1) addressing the influence of environmental contaminants (e.g., xenoestrogens) on the gut microbiome of a top-tier predator, 2) utilizing novel methods for sampling and manipulating the microbiome of reptilian eggshells, 3) documenting taxonomic variation in the microbiome through the diet of mothers and potential phenotypic effects on offspring, and lastly, 4) evaluating the scientific literature for vertical transmission of microbiota in wildlife and highlighting knowledge gaps. These studies will provide a foundation for future researchers to integrate microbiology, ecology, and physiology.

Study Species

Alligator mississippiensis

The American alligator (*Alligator mississippiensis*) is a long-lived, top-tier consumer that is mostly carnivorous and lays clutches with many eggs. The American alligator has been used as a model to study environmental health and the effects of contamination, such as EDCs (Finger and Gogal 2013; Norris and Lopez 2010). Due to its longevity and trophic position, EDC concentrations and physiological effects may be biomagnified (or have increased deposition) in older, more mature individuals (Y E et al. 2020; Finger and Gogal 2013; Roe et al. 2004; Heinz,

Percival, and Jennings 1991). Previous studies have examined the influence of diet (Bloodgood et al. 2020; Jiang et al. 2017), spatial variation (Littleford-Colquhoun et al. 2019; Kohl et al. 2017), and gestation (Trevelline, MacLeod, et al. 2019) on the gut microbiome of various reptiles. Other studies have examined the influence of endocrine-disrupting contaminants (EDCs) on the microbiome and how they relate to health (Kieran et al. 2020). These characteristics make alligators an excellent model to address whether EDCs might influence the gut microbiome and thus, downstream physiological processes. Specific benefits of this species are outlined in chapter 1.

Anolis sagrei

The brown anole (*Anolis sagrei*) is a lower-tier consumer that is mostly insectivorous and lays one egg at a time, many times throughout the reproductive season. Additionally, the brown anole is native to Cuba and The Bahamas, but invasive in much of the Southeastern US. The brown anole is well-suited for answering questions regarding maternal effects, as embryos are greatly influenced by environmental variation (Hall and Warner 2018; Warner et al. 2012) and females have a high reproductive rate (Lee et al. 1989) and produce about one egg per week, allowing for single eggs to be collected. Very little is known regarding the brown anole microbiome, including along the gastrointestinal tract, cloacae, and on eggshells. Because we know so little about what shapes the brown anole microbiome and because they are a readily studied species in evolutionary biology, results from research in this area have the potential to be highly impactful. Specific benefits of this species are outlined in chapter 2 and 3.

Chapter 1

Xenobiotic estradiol-17ß alters gut microbiota of hatchling American Alligators (*Alligator mississippiensis*)

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Significance

The community structure of the gut microbiome is sensitive to environmental factors and is responsible for modulating various health parameters of many organisms (e.g., immune function, digestive efficiency, circulating hormone levels). For example, xenoestrogens that infiltrate ecosystems and bioaccumulate in food chains can influence the gut microbiome in ways that might negatively impact the health and persistence of wildlife populations. Our experimental work shows that increased exposure to estradiol reduces the diversity, richness, and evenness of the gut microbiome in the American alligator. This study provides new insight into the effects of environmental estrogens on critical microbial communities that reside within important top-tiered predators in many aquatic ecosystems.

Summary

Environmental estrogens pose serious concerns for ecosystems through their effects on organismal survival and physiology. The gut microbiome is highly vulnerable to environmental influence, yet the effects of estrogens on gut homeostasis are unknown because they are poorly studied in wildlife populations. To determine the influence of environmental estrogens (i.e., xenoestrogens) on the diversity and abundance of gut microbiota, we randomly assigned 23 hatchling American alligators

(*Alligator mississippiensis*) to three ecologically relevant treatments (control, low, and high estrogen concentrations) for ten weeks. We predicted that xenoestrogen exposure would decrease microbial diversity and abundance within the digestive tract and that this effect would be dose dependent. Microbial samples were collected following diet treatments and microbial diversity was determined using 16S rRNA gene-sequencing. Individuals in estrogen-treatment groups had decreased microbial diversity, but a greater relative abundance of operational taxonomic units than those in the control group. Additionally, this effect was dose-dependent; as individuals were exposed to more estrogen, their microbiota became less diverse, less rich, and less even. Findings from this study suggest that estrogen contamination can influence wildlife populations at the internal microbial level, which may lead to future deleterious health effects.

Introduction

The environment plays an integral role in shaping patterns of development, survival, and reproduction (S. F. Gilbert and Epel 2015; West-Eberhard 2003; Dennis Charles Deeming, Deeming, and Ferguson 1991), but contaminants can negatively affect these components of fitness through their influence on important physiological processes. Endocrine-disrupting contaminants (EDCs), for example, can enter the environment through agricultural run-off and spills, disrupting organismal processes such as endocrine physiology (e.g., gene expression) (Kazeto, Place, and Trant 2004) and gonadal development (Sparling 2016; Jobling et al. 1998; Jr et al. 1994). Synergism of multiple EDCs in ecosystems can also exacerbate the complexity of effects on physiology (Swan *et al.*, 2005). Consequently, these compounds can have down-stream effects that can harm individual and population health (Finger and Gogal 2013; Milnes and Guillette 2008).

EDCs include compounds such as pesticides, fungicides, and pharmaceuticals (Sparling 2016; Finger and Gogal 2013; Guillette Jr, Edwards, and Moore 2007; Guillette and Iguchi 2003), many of which are biochemically similar to estrogen and arise from a range of natural and synthetic sources. Since estrogen receptors are present throughout the body (Deroo and Korach 2006), exposure to xenoestrogens can disrupt synthesis, transport, storage, and clearance of hormones (Sparling 2016; Milnes and Guillette 2008; Guillette Jr, Edwards, and Moore 2007). Estradiol-17ß (E2) is one of the most active estrogens and plays a key role in regulating growth and reproductive function (X. Yang et al. 2017; Fernandez and Russo 2010; Simpson 2003). Because E2 does not degrade under anaerobic conditions [half-life of 2-3 days in river water (Ying, Kookana, and Dillon 2003; Ying, Kookana, and Ru 2002)], it can remain active in certain environments and is often detectable in treatment plant effluents and surface water (Adeel et al. 2017; Ying, Kookana, and Ru 2002; Kuch and Ballschmiter 2001; Ternes et al. 1999; Desbrow et al. 1998). Indeed, xenobiotic exposure to E2 has been shown to influence animal health and physiology in aquatic systems (Miles-Richardson et al. 1999; Toft and Baatrup 2001; Irwin, Gray, and Oberdörster 2001; Clark, Norris, and Jones 1998; Palmer and Palmer 1995). Exposure to concentrated amounts of E2 can occur directly from the environment or through consumption of bioaccumulated prey items and influence bodily function at the cellular level. In fact, gut microbial activity can drive estrogen metabolism (Plottel and Blaser 2011; Adlercreutz and Järvenpää 1982; Eriksson 1970).

The gut microbiome modulates estrogen levels within the body by secreting ß-glucuronidase (GUS) enzymes, which enable estrogen to become biologically active (Ervin et al. 2019; Baker, Al-Nakkash, and Herbst-Kralovetz 2017; Plottel and Blaser 2011). Additionally, the presence of estrogen receptors in the epithelia of the gastrointestinal (GI) tract (X. Yang et al. 2017) further indicate that the gut may be an important site for metabolizing estrogen. The

"estrobolome," or the gene repertoire of gut microbiota that metabolize estrogens, influences the levels of circulating and excreted estrogens, as well as estrogen metabolites (Plottel and Blaser 2011). The liver is responsible for conjugating E2 to its inactive form (estradiol-17-glucuronide). However, GUS enzymes secreted by bacteria in the GI tract deconjugate E2, which allows estrogen in its biologically available form to recirculate through the bloodstream and possibly contribute to hormonal disorders and diseases at high levels (Ervin et al. 2019).

An estrobolome enriched in GUS-producing genes can promote estrogen metabolite deconjugation, leading to more unbound estrogens in the plasma and bodily tissues to potentially cause damage (Ervin et al. 2019). Additionally, ethinyl estradiol (a synthetic estrogen) can alter intestinal flora in a sex-dependent and transgenerational manner in rodents by increasing proportions of potentially pathogenic bacteria (Javurek et al. 2016). Estradiol has been show to decrease gut microbiota diversity in zebrafish (Catron et al. 2019). Indeed, this imbalance of gut microbiota from individuals exposed to contaminants is not uncommon (Aguilera, Gálvez-Ontiveros, and Rivas 2020; Evariste et al. 2019). A symptomatic effect of contaminant exposure, either through direct facilitation of a toxin in the gut or by a cascade of physiological responses that influence microbiota composition, is a decrease in microbial diversity (Aguilera, Gálvez-Ontiveros, and Rivas 2020). This decrease in diversity, including compositional and functional diversity, is linked to a variety of deleterious health effects (Carding et al. 2015). However, the direct influence and consequences of xenoestrogens on gut microbial diversity is largely unknown.

American alligators (*Alligator mississippiensis*; Order Crocodilia) are an effective model to study environmental health and the effects of EDCs (Y E et al. 2020; Finger and Gogal 2013; Norris and Lopez 2010). *Alligator mississippiensis* is a long-lived, top-trophic predator, meaning that EDC concentrations and physiological effects may be biomagnified (or have increased

deposition) in relatively old individuals (Y E et al. 2020; Finger and Gogal 2013; Roe et al. 2004; Heinz, Percival, and Jennings 1991). Additionally, like other reptiles, environmental conditions mediate development (Joanen and Mcnease 1989), growth (Lance 2003), and homeostasis (Seebacher, Elsey, and Trosclair III 2003) in *A. mississippiensis*. These characteristics make alligators an excellent model to address whether EDCs, including environmental E2, might influence GI microbiota. Past studies have identified significant microbial variation within the GI tract of alligators (Keenan, Engel, and Elsey 2013) and the influence of heavy metals on microbiota composition (Kieran et al. 2020); however, nothing is known regarding the physiological influence of xenoestrogens on the alligator gut microbiome.

The aim of this study is to investigate whether xenobiotic estrogens, specifically E2, can influence the diversity and abundance of the gut microbiota in hatchling alligators. Given the decreased microbial diversity following E2 exposure documented in other studies, we predict that 1) xenoestrogen exposure will decrease microbial diversity and abundance within the digestive tract and that 2) the effect of xenoestrogens on the gut microbial community will be dose-dependent, whereby higher concentrations will have a greater impact than lower concentrations. Experimental tests of these predictions will provide a novel assessment of how estrogens can influence important microbiota in wildlife populations.

Methods

Transport and housing

Twenty-three hatchling (<1 year old) *A. mississippiensis* were obtained from Rockefeller Wildlife Refuge (RWR; Grand Chenier, LA, USA) and transported to Auburn University's Aviary Facility (Auburn, AL) on 4 March 2019. Eggs were collected from natural nests at RWR and incubated

under lab conditions at an average of 30.6 °C. Upon hatching, individuals were communally housed in controlled environmental chambers. Alligators were transported via containers drilled with holes to allow for air circulation and were blindfolded to potentially reduce stress (Finger *et al.*, 2018). Upon arrival to Auburn University, alligators were individually housed in 23 clear, plastic tanks (30 cm x 44 cm x 66 cm). Each tank was equipped with an individual UV light and one wooden basking platform and filled with approximately 17-18 liters of water. Photoperiod was kept at a 12-12 hr light-dark cycle and air temperature was maintained above 25 °C. Water temperature was documented twice a day via two digital temperature probes randomly placed in six tanks. Water in tanks was replaced once per week a day after feeding.

Prior to E2 exposure, alligators were housed for 10 weeks to allow for dietary transition (Kohl et al. 2017). During this time, individuals were fed thawed day-old pinky mice (weighing 1-2 g; The Big Cheese Rodent Factory, Fort Worth, TX, USA) twice a week. Mice were thawed for 15 minutes prior to feeding and were then placed into tanks. If not eaten, mice were removed the following day during cleaning.

Individuals were weighed once per week following arrival and throughout the treatment period. Additionally, 6 to 8-month-old crocodilians can eat about 4% of their body weight (in grams) per week under captive conditions (Webb, Hollis, and Manolis 1991). Thus, we fed alligators in our study ~5% of their body weight per week to eliminate potential effects of under- or over-feeding. Importantly, digestive efficiency may be low when the stomach is repeatedly filled to capacity, as observed under captive conditions (Webb, Hollis, and Manolis 1991).

Experimental design and sample collection

On 31 May 2019, individuals were randomly assigned to three treatments. These treatments included individuals with no estradiol exposure (control treatment, n=7), individuals that received 0.5 µg/kg E2 (low dose treatment, n=8), and individuals that received 1 µg/kg E2 (high dose treatment, n=8). Alligators were visually sexed prior to treatment assignment and similar distributions of males and females were placed into the groups. Sex was confirmed during dissection after the experiment concluded. Dosage ranges were chosen to mimic ecologically relevant concentrations of E2 at polluted sites [351-957 ng/L (approximately 0.957 µg/kg) in dairy farm wastewater and 1,000-1,500 ng/L in swine farm effluents (approximately 1.5 µg/kg) (Adeel et al. 2017; Li Yx and Lin 2010)]. These concentrated animal feeding operations have been shown to influence the physiology of aquatic wildlife through pollution of wastewater that drains into natural ponds and streams (Orlando *et al.*, 2004).

Estradiol-17ß (Sigma Aldrich) was prepared by dissolving the powdered form into peanut oil and diluting to desired concentrations. Mice were injected with 0.5 µg/kg, 1 µg/kg, or a peanut oil control at a volume of 1 mL/kg using a 1 mL syringe. Any associated estrogen in pinky mice was considered to not influence the results of this study as estrogen concentration in fetal mice is very low (Saal et al. 1997) and would be equal across treatments. Alligators were blindfolded and then fed by oral gavage to ensure all dosages of estradiol were consumed. This involved inserting pinky mice directly into the esophagus and massaging the throat gently with their mouth held closed, a technique used in previous studies (Finger, Hamilton, et al. 2018). Individuals were closely monitored following feeding and regurgitation was not observed. Alligators were orally gavaged with two pinky mice injected with E2 each week; thus, 20 total doses of a control or E2 were given to hatchlings.

Following the ten-week dosage period, individuals were weighed to the nearest 0.1 g and euthanized via decapitation and subsequent cervical dislocation and pithing (Nevarez et al. 2014). Twenty mL of heparinized blood samples were collected following decapitation and centrifuged; plasma was extracted to analyze blood E2 concentrations. Microbial samples were aseptically excised along the digestive tract using sterile scalpel blades and immediately frozen at -80 °C. Samples were collected along three sections of the gastrointestinal (GI) tract including stomach, duodenum, and colon (Keenan, Engel, and Elsey 2013). Samples from the duodenum were prioritized for sequencing and further analyses because of this region's important role in nutrient absorption. Additionally, destructive sampling was selected over fecal sampling due to the lack of GI tract microbiota representation from fecal microbial community composition in *A. mississippiensis* (Keenan, Engel, and Elsey 2013).

The day following sample collection, total nucleic acids were extracted using Zymo Research *Quick*-DNA Fecal/Soil Microbe Miniprep kit, following the manufacturer's instructions. Nucleic acid yield was quantified using Nanodrop and stored prior to 16S rRNA gene-sequencing.

Sample analysis and microbial inventories

Heparinized blood samples were collected during dissections and all blood samples were stored on ice until they were centrifuged approximately 1–4 hrs following collection; plasma was then stored at -80 °C. Hormonal steroids were thawed and extracted using 3 mL of diethyl ether and dried down with nitrogen gas. Plasma was then resuspended in a phosphate buffer solution and aliquoted in duplicate (200 µL each) into the respective tritiated hormone and antibody. Plasma estrogen levels were quantified using two ELISA estradiol kits (Caymen Chemical) following the

manufacturer's instructions. Extraction and radioimmunoassay procedures followed previously established protocols (Mendonça *et al.*, 1996). Values were corrected for volume of plasma and are reported in picograms per milliliter of plasma.

To determine the role of treatment on levels of E2 in the bloodstream, plasma estradiol concentrations from individuals were analyzed using a linear model with treatment as an independent variable and alligator mass and sex as covariates. If interactions were not significant, they were excluded from the model. Additionally, effects of E2 exposure were analyzed using a generalized linear model with alpha diversity metrics (see below) as dependent variables with mass and sex included as covariates. Because all hatchlings were hatched and housed together at RWR, clutch ID was unknown and thus, not incorporated into statistical models. All statistical analyses were performed using RStudio software version 1.0.153 (R Development Core Team 2018) with the package *lme4* (Bates et al. 2007, 4), and resulting figures were created utilizing color-blind friendly palettes. Raw means ± *SE* are reported for treatment groups where linear model regression coefficients are β.

Microbial samples were sent to the University of Georgia for 16S rRNA gene-sequencing in triplicate. Gene libraries for the 16S rRNA gene were prepared by the 2-step Adapterama Quadruple-index PCR method (Glenn et al. 2019) using the primers S-DBact-0341-b-S-17 (Bakt_341F) CCTACGGGNGGCWGCAG and S-DBact-0785-a-A-21 (Bakt_805R) GACTACHVGGGTATCTAATCC (Klindworth et al. 2013). This method resulted in 46 base-pair reads. Sequences were demultiplexed via Mr. Demuxy software [https://pypi.org/project/Mr_Demuxy/1.2.0/; (Glenn et al. 2019)] and imported into QIIME2 version 2020.2 (Caporaso et al. 2010; Bolyen et al. 2019) where they were denoised and chimeras removed using DADA2 (Callahan et al. 2016). Feature tables were filtered and classified into

operational taxonomic units (OTUs) using sklearn on a pre-trained 99 OTU GreenGenes v 13.8 database (DeSantis et al. 2006). Any OTUs identified in the negative control sample were considered contaminants and thus removed from the following analyses (Salter et al. 2014); however, contamination was not present in any of the sequence files. Unassigned OTUs, which made up 0.005% of total sequences, were also removed from the dataset. Because samples were sequenced in triplicate, we used the average of OTU absolute abundances in all three files for downstream analyses. Sequences and datasets were used to calculate rarefaction curves which revealed high to moderate sample coverage (Supplementary Figure 1.1).

Alpha diversity was identified using several measures: OTU absolute abundance, Shannon's diversity index, and Pielou's evenness index (Pielou 1966). OTUs were grouped according to genus and phylum and analyzed using a linear fixed effects model with OTU as a dependent variable and treatment group as an independent variable. Mass and sex were also included as covariates in the analysis. If interactions were not significant, they were removed from the models. Other alpha diversity (e.g., phyletic and genera relative abundances) measures were analyzed using linear fixed effects models as well, however sex and mass were not included as covariates. Beta diversity was compared using a non-metric multi-dimensional scaling analysis (NMDS) to calculate the level of similarity between bacterial community composition using the Bray-Curtis dissimilarity index in the R *vegan* package (Oksanen et al. 2007), where each point in the plot is an individual sample. This metric was quantified using an analysis of similarity (ANOSIM, permutations=999). All 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under Accession Number PRJNA831851. All scripts and datasets have been uploaded to GitHub (https://github.com/kmm0155/Alligator-Gut-Microbiome-Murphyetal). The main dataset used in statistical models can be found as Supplementary Table 1.

Results

Plasma estrogen

Individuals in the high treatment group had an average of 143 pg/mL (*SE* = 51.05) more E2 in their plasma than control individuals (*P*=0.01; Fig. 1.1). No significant difference was detected between low dose and control groups (β = 41.08, *SE* = 51.48, *P* = 0.44), nor between low and high dose groups (β = 102.06, *SE* = 51.42, *P* = 0.06), but there was a trend for the high dose individuals to have a higher concentration of E2 than those in the low treatment. Additionally, females had 163 pg/mL more E2 in their plasma than males across treatment groups (*SE* = 43.29, *P*=0.001). Mass was also significantly correlated with plasma E2 levels (β = 2.16, *SE* = 0.85, *P* = 0.02; Supplementary Fig. 1.2).

Microbial sequences

A total of 10,899 OTUs were utilized for analyses following blank sample and unassigned OTU removal. Individuals in the low-dose treatment group had 133 more bacterial OTUs within the GI tract than control treatment individuals (*SE* = 61.78, *P* = 0.04; Table 1.1). However, high treatment individuals did not differ in OTU absolute abundance from control treatment individuals (β = 91.82, *SE* = 61.30, *P* = 0.15; Fig. 1.2A). No significant difference in number of OTUs were detected between high and low dose treatments (β = 41.01, *SE* = 60.72, *P* = 0.51). Additionally, OTU absolute abundances were not correlated with plasma E2 values (β = 0.26, *SE* = 0.25, *P* = 0.31) or associated with alligator body mass (β =0.39, *SE* = 1.20, *P* = 0.74) and sex (β = 45.96, *SE* = 61.97, *P* = 0.47). However, the generalized linear model revealed a correlation between E2

exposure and OTU absolute abundance where xenobiotic exposure to E2 increased abundances (β = 112, *SE* = 51.6, *P* = 0.043; Table 1.2).

Three bacterial phyla were detected across the 23 individual alligators: Proteobacteria (51% of absolute phyla across treatment groups), Fusobacteria (29% of absolute phyla across treatment groups), and Firmicutes (20% of absolute phyla across treatment groups). The high treatment group had a significantly greater relative abundance of Proteobacteria than the control treatment group (β = 164.8, *SE* = 54.27, *P* = 0.007; Table 1.1). Additionally, the low treatment group had more Proteobacteria than the control group (β = 109.5, *SE* = 54.27, *P* = 0.057; Table 1.1). The high and low treatment groups did not differ in terms of relative abundance of Proteobacteria (*P* = 0.305).

There were no bacterial groups that were present in one treatment group and absent in another. Bray-Curtis NMDS showed different patterns of clustering of samples across treatment groups (Fig. 2B). However, bacterial community structure did not differ between groups (*R* = 0.026, *P* = 0.28), suggesting that microbiomes of each treatment group were similar.

Four bacterial genera were detected across treatment groups: *Helicobacter* (64% of absolute genera across treatment groups), *Clostridium* (25% of absolute genera across treatment groups), *Cetobacterium* (11% of absolute genera across treatment groups), *Defluviitalea* (0.03% of absolute genera across treatment groups). Again, the high treatment group had a significantly greater relative abundance of *Helicobacter*, a Proteobacterium, than the control treatment group (β = 163.3, *SE* = 53.57, *P* = 0.006; Fig. 1.2C).

Based on Shannon's diversity index, the high treatment group was less rich and less even than the control treatment (β = 0.63, *SE* = 0.22, *P* = 0.01; Table 1.1 and Fig. 1.3A). However, high treatment individuals had a greater Shannon's index than low treatment individuals (β = 0.45, *SE*

= 0.22, P = 0.06; Table 1.1 and Fig. 1.3A). Mass had a significant effect on Shannon's index, whereby heavier individuals had a lower Shannon's index than those that weighed less (β = 0.012, SE = 0.005, P = 0.041). Additionally, a significant interaction was detected between body mass and sex when comparing Shannon's index (β = 0.016, SE = 0.007, P = 0.048), whereby Shannon's index was negatively correlated with body mass; however, this effect is not seen as strongly in males. Plasma E2 values were also negatively correlated with Shannon's index (β = 0.002, SE = 0.0009, P = 0.014). Analyses of Pielou's index revealed similar results to those for Shannon's diversity index. The high treatment group was less rich and diverse than the control group (β = 0.15, SE = 0.05, P = 0.009; Table 1.1 and Fig. 1.3B), but no differences were detected between low treatment and control groups (β = 0.05, SE = 0.05, P = 0.38) nor between low and high treatment groups (β = 0.10, SE = 0.05, P = 0.06). Plasma E2 levels were also negatively correlated with Pielou's index (β = 0.0005, SE = 0.0002, P = 0.013). Additionally, mass (β = 0.001, SE = 0.001, P = 0.28) and sex (β = 0.05, SE = 0.05, P = 0.36) did not influence Pielou's index.

Table 1.1. Effect of estradiol-17β (E2) treatment on alpha diversity metrics in the gut microbiome and E2 concentrations in plasma of hatchling *Alligator mississippiensis*. Sex and mass were included as covariates in the linear fixed effects model. Effect sizes (β) utilize the control as a reference group. Statistically significant *p*-values are in bold face. OTU = operational taxonomic unit.

Dependent variable	Control	Low treatment		High treatment		Overall model values
	Mean	Mean	*β ± SE*	Mean	*β ± SE*	*F-value, DF, P-value*
OTU absolute abundance	400	537	133±61.78	476	92±61.27	2.10, 20, 0.12
Proteobacteria	134	243	109.5±54.3	299	164.8±54.3	4.72, 20, **0.021**
Helicobacter sp.	134	240	106.3±53.6	297	163.3±53.6	4.74, 20, **0.021**
Shannon's diversity index	2.74	2.606	-0.178±0.22	2	-0.63±0.22	3.12, 20, **0.035**
Pielou's evenness index	0.751	0.694	-0.047±0.05	0.611	-0.15±0.05	4.01, 20, **0.009**
Blood plasma E2 (pg/mL)	470	525	41.08±51.5	578	143±51.05	5.97, 20, **0.003**

Table 1.2. Effect of sex, mass, and estradiol-17β (E2) treatment using a generalized linear fixed effects model on alpha diversity in the gut microbiome of hatchling *Alligator mississippiensis*. Effect sizes are represented as 'β'. Statistically significant *p*-values are in bold face. OTU = operational taxonomic unit.

Dependent variable	Sex effect		Mass effect		E2 treatment effect	
	$\beta \pm SE$	*P* value	$\beta \pm SE$	*P* value	$\beta \pm SE$	*P* value
OTU absolute abundance	-81.2±47.9	*P*=0.11	1.14±0.99	*P*=0.26	112±52	***P*=0.043**
Shannon's diversity index	-2.35±1.07	***P*=0.04**	-0.01±0.006	***P*=0.03**	-0.41±0.21	*P*=0.06
Pielou's evenness index	0.03±0.04	*P*=0.54	-0.0003±0.001	*P*=0.77	-0.01±0.05	*P*=0.052

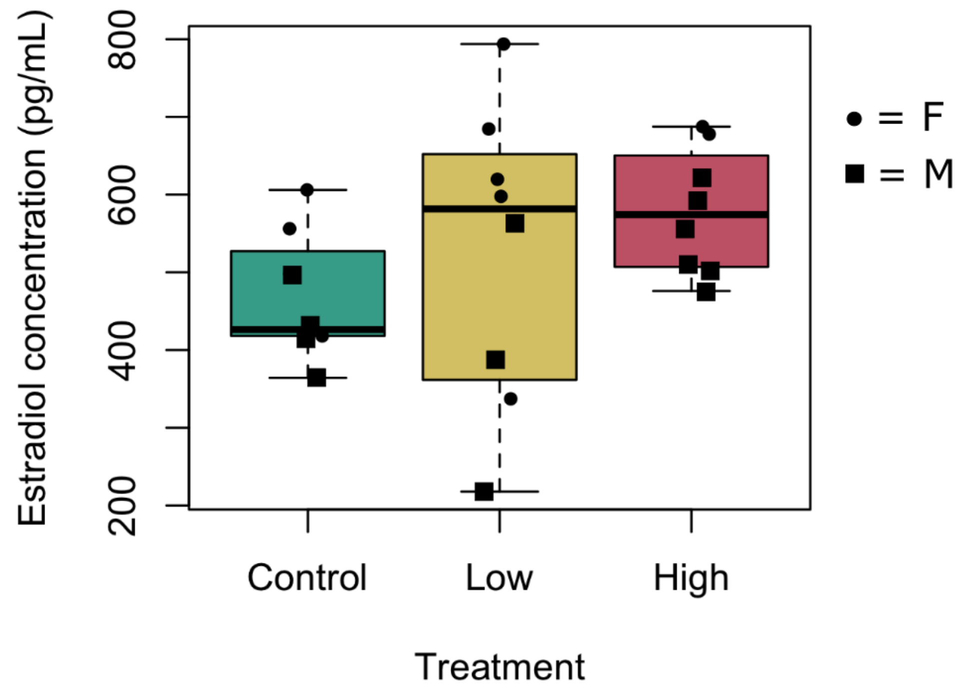


Figure 1.1. Estradiol-17ß (E2) concentration in plasma of hatchling *Alligator mississippiensis* exposed to no E2 (Control) and two concentrations of E2 (Low and High) are represented as boxes, while the whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5x of the interquartile range. The bold line represents median values within the box. Females are represented as circles and males are represented as squares.

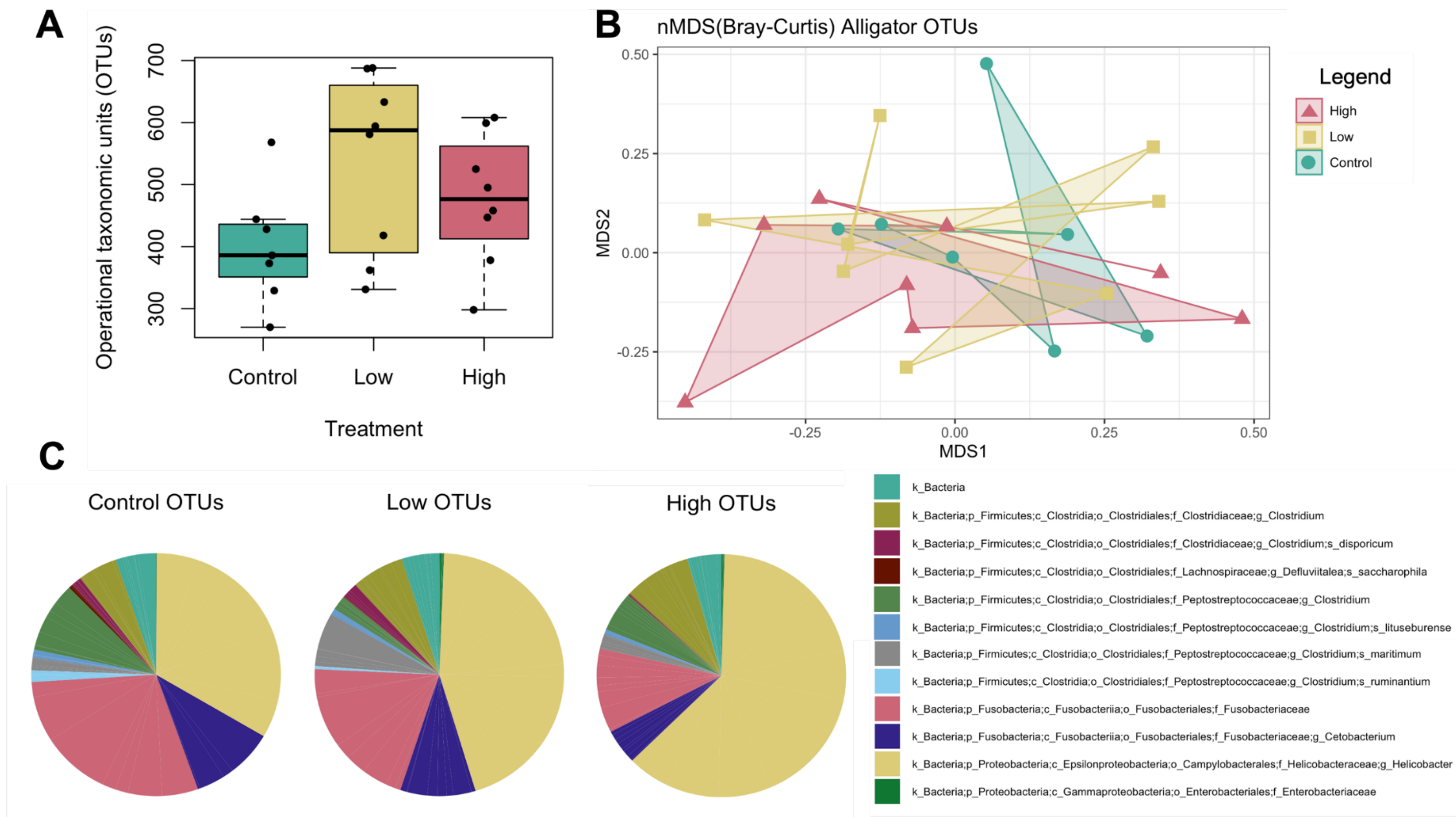


Figure 1.2. Variation in microbial communities and abundance across three treatment groups (i.e., level of exposure to estradiol-17ß) in hatchling *Alligator mississippiensis*. (A) Differences in microbial operational taxonomic units (OTUs) among treatments. The

whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents median values within the box. (B) Non-metric multidimensional scaling (NMDS) plot of all individuals. The green circles in the NMDS plot are for individuals in the control treatment, yellow squares are for individuals in the low estradiol treatment, and red triangles are for individuals in the high estradiol treatment. Lines denote vector overlays on NMDS ordination, indicating the directionality and strength of change (line length) for a specific parameter. (C) Differences in bacterial OTU relative abundances among treatment groups (i.e., Control, Low, and High) where kingdom (k), phylum (p), class (c), order (o), family (f), genus (g), and (s) are indicated in the Figure legend.

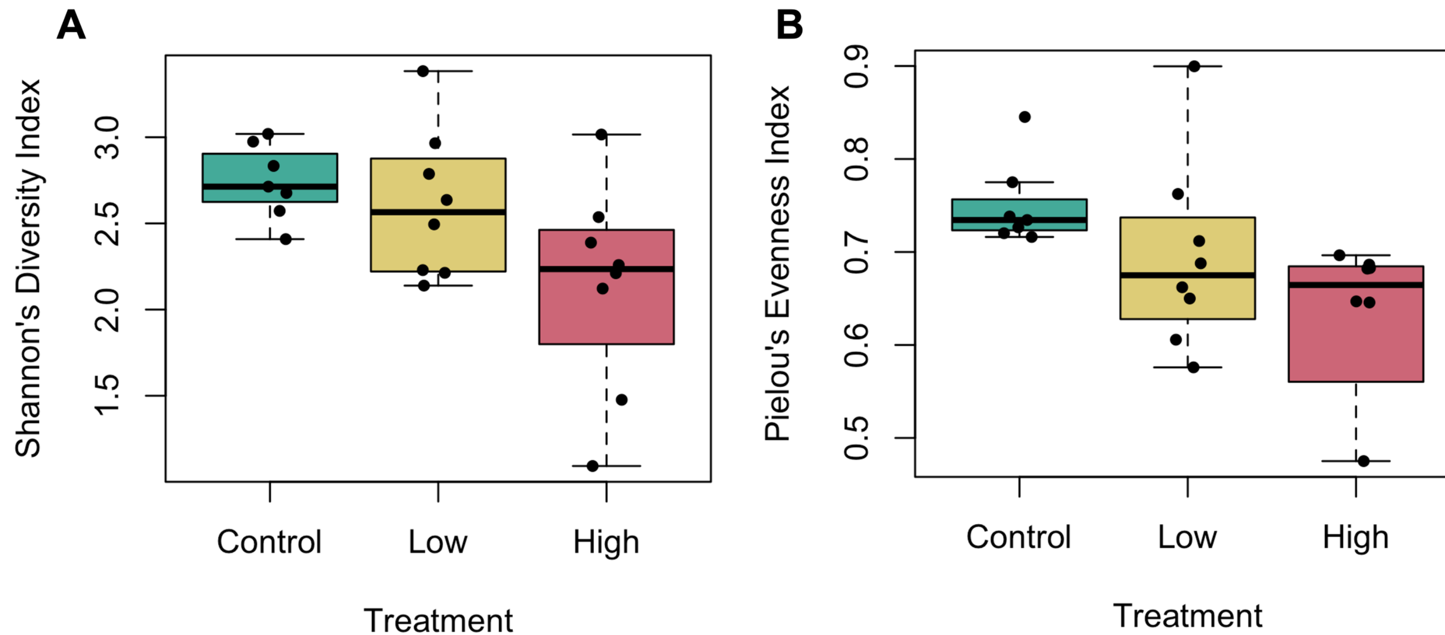
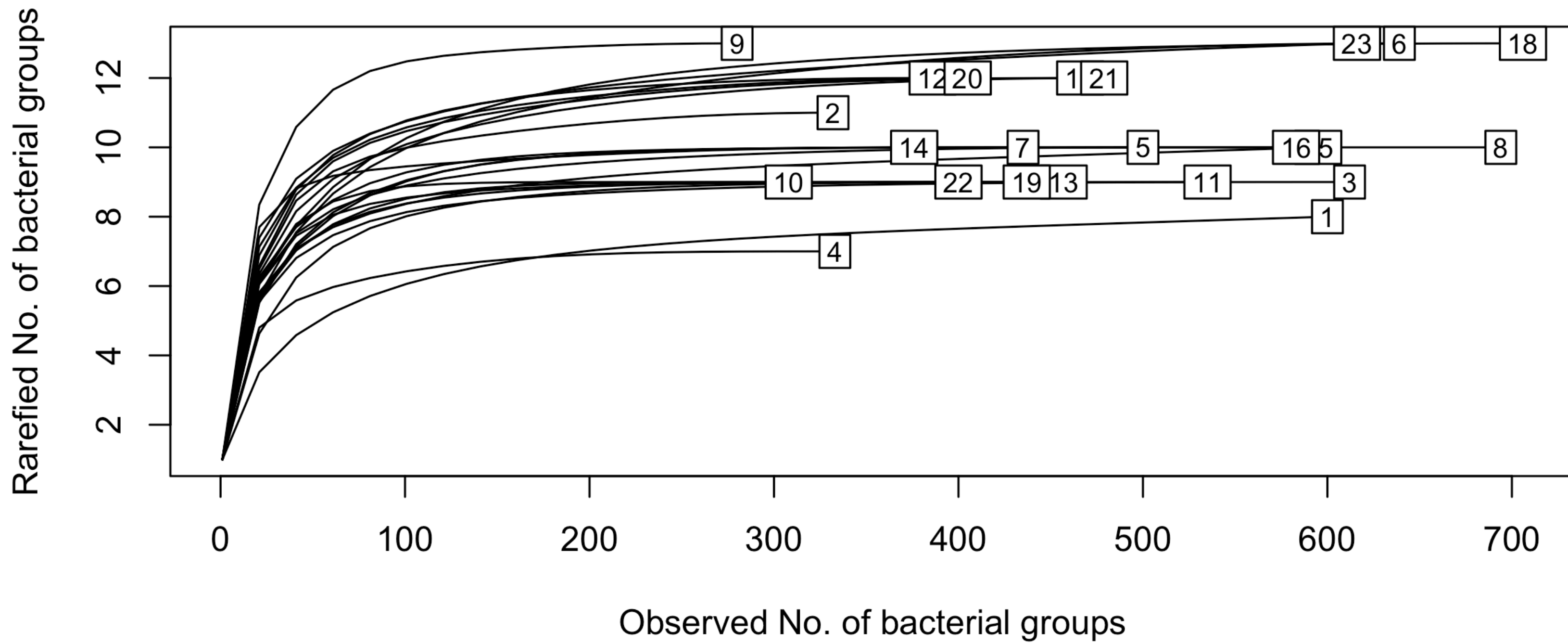


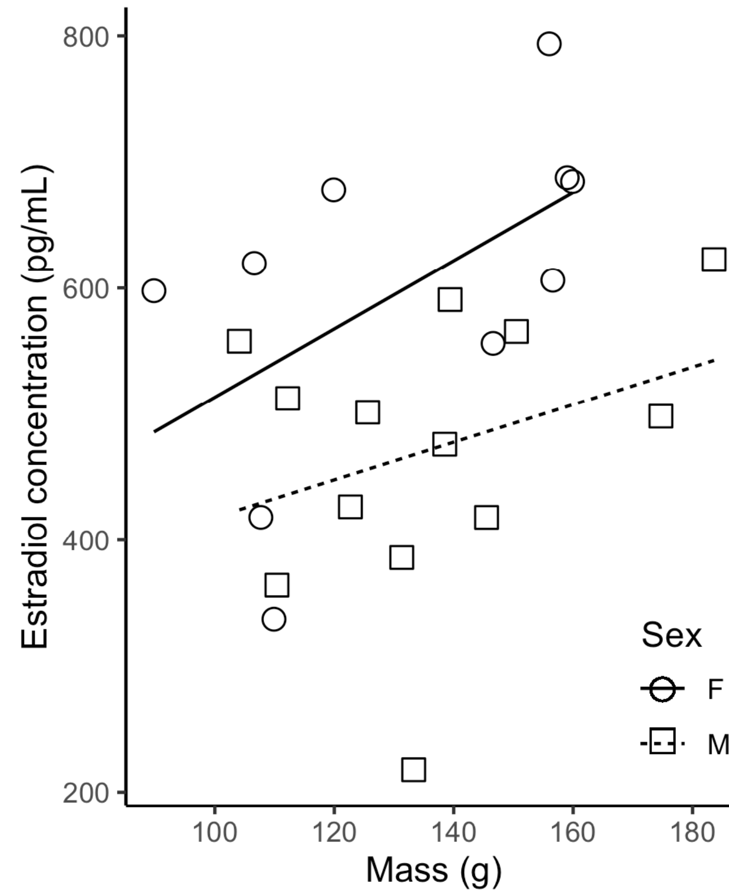
Figure 1.3. Variation in Shannon's diversity and Pielou's evenness indices across three treatment groups (i.e., level of exposure to estradiol-17ß) in hatchling *Alligator mississippiensis*. (A) Shannon's diversity index scores and (B) Pielou's evenness index scores for each treatment group are represented as boxes, while the whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5x of the interquartile range. The bold line represents median values within the box.

Supplementary Table 1.1. Sex, mass, Operational taxonomic unit (OTU), Shannon's diversity index, and Pielou's evenness index distribution by sample (i.e., individual ID) and treatment for the 23 hatchling *Alligator mississippiensis* used in this study.

Individual ID	Treatment	Sex	Mass (g)	OTU absolute abundance	Shannon's diversity Index	Pielou's evenness index
D	Control	F	107.7	329	2.68	0.72
G	Control	F	156.6	428	2.57	0.72
I	Control	M	145.5	270	2.97	0.85
M	Control	M	122.7	373	2.83	0.73
N	Control	M	110.4	444	2.71	0.73
Q	Control	F	146.6	568	2.41	0.74
U	Control	M	174.7	386	3.02	0.78
B	Low	M	131.3	331	2.64	0.65
F	Low	F	89.8	633	2.23	0.58
H	Low	F	159.9	687	2.21	0.61
O	Low	F	109.9	362	3.38	0.90
P	Low	M	150.5	581	2.49	0.66
S	Low	F	106.6	688	2.79	0.69
T	Low	F	156	418	2.14	0.71
X	Low	M	133.3	594	2.97	0.76
A	High	F	159	599	1.09	0.37
C	High	M	183.6	608	2.26	0.68
E	High	M	139.4	495	2.54	0.70
K	High	M	104.1	298	2.21	0.69
L	High	M	112.2	525	1.48	0.48
R	High	M	125.6	447	2.39	0.65
V	High	F	119.9	458	3.02	0.65
W	High	M	138.5	378	2.12	0.68



Supplementary Figure 1.1. Rarefaction curve of rarified number of bacterial groups by observed number of bacterial groups. Labels are numerically associated with individual hatchling *Alligator mississippiensis* in the dataset.



Supplementary Figure 1.2. Relationships between blood plasma estradiol-17ß (E2) concentration by body mass of hatchling *Alligator mississippiensis*, where each datapoint is represented by individual sex. Open circles and the solid black line are for females, while open squares and dashed black line are for males.

Discussion

Concentrations of EDCs, including environmental estrogens such as E2, in aquatic ecosystems are major determinants of population health for a variety of species and potentially have strong ecosystem-wide effects. Physiology at various life-history stages can be influenced by xenoestrogen pollutants and in turn, can have important consequences on reproduction. E2 has been shown to influence gut homeostasis (Y. Liu et al. 2016). However, these associations are not well understood, particularly in wildlife populations. In this study, we administered E2 to hatchling alligators and demonstrated that xenoestrogen treatment decreased microbial diversity in the GI tract. However, this pattern does not support part of our first prediction where E2 increased gut bacterial relative abundances, particularly in specific groups of Proteobacteria. Interestingly, the low treatment group had more OTUs than either the control or high treatment groups. This effect was also dose-dependent, whereby increased exposure to E2 reduced the diversity, richness, and evenness of microbiota.

Blood plasma analysis revealed that treatment individuals had higher E2 concentrations than control individuals. Notably, females had greater E2 blood plasma levels than males and mass was positively correlated with E2 values. These findings are similar to previous studies in *A. mississippiensis*, where EDC exposure elevated E2 blood plasma concentrations that were also associated with both sex and size (Milnes et al., 2002). These data suggest that xenobiotic E2 may 1) increase blood plasma E2 regardless of sex, 2) increase blood plasma E2 in females at less than a year of age, and 3) increase overall mass of individuals following short-term (i.e., 10 weeks) exposure.

Similar to previous studies in zebrafish and rodent models (Javurek *et al.*, 2016; Liu *et al.*, 2016), E2 treatment affected bacterial diversity in alligators. For example, experimental exposure

of male zebrafish to E2 and bisphenol A (BPA) reduced Shannon's diversity index in comparison to untreated males (Liu *et al.*, 2016). Moreover, this work demonstrated a dominating-effect of bacterial phylum CKC4 along with decreased diversity (Y. Liu et al. 2016). Likewise, we saw an increase in one particular bacterial phylum (i.e., Proteobacteria) in low and high treatment individuals (Figure 1.2). This decrease in bacterial diversity could be attributed to the interspecies competition of bacteria in the GI tract and/or E2 decreasing nutrient availability, amount, etc. whereby some species can no longer thrive (Baker, Al-Nakkash, and Herbst-Kralovetz 2017). Additionally, our study revealed a negative correlation between mass and Shannon's diversity index. Heavier individuals seemed to have less rich and even microbial gut communities than their lighter counterparts. However, this finding did not remain when analyzing Pielou's evenness index, suggesting that the relationship between mass and microbial diversity may depend on which diversity index is used by researchers.

The bacterial taxon that dominated the GI tract of individuals in the high treatment group was a Proteobacteria genus, *Helicobacter.* This often pathogenic genus is opportunistic in that following a disruption in homeostatic bacterial GI communities, *Helicobacter* can thrive and dominate intestinal environments. This increase in abundance, or the imbalance of *Helicobacter* with other bacterial taxa, contributes to a disrupted microbiota community. Changes in microbiota community composition have been shown to cause inflammatory responses and exhibit metabolic profiles that can negatively influence gut epithelial health (Baker, Al-Nakkash, and Herbst-Kralovetz 2017; Turnbaugh et al. 2006). *Helicobacter pylori* is responsible for chronic gastritis and peptic ulcers and is considered a pathogenic bacteria that is linked to gastric cancer in humans (Lofgren et al. 2011; Ohtani et al. 2007; Graham 1991). Surprisingly, other studies have shown that E2 acts bateriostatically on *H. pylori in vitro* (Hosoda et al. 2011) and that *H. pylori* can absorb

and hold E2 within its membrane (Hosoda et al. 2009). All *Helicobacter* species are associated with vertebrate hosts, show high host-specificity, and are mostly associated with the gastric, enteric, and hepatobiliary tracts (M. J. Gilbert et al. 2019; 2014).

Given the potential interaction between E2 and *Helicobacter*, and that E2 treatment increased *Helicobacter* abundance in our study, we suggest two main reasons for this observed change. First, Proteobacteria increase is often an indicator of disease or illness (Rizzatti et al. 2017). Indeed, treatment with pesticides can increase pathogenic bacteria (including *Helicobacter*) levels in rats (F. Yang et al. 2019). Other agricultural pollutants, such as glyphosate-based herbicides, decrease potentially beneficial microbes in young Japanese quail (Ruuskanen et al. 2020). E2 treatment in our study increased Proteobacteria species like *Helicobacter*, which suggests an imbalanced gut microbiota profile. Second, as carnivores and carrion-eaters, crocodilians are known to carry pathogenic bacteria within their GI tracts (Lin et al. 2019; Willson et al. 2019; Keenan, Engel, and Elsey 2013). The abundances of these internal pathogenic bacteria, like *Helicobacter*, might assist with "inoculating" individuals against pathogens consumed through carrion and any bacterial toxins produced (Roggenbuck et al. 2014). We also identified *Clostridium* sp. in our study which has been associated with pathogenesis. A recent study by Kieran et al. (2020) shows increases in *Clostridium* due to Selenium (Se) exposure in sexually immature *A. mississippiensis* following their seven-week experiment. Similar to E2, Se is necessary for endocrine function, but can induce toxicity at high levels (Janz et al. 2010).

One of the most interesting results from our study shows that exposure to a relatively low concentration of E2 increases microbiota absolute abundances but does not influence microbiota diversity. This dose-dependent effect suggests that varying concentrations of E2 may influence microbial community composition, in terms of alpha and beta diversity, in different ways. We

suggest two reasons for why this may be: 1) Perhaps there is a three-way interaction occurring between genetics of individuals, their associated microbiota, and bodily concentrations of E2. Previous studies illustrate that xenoestrogens can affect alligator physiology , and that these physiological effects can influence gut microbiota community structure or vice versa. We suggest, however, that there may be reciprocal effects between the concentration (and potentially exposure time) of E2 and gut microbiota where gut microbiota may influence E2 concentrations in wildlife. Indeed, microbiota can metabolize estrogens to their biologically-active form (Ervin et al. 2019; Baker, Al-Nakkash, and Herbst-Kralovetz 2017; Plottel and Blaser 2011). It has been suggested that microbiota can thereby influence the circulating levels of estrogen in the body (Plottel and Blaser 2011); however, no study thus far has demonstrated that altering gut microbiota patterns can change E2 plasma concentrations over time. Additionally, the term "endobolome" has been used to define microbial genes and their pathways that are involved in metabolism of steroid hormones and EDCs (Aguilera, Gálvez-Ontiveros, and Rivas 2020). Because our study did not analyze enhanced gene pathways or metabolites produced by the gut microbiota, we cannot conclude whether this change in community diversity altered circulating E2 levels through these mechanisms. 2) Perhaps genetic contributions to variation in the gut microbiome are reduced at a high concentration of E2, and genetic influences are only observed when exposed to E2 at low concentrations. Such interactive effects between genotype and E2 exposure could have different influences on abundance and diversity of gut microbiota. Indeed, this bi-phasic effect of EDCs on physiology is not unknown, and is attributed to the up- or down-regulation of genes by EDC compounds and to the response to natural hormones in the body [leading to the production of more or less hormone (Vandenberg et al. 2012)]. Previous studies have also demonstrated that following exposure to E2 alone, *A. mississippiensis* develops the same reproductive abnormalities observed

in natural populations subject to complex mixtures of EDCs (Hale and Parrott, 2020). Thus, synergism of multiple EDCs in ecosystems may influence the gut microbiota in ways that E2 alone would. This idea may be addressed in future studies and would contribute to our knowledge of the complex interactions between the gut microbiota, EDCs, and population health.

A limitation of our study is that we cannot identify whether gut microbiota are modulated directly by E2 itself versus an E2-induced physiological response by an individual alligator. Environmental chemicals can induce changes in the gut microbiome, although many of these regulating mechanisms remain unknown (Chiu et al. 2020). For example, carbendazim, a widely-used fungicide, induces a change in gut microbiota composition by increasing lipid absorption in the intestine which can cause multi-tissue inflammatory responses (Jin et al. 2018). If environmental E2 is contributing to the already circulating pool of estrogen in the body, this influx could overwhelm the liver (whose role is conjugating unbound E2 to its inactive form, estradiol-17-glucuronide) and the estrogen that the liver can conjugate is then unbound by GUS enzymes in the GI tract. Thus, individuals would have higher blood E2 concentrations, which we also found in our study between high treatment group individuals and the controls. This would contribute to the idea that it may be the body's physiological response, mediated by transcriptional factors, to an increase in circulating E2 rather than the interaction between GUS enzymes and bacteria growth. Additionally, we prioritized a region of the digestive tract that is not as rich and diverse in microbiota as compared to the lower GI tract communities in alligators (Keenan, Engel, and Elsey 2013). If samples taken from the colon of alligators used in this study had been sequenced, we might have seen even greater differences across treatment groups. It should be noted that the alligators' young age may have contributed to fewer OTUs present than those shown in other studies (Kieran et al. 2020; Keenan, Engel, and Elsey 2013). Indeed, the number of OTUs present

in human GI tracts is low at birth and bacterial diversity increases with age across populations (Yatsunenko et al. 2012). Additionally, the gut microbiome is thought to develop through environmental exposure in reptiles (Colston and Jackson 2016).

Captive housing conditions may have lowered the diversity of bacterial communities across time as well. Captive housing significantly alters bacterial communities in lizards (Kohl et al. 2017), and the same may apply for alligators; however, farm-raised alligators have similar bacterial variability or community structure compared to their wild counterparts (Keenan, Engel, and Elsey 2013). Additionally, during the acclimation period prior to xenobiotic E2 exposure, individuals were not subject to force-feeding. During the treatment period, hatchlings were fed by oral gavage. This difference in feeding technique may attribute some variation in gut microbiota communities where previous studies have shown that exposure to acute stress may alter the composition, function, and metabolic activity of gut microbiota (Karl et al., 2018). However, because control individuals were also subject to this change in feeding technique, potential variation due to stress should be accounted for. Lastly, because individuals were hatched and housed under communal conditions at RWR prior to transportation to Auburn University, clutch ID was unknown and could not be incorporated as a random effect in statistical models. Perhaps variation in our results could be attributed to *in ovo* bacterial communities acquired from maternal sources that may have influenced the colonization and development of hatchling gut microbiota (Trevelline *et al.*, 2018). Future studies should incorporate these factors into the experimental design and statistical analyses.

Studies of captive wildlife can provide important insights into the functional roles of microbiota in disease and health (Williams et al. 2018). However, sampling from wildlife populations in their natural environments unravels variation in ecologically relevant contexts

(Amato 2013). Controlled laboratory experiments, such as ours, can deepen our understanding of direct relationships observed in natural environments. Our study serves as an important link between EDCs (e.g., xenoestrogens) and gut microbiota and exemplifies how future research might expand upon the influence of such changes on health of wildlife populations.

Chapter 2

Methods for sampling and manipulating the microbiome of reptile eggs at oviposition

Manuscript in-preparation for submission to *Biology Letters*

Introduction

Effective sampling methods for microbiome studies have been widely debated (Sare et al., 2020; Pollock et al. 2018; Kong et al. 2017; Videvall et al. 2017; Goodrich et al. 2014), particularly those used for understanding the evolutionary biology of the host (Hird 2017). To ensure accurate representation of the microbiome in a collected sample, different methods are employed depending on the tissue or surface type. Importantly, however, sampling techniques or methods can alter the diversity and abundance of microbes that are collected in samples (Pollock et al. 2018; Vogtmann et al. 2017; Ingala et al. 2018), which can provide an inaccurate or inconsistent understanding of host microbiota. This is especially important when evaluating the microbiome as an indicator of health or when applied towards wildlife conservation (Trevelline, Fontaine, et al. 2019; Williams et al. 2018). Many studies in these fields use methods that are non-destructive or minimally invasive to hosts, but whether these approaches provide unbiased results that differ from destructive methods is poorly understood [e.g., fecal vs intestinal sampling methods (Ingala et al. 2018)]. In addition, studies of microbial transfer from mothers to offspring at birth have generally used non-destructive methods, but the efficacy of these approaches are rarely compared to other approaches.

Maternal transfer of microbes to offspring has been critical in understanding health in humans (Mueller et al. 2015) and in wildlife populations (Grosser et al. 2019; Zhou et al. 2017)

because these maternal effects can have lasting positive effects on offspring [e.g., stimulates metabolic processes (Kimura et al. 2020), primes the immune system (Maradiaga et al. 2018)]. Although vertical transmission of microbiota from mothers to offspring is well documented in viviparous vertebrates (mammals: Woodruff et al. 2022; Grzeskowiak et al. 2022; Jasarevic et al. 2017); some reptiles: Kohl et al. 2017), most vertebrates are oviparous, and consequently, little is known about how microorganisms colonize eggs (Nyholm 2020) and their impacts on development. Nevertheless, some studies demonstrate a broad diversity of bacteria inhabiting eggshells in birds (van Veelen, Salles, and Tieleman 2018; Martínez-García et al. 2016), non-avian reptiles (Sarmiento-Ramírez et al. 2014), and amphibians (Hughey, Delia, and Belden 2017), as well as demonstrate potential maternal transfer of microbiota to the eggshell (van Veelen, Salles, and Tieleman 2018). Thus, effective sampling at this early stage is critical for understanding how microbial communities may be passed from mother to offspring in oviparous organisms, and methods for manipulating the microbiome (without harming offspring) are necessary for identifying its functional significance.

Sampling of eggs is often accomplished via swabbing. This non-invasive method is simple, enables researchers to quickly obtain samples in field or laboratory settings, and has little to no impact on embryo development or egg survival (Stéphanie Grizard et al. 2014; Martín-Gálvez et al. 2011). However, swabbing requires manually rubbing the swab over portions of the egg, which could result in incomplete sampling and poor downstream characterization of the eggshell microbiome. A second approach involves crushing the eggshell to enable adequate sampling depth; this approach is also relatively simple, but it is destructive and identifying whether bacteria sequences are derived from the host versus other sources can be difficult (Stephanie Grizard 2015). Sonication [i.e., agitation of microbes into a solution using high-frequency sound waves (Kwon

and Jewett 2015; Shrestha, Holland, and Bundy 2012)] of eggshells following hatching has also been used, but this approach does not provide a representation of the eggshell microbiome immediately after oviposition, and hence, is not suited for studies of maternal transfer during early egg incubation. While sonication likely provides more thorough sampling of the microbiome than swabbing, its destructive nature also limits studies aimed at understanding the influence of microbes on development because it could reduce embryo survival if performed before hatching.

Current investigations of eggshell microbiomes rarely include more than one sampling technique and no comparison of methods currently exists for oviparous vertebrates (Stephanie Grizard 2015). Moreover, to quantify how aspects of the microbiome (e.g., bacterial abundance, diversity) relate to embryo survival or offspring phenotypes, non-destructive sampling methods must be developed. Direct manipulation of the eggshell microbiome will also be critical in understanding its functional consequences. Thus, experimental protocols that manipulate the microbiome with minimal impact on egg survival will be particularly useful. One approach to address this issue is to compare egg survival of bacteria-free vs unmanipulated control groups. Antimicrobial treatment of eggshell surfaces has been shown to limit pathogen transmission, leading to higher survival rates (Grotmol and Totland 2000), and is often employed in aquaculture hatcheries for disease mitigation (Swaef et al. 2016). In amphibian species, disinfection of egg masses has been used to manage pathogens in captive populations (Ujszegi, Molnár, and Hettyey 2021). Calcified eggshells (e.g., many reptile eggs) have also been subject to disinfection, namely in the poultry industry (Olsen et al. 2017; Musgrove et al. 2005). Manipulation of the eggshell microbiome in wild species may illuminate its influence on developing embryos and hatchlings, but methods for doing so in non-avian reptiles have not been explored.

The overarching goal of this study was to develop effective protocols that address the two primary issues discussed above. Specifically, we performed a series of experiments using the brown anole lizard (*Anolis sagrei*) to provide useful guidelines for effective/non-destructive methods for sampling and manipulating the microbiome on eggshells. We first aimed to examine the effectiveness of swabbing and sonication for measuring eggshell microbial abundance and diversity. We also quantify the effects of these two sampling methods on egg survival. Second, we compared the efficacy of bleach, tryptic soy broth (TSB), and water (as a control) in manipulating bacterial abundance on the surface of freshly laid eggs, and we quantified their influence on egg survival and hatchling phenotypes. We provide detailed descriptions of our experimental protocols, which will help guide future studies of host-microbe interactions in oviparous reptiles, with specific focus on methods needed for quantifying maternal transfer of the microbiome to offspring, as well as the functional significance of eggshell microbiota.

Methods

Study species

The brown anole lizard (*Anolis sagrei*) has many characteristics that are well suited for addressing our aims. Brown anoles are relatively small (~ 2-5 grams) and easily housed in captivity. Protocols for their captive propagation are well established and they readily reproduce in captivity (Warner 2014; Sanger et al. 2008). Female *A. sagrei* produce about one egg per week over a long reproductive season (April-October); this high reproductive rate allows for large sample sizes for egg experiments. The small eggs of this species are relatively robust to movement (Hulbert et al. 2017) and submersion in water (Losos, Schoener, and Spiller 2003), which could reduce the

impacts of egg manipulations (e.g., swabbing, sonication, bacterial removal via submersion in different solutions) on embryo survival.

Animal collection and housing

From 2019 – 2022, a total of 303 *A. sagrei* (202 females and 101 males) were collected from Palm Coast, FL. An average of 60 individuals were collected during each season (40 females and 20 males; Supplementary Table 2.1) and then transported to Auburn University, Auburn, Alabama, USA. For all experiments below, two females and one male were housed per cage. Cages were equipped with a bamboo stick for perching and basking, artificial foliage, and a nesting pot filled with moist potting soil. Cages were illuminated overhead with Reptisun 5.0 UVB bulbs (ZooMed Inc.) and kept on a 12:12 light-dark cycle. Each lizard was fed two crickets (dusted with calcium and vitamins) every other day and misted with water daily. Nesting pots (4-inch diameter, 4-inch tall) were placed in cages and eggs were collected (see details below for each experiment) and allocated to different treatments for three different experiments (Table 2.1).

1) Eggshell bacterial sampling method

In May 2019, eggs were obtained by emptying one nesting pot at a time into a clean, but not aseptic, plastic container using sterile gloves. Soil was searched for eggs by gently rocking soil back and forth in the plastic container. After eggs were collected, the soil was misted with DI water and placed back into the nesting pots. A preliminary experiment was conducted to examine the influence of probe sonication on egg survival. Eggs obtained on 7–10 May 2019 were placed either in a 50 mL conical tube (VWR) with 10 mL of autoclaved 1X PBS solution for 5 minutes as a control for egg survival (n = 3) or were exposed to a probe sonicator (n = 3). Probe sonication was

done in three 1-min pulses at 20 kHz. Although two of the three eggs eventually hatched, the high-intensity sonication removed the calcium-lining on the shells of all the eggs. Because the damaging effect of this approach on the eggshell was immediately obvious, we decided to use a water bath sonicator (VWR, Ultrasonic Cleaner) in our subsequent experiments for microbiome sampling without damaging the protective calcium-lining of the eggshells.

Eggs obtained on 16–22 May 2019 (n = 12) were allocated to one of three groups (Table 1; n = 4 per treatment) to examine the efficacy of swabbing and sonication for sampling the eggshell microbiome: (1) Eggs swabbed, and then the swab was placed in sterile 1X PBS; (2) Eggs placed directly in sterile 1X PBS and sonicated; (3) Egg placed directly in sterile 1X PBS, but not sonicated (control for submersion in 1X PBS). For group 1, eggs were swabbed for 20 seconds (wearing sterile gloves) over the complete surface of the eggshell in one round using a single Puritan sterile flocked swab. The swab was immediately placed in 50 uL of 1X PBS in a sterile 1.5 mL Eppendorf tube and set on ice until DNA extraction. In groups 2 and 3, eggs were immediately placed in sterile 1X PBS and those in group 2 were immediately transported to a water bath sonicator and sonicated for 5 minutes. Following these procedures, eggs were removed from tubes with sterile forceps. Swabs in 1X PBS 1.5 mL Eppendorf tubes and the 1X PBS solution in 1.5 mL Eppendorf tubes that eggs were suspended in were then placed on ice until immediate DNA extraction. Eggs in group 3 were submerged in a 1X PBS solution for approximately the same length of time as the sonicated eggs; however, they were not exposed to sonication. Only samples in groups 1 and 2 were used for downstream microbiome sequencing (n=4/treatment for sequencing; Table 1).

Following eggshell sampling, eggs were weighed and placed in petri dishes layered with moist (-150 pKa) vermiculite at a constant 28 °C. Eggs were checked daily for hatchlings and

when a hatchling was found, snout-vent length (SVL), tail length (TL), mass, and sex were recorded. Because of the relatively small sample sizes used for quantifying bacteria, additional eggs were allocated into the treatments from 22 April to 1 May 2020 (n = ~15 more eggs per treatment; Table 2.1) following the same sampling methods described above to measure egg survival. However, we added one additional control treatment where eggs were not swabbed or sonicated (see treatment 4 in Table 2.1). Microbiome samples were not collected for these additional eggs in 2020, and only egg survival and hatchling mass, snout-vent length (SVL), and tail length (TL) were measured.

2) Eggshell bacterial manipulation

We performed two experiments aimed to identify effective non-invasive methods to manipulate the microbiome on eggshells. For the first experiment (3 May to 10 June 2020), eggs were obtained by emptying one nesting pot at a time into a clean, but not aseptic, plastic container. Soil was searched for eggs by gently rocking soil back and forth in the plastic container. After eggs were collected, the soil was misted with DI water and placed back into the nesting pots. Eggs were randomly allocated into one of five treatment groups (n = 8-13 eggs per treatment; Table 1) to examine the effect of antimicrobial solutions on egg survival: (1) A control group (no external eggshell treatment); (2) One time submersion of an egg in 5 mL of autoclaved water for 30 seconds (control for submersion in a solution); (3) One time submersion of an egg in 5 mL of 10% bleach for 30 seconds; (4) One time submersion of egg in 5 mL of 20% bleach for 30 seconds; (5) One time submersion of an egg in 5 mL of 70% ethanol for 30 seconds. Eggs were weighed and placed in petri dishes layered with moist (-150 pKa) vermiculite at a constant 28 °C. Egg survival was

measured by whether the embryo survived to hatching. Upon hatching, individuals were weighed and measured (mass, SVL, TL).

For the second experiment (May 2021 and May 2022), nesting pots were checked for eggs three times per week and eggs collected were randomly allocated to four treatments: (1) A control treatment (unmanipulated eggs); (2) A procedural control where eggs were submersed in sterilized water for 30 seconds; (3) Submersion of eggs in 20% bleach for 30 seconds as an 'antimicrobial' treatment; (4) Submersion in 5 mL of tryptic soy broth (TSB) for 30 seconds as a 'bacterial growth promoting' treatment. To ensure that treatments were effective, eggshell swabs were collected (A) prior to and (B) immediately following treatment by swabbing eggs for 30 seconds, ensuring coverage of the entire egg surface. All swabs were individually placed in sterile 1.5 mL Eppendorf tubes with 500 µL sterile 1X PBS and vortexed for 15 seconds following an adapted protocol (D'Alba et al. 2010). This microbial suspension was serially diluted to 10^{-1} and 0.1 mL of the solution was plated onto diluted trypticase soy agar (TSA, 10%) and incubated at 28 °C. Colony Forming Units (CFU) per egg were enumerated at 24 hrs. All eggs were incubated in autoclaved vermiculite moistened with autoclaved water (-150 kPa) at 28 ºC until hatching. Hatchlings were weighed and measured (mass, SVL, TL).

DNA extraction, microbial inventories, and statistical analyses

For the bacterial sampling experiments, DNA was extracted from all microbiome samples (n = 4 eggshell swabs, n = 4 1X PBS solution that underwent whole-egg sonication) using *Quick*-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research) per the manufacturer's instructions. DNA amount (ng/µL) was quantified using Qubit fluorometer (Invitrogen, Qubit 4 Fluorometer).

To examine the influence of sampling method on DNA concentration, a linear model was used in R Studio software (Version 1.0.153; R Development Core Team 2018).

Extracted DNA was sent to a next-generation sequencing facility (University of Illinois-Chicago, Chicago, IL, USA) for 16S rRNA amplicon sequencing through Illumina MiSeq sequencing platforms using the primers X. This method resulted in 16 demultiplexed, base-pair reads. Microbial sequences were analyzed using QIIME 2 version 2020.2 (Caporaso et al. 2010; Bolyen et al. 2019) and were denoised and chimeras removed using DADA2 (Callahan et al. 2016). *Sklearn* was used to filter and classify feature tables into operational taxonomic units (OTUs) on a pre-trained 99 OTU GreenGenes v 13.8 database (DeSantis et al. 2006). Operational taxonomic units (OTUs) present in the 'blank' sample were considered contaminants and removed from the dataset (Salter et al. 2014); however, contamination was not present in any of the sequence files. Sequences were annotated and rarefaction curves calculated, elucidating sample coverage. Datasets were used to calculate rarefaction curves which revealed high to moderate sample coverage (Supplementary Fig. 2.1).

We calculated several measurements of alpha diversity: the Shannon index, Faith's phylogenetic diversity (Faith 1992), and absolute abundance of OTUs. R Studio was used to compare the alpha diversity measurements using a linear mixed-effects model with alpha diversity as a dependent variable and sampling method as an independent variable. Cage number was included as a random effect. Beta diversity was compared among treatment groups using a non-metric multi-dimensional scaling analysis (NMDS) to calculate the level of similarity between bacterial community composition using the Bray-Curtis dissimilarity index in the R *vegan* package (Oksanen et al. 2007). Bacterial community compositions (Bray-Curtis distance metric) was

analyzed using an analysis of similarity (ANOSIM, permutations=999). All 16S rRNA sequences will be deposited in the Sequence Read Archive (SRA).

For each experiment, egg hatching success was analyzed with generalized linear mixed models with a binomial error distribution; treatment and egg mass were independent variables. Linear mixed effects models were used to quantify the effects of eggshell treatment on DNA concentration for experiment 1. Two separate linear mixed effects models were used to analyze colony forming units (CFUs) for experiment 2 where (1) CFUs after treatment (timepoint B) were compared across treatment groups; this analysis included CFUs before eggshell treatment (timepoint A) as a covariate. For the second model, data collected at timepoints A and B and compared across treatment groups. Linear mixed models were also used to quantify treatment effects on hatchling morphology (e.g., mass, SVL, TL) using egg mass as a covariate. All interaction terms between treatment and egg mass were not statistically significant and were removed from final analyses. Cage number was used as a random factor in all models. All scripts have been uploaded to GitHub (https://github.com/kmm0155/Murphyetal2023_Methods).

Results

Eggshell bacterial sampling method

Of the 81 eggs that were incubated over 2019 and 2020, 62 survived to hatching (77.5%). Of the 18 eggs that underwent sonication via the water bath sonicator, only 5 survived (27.8%); thus, egg survival was statistically different among treatments (β = -4.34; *SE* =1.79; *P* = 0.015; Figure 2.1A). Of the 62 hatchlings, sonication reduced hatchling mass by 0.04 g (0.012 *SE*; P = 0.003; Figure 2.1B), SVL by 3.32 mm (1.43 *SE*; *P* = 0.028; Figure 2.1C), and TL by 4.42 mm (1.27 *SE*; *P* = 0.002) compared to control treatments. Hatchling sex was not influenced by egg

treatment ($P \geq 0.05$). Egg mass was significantly correlated with hatchling mass ($\beta = 0.45$; $SE = 0.15$; $P = 0.005$), but not with SVL ($\beta = 26.53$; $SE = 17.96$; $P = 0.15$) or TL ($\beta = 29.03$; $SE = 14.97$; $P = 0.063$).

Sonicating eggs yielded 0.63 ng/µL more DNA than swabbing eggs ($SE = 0.186$; P = 0.027). A total of 99,588 OTUs were utilized for analyses following blank sample and unassigned OTU removal. Sampling method did not influence OTU absolute abundance ($\beta = 875.50$; $SE = 850.82$; $P = 0.362$; Fig. 2.2A). Although the effect of sampling method on microbiome diversity based on Shannon's Diversity Index was not statistically supported ($\beta = -0.62$; $SE = 0.23$; $P = 0.053$; Fig. 2.2B), eggshells that were sonicated resulted in notably greater diversity than those that were swabbed. Faith's phylogenetic diversity and evenness followed similar trends ($\beta = -2.33$; $SE = 1.40$; $P = 0.172$). Bacterial taxa of samples collected by swabbing differed from those collected by sonication (ANOSIM = 0.98; $P = 0.027$), indicating that sampling methods affect characterization of community structure (Fig. 2.2C).

We identified 3 bacterial phyla (14% of observed phyla; Fig. 2.2D) and 13 bacterial genera (15% of observed genera) that significantly differed in abundances between the sonication and swabbing methods. The most abundant groups identified were *Proteobacteria, Bacteroidetes,* and *Actinobacteria*. The sonication method had higher abundances of both *Bacteroidetes* (20%) and *Firmicutes* (4%) when compared to the eggs that had been swabbed (11% *Bacteroidetes*, 1% *Firmicutes*). However, eggs that had been sonicated had a lower abundance of *Proteobacteria* (50%) than that of swabbed eggs (67% *Proteobacteria*). Additionally, 4 bacterial genera were only

identified via the swabbing method (5% of observed genera), while 7 bacterial genera were only identified via sonication (8% of observed genera).

Eggshell bacterial manipulation

Egg survival differed between treatments in 2020 (Figure 2.3A); eggs submerged in ethanol had lower survival than those submerged in 20% bleach, but this difference was non-significant ($\beta = -3.11$; $SE = 1.62$; $P = 0.055$). Overall, of the 31 eggs that survived to hatching (57.4%), there was a significant difference in hatchling size where eggs treated with 20% bleach were 0.026 g heavier ($SE = 0.009$; $P = 0.024$; Figure 2.3B) and 20% longer in SVL than controls ($\beta = 0.99$; $SE = 0.35$; $P = 0.024$; Figure 2.3C); tail length was not affected ($\beta = 2.31$ $SE = 1.17$; $P = 0.096$). Treatment did not affect hatchling sex ($P \geq 0.05$). Egg mass was not significantly correlated with hatchling mass ($\beta = 0.44$; $SE = 0.19$; $P = 0.057$), SVL ($\beta = 6.27$; $SE = 7.44$; $P = 0.43$), or TL ($\beta = -1.15$; $SE = 26.13$; $P = 0.966$).

In 2021 and 2022, egg survival did not differ among eggs that were submersed in water, 10% bleach, or tryptic soy broth (TSB) for 30 seconds ($P \geq 0.05$; Figure 2.4A). Unlike hatchlings from 2020, eggshell treatment did not affect hatchling mass or SVL ($P \geq 0.05$; Figure 2.4B and C). However, eggshell treatment with 20% bleach increased tail length by 2.07 mm ($SE = 0.93$; $P = 0.032$). Treatment did not affect hatchling sex ($P \geq 0.05$). Egg mass was significantly correlated with hatchling mass ($\beta = 0.58$; $SE = 0.12$; $P < 0.01$), but not with SVL ($\beta = 8.67$; $SE = 6.79$; $P = 0.21$) or TL ($\beta = 27.06$; $SE = 17.43$; $P = 0.129$).

Colony forming units (CFUs) differed between treatment groups (see below); however, 3 of the 17 control eggs (17.6%), 1 of the 14 eggs submerged in autoclaved water (7.1%), 1 of the 7 eggs treated with bleach (14.3%), and 4 of the 8 (50%) eggs treated with TSB had CFUs that were

too numerous to count and were thus removed from the analyses. CFUs were reduced in all treatments after the initial swab, but the magnitude of the decline differed among treatments. Samples from eggs treated with water had CFUs than control samples (β = 149.28; *SE* = 42.52; *P* = 0.004; Figure 2.5) and samples from the TSB treatment (β = 321.40; *SE* = 83.47; *P* = 0.002; Figure 2.5). CFU counts were not affected by bleach treatment (β = -40.06; *SE* = 55.87; *P* = 0.487; Figure 2.5) or the covariate (CFU count prior to eggshell treatment) in the model (β = 0.04; *SE* = 0.10; *P* = 0.685).

CFU counts did not differ among treatment groups prior to treatment (*P* > 0.05; Table 2.2). When data was subset to just CFUs following treatment and despite reduced sample sizes, the number of CFUs also increased when eggs were submerged in autoclaved water compared to unmanipulated controls following eggshell treatment (i.e., timepoint B; β = 141.04; *SE* = 40.43; *P* = 0.003; Table 2.2, Supp. Figure 2.2). Similar to the earlier model, there was a greater increase in CFU count when eggs were submerged in TSB compared to unmanipulated controls (β = 241.60; *SE* = 70.30; *P* = 0.003; Table 2.2, Supp. Figure 2.2). Bleach (20%) did not have a significant effect on CFU counts when compared to unmanipulated controls (β = -45.54; *SE* = 50.82; *P* = 0.383; Table 2.2, Supp. Figure 2.2). However, when treatment groups were compared individually between timepoint A (prior to eggshell treatment) and timepoint B (following eggshell treatment), significant effects on CFU counts were observed. Interestingly, the no manipulation controls differed between timepoints A and B where following a second swabbing of the eggshell decreased CFU counts (β = -208.70; *SE* = 48.80; *P* = $7e^{-4}$; Table 2.2, Supp. Figure 2.2). This significant decrease was also observed when eggshells were treated with 20% bleach (β = -399.32; *SE* = 54.27; *P* = $3e^{-4}$; Table 2.2, Supp. Figure 2.2). Submersion in autoclaved water did not influence CFU counts when compared between timepoints A and B (β = -83.77; *SE* = 59.38; *P* = 0.184;

Table 2.2, Supp. Figure 2.2) as well as treatment with TSB (β = -192.67; *SE* = 195.73 *P* = 0.505; Table 2.2, Supp. Figure 2.2).

Table 2.1. Egg allocation to treatments for each experiment. For the eggshell bacterial sampling experiment (2019-2020), *A. sagrei* were collected in 2019 and only eggs in treatments 1 and 2 were used for 16S rRNA gene-sequencing. In 2020, the experiment was repeated with treatment group 4 and no samples were sequenced. The total number of eggs (n) for 2019 and 2020 are provided. For the eggshell bacterial manipulation experiment (2020), *A. sagrei* were collected in 2020 and only egg survival and hatchling phenotypes (i.e., mass, SVL, TL) were evaluated. For the second bacterial manipulation study (2021-2022), *A. sagrei* were collected in 2021 and egg survival and hatchling phenotypes (i.e., mass, SVL, TL) measured. In 2022, a similar experiment was repeated, but we included a bacterial growth promoting treatment using tryptic soy broth (TSB), and we also counted bacterial Colony Forming Units (CFU) for samples in all treatments.

Treatment	Eggshell bacterial sampling (2019-2020)	Eggshell bacterial manipulation (2020)	Eggshell bacterial manipulation (2021-2022)
1	swab, then place swab in sterile 1X PBS (n= 23)	no external eggshell treatment (control; n=11)	no external eggshell treatment (control; n=16, n= 17 for CFU)
2	egg placed directly in sterile 1X PBS for sonication (n=18)	one time submersion of an egg in 5 mL of autoclaved water for 30 seconds (control; n=11)	one time submersion of an egg in 5 mL of autoclaved water for 30 seconds (control; n=13, n= 14 for CFU)
3	egg placed directly in sterile 1X PBS, not sonicated (control; n= 19)	one time submersion of egg in 5 mL of 10% bleach for 30 seconds (n=13)	one time submersion of egg in 5 mL of 20% bleach for 30 seconds (n=14, n= 7 for CFU)
4	no swabbing or sonicating (control; n= 21)	one time submersion of egg in 5 mL of 20% bleach for 30 seconds (n=8)	one time submersion of egg in 5 mL of TSB for 30 seconds (n=17, n= 8 for CFU)
5	---	one time submersion of an egg in 5 mL of 70% ethanol for 30 seconds (n=11)	---

Table 2.2. Colony forming units (CFUs) from eggshell bacterial manipulation experiment (2022). CFU differences (β) between treatment groups and no manipulation controls are reported with standard error (SE). Comparison between timepoints A (prior to eggshell treatment) and B (immediately following eggshell treatment). Timepoint A is the reference for comparison. Significant p-values are in bold face.

Treatment	Prior to eggshell treatment (β±SE, *P*)	Following eggshell treatment (β±SE, *P*)	Comparison between timepoints A and B (β±SE, *P*)
Control (no manipulation)	---	---	-208.70 ± 48.80, $\mathbf{7e^{-4}}$
Control (autoclaved water)	17.20 ± 80.91, 0.834	141.04 ± 40.43, **0.003**	-83.77 ± 59.38, 0.184
20% Bleach	141.44 ± 102.91, 0.187	-45.54 ± 50.82, 0.383	-399.32 ± 54.27, $\mathbf{3e^{-4}}$
Tryptic soy broth (TSB)	214.91 ± 102.59, 0.052	241.60 ± 70.30, **0.003**	-192.67 ± 195.73, 0.505

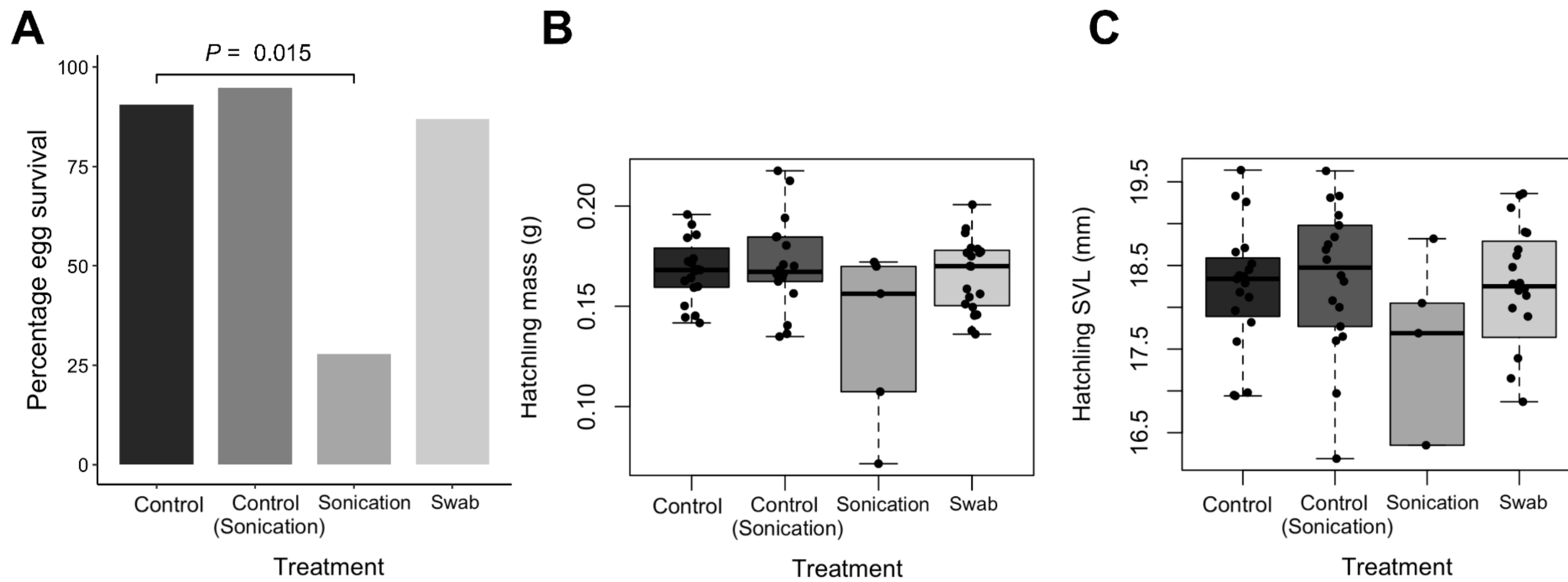


Figure 2.1. Egg survival and hatchling phenotypes across treatments in eggshell bacterial sampling (2019 – 2020) experiment. (A) Percentage egg survival among eggshell bacterial sampling methods. *P* = significant difference egg survival between control and sonication treatment group. (B) Hatching mass (g) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (C) Hatchling snout-vent length (SVL; mm) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

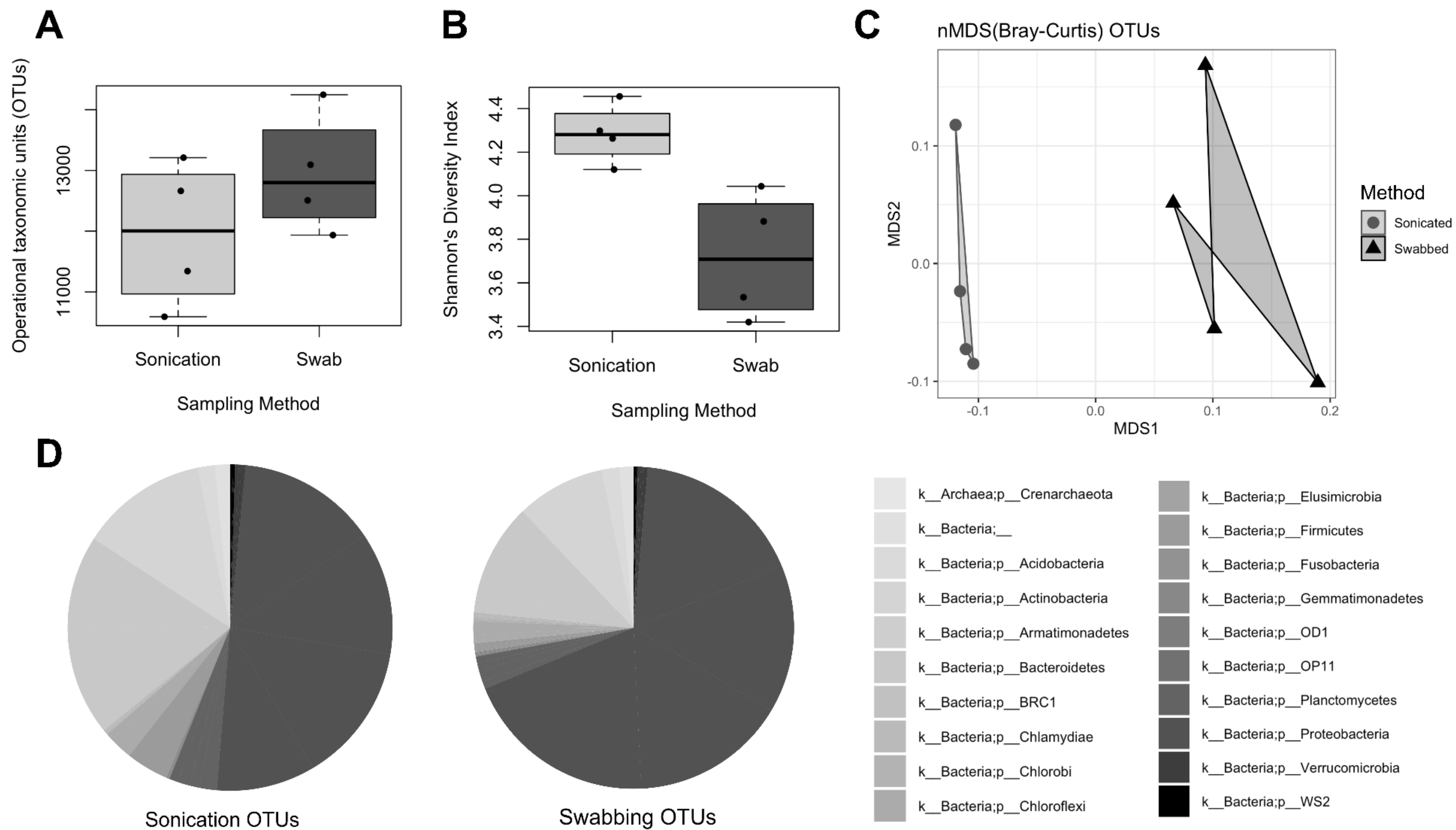


Figure 2.2. Variation in microbial communities and abundance across treatment groups in the bacterial sampling experiment (2019). (A) Differences in microbial operational taxonomic units (OTUs) among treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (B) Differences in Shannon's diversity

index among treatment groups. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (C) Non-metric multidimensional scaling (NMDS) plot of all individuals. The gray circles in the NMDS plot are for eggs in the sonicated group and the black triangles are for eggs in the swabbing group. Lines denote vector overlays on NMDS ordination, indicating the directionality and strength of change (line length) for a specific parameter. (D) Differences in bacterial relative abundances of phyla among treatment groups.

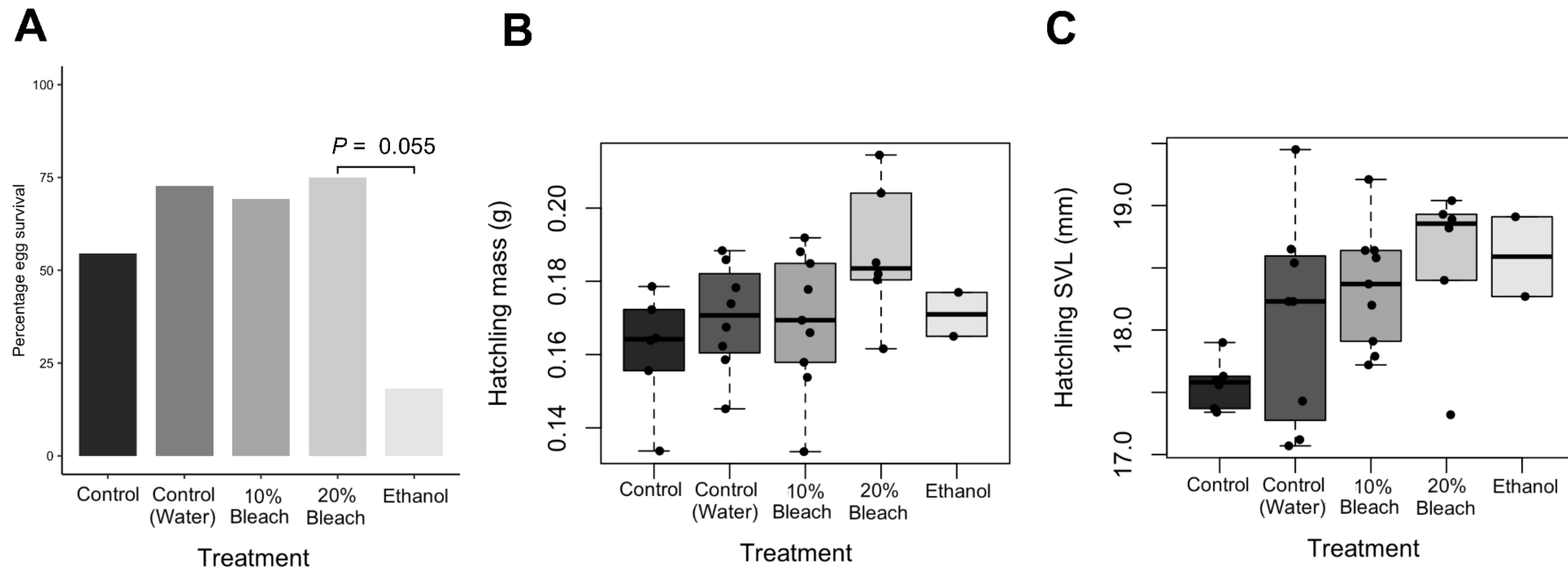


Figure 2.3. Egg survival and hatchling phenotypes across treatments in eggshell bacterial sampling (2019 – 2020) experiment. (A) Percentage egg survival among eggshell bacterial manipulation methods. *P* = significant difference egg survival between 20% bleach and ethanol treatment group. (B) Hatching mass (g) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (C) Hatchling snout-vent length (SVL; mm) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

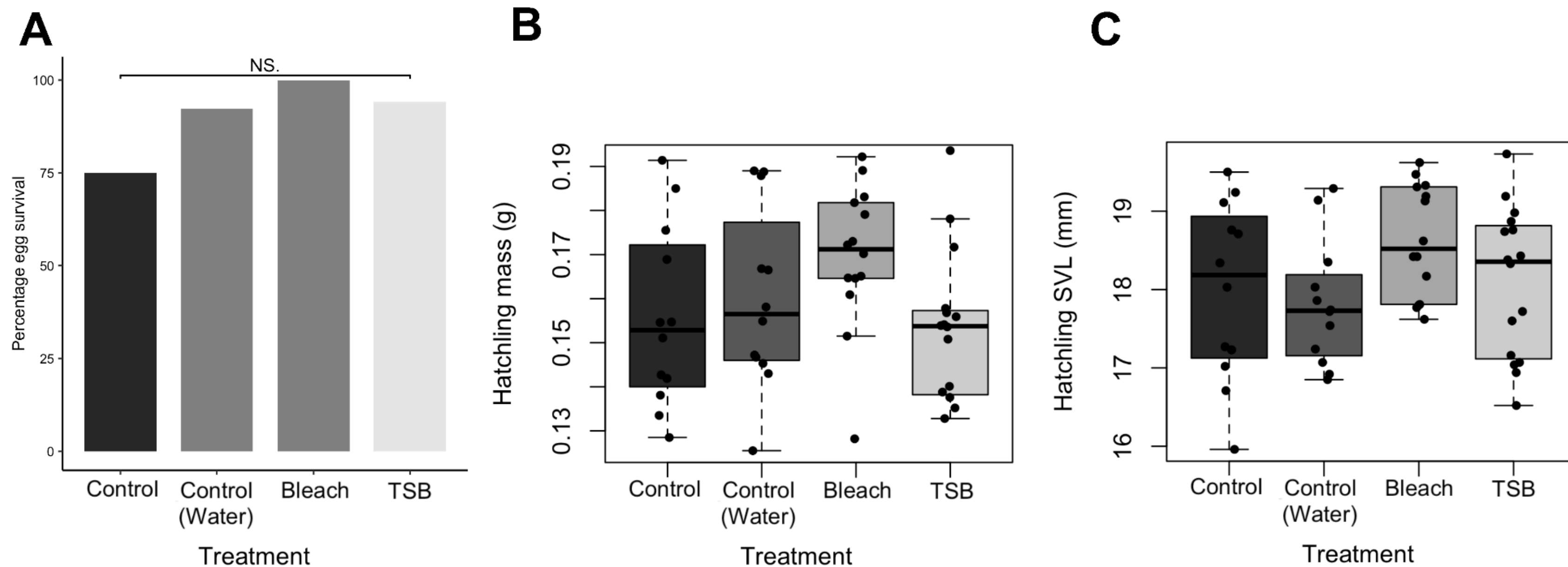


Figure 2.4. Egg survival and hatchling phenotypes across treatments in eggshell bacterial manipulation experiment (2021 – 2022) experiment. (A) Percentage egg survival among eggshell bacterial manipulation methods. NS. = not significant difference in egg survival across treatment groups. (B) Hatching mass (g) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (C) Hatchling snout-vent length (SVL; mm) across treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

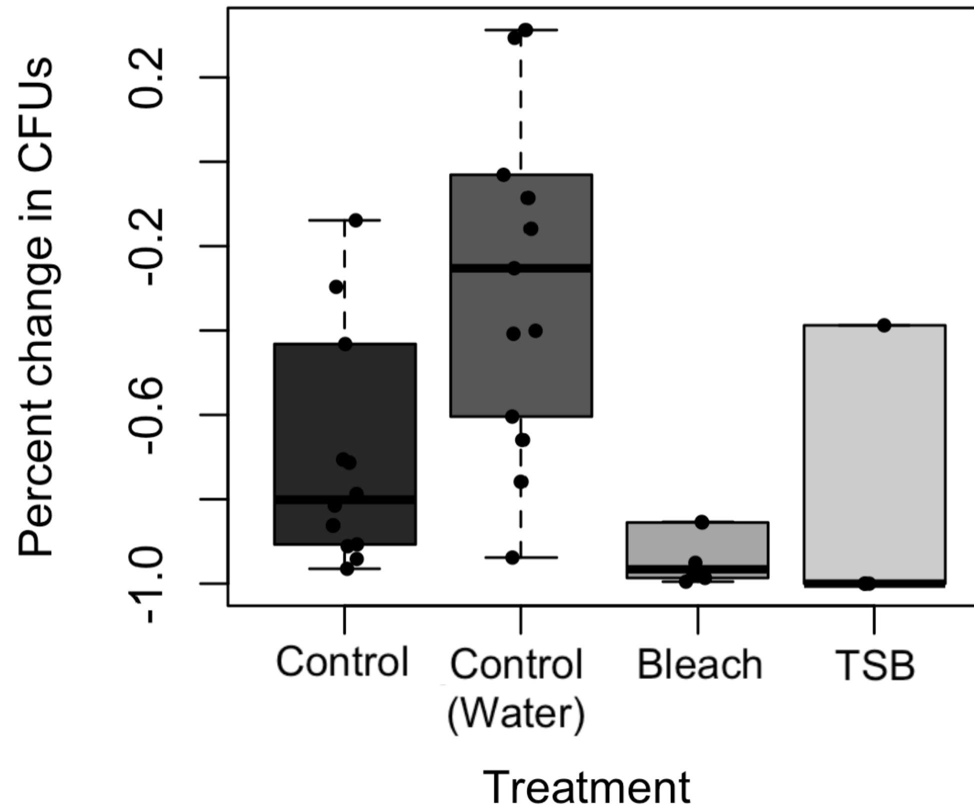
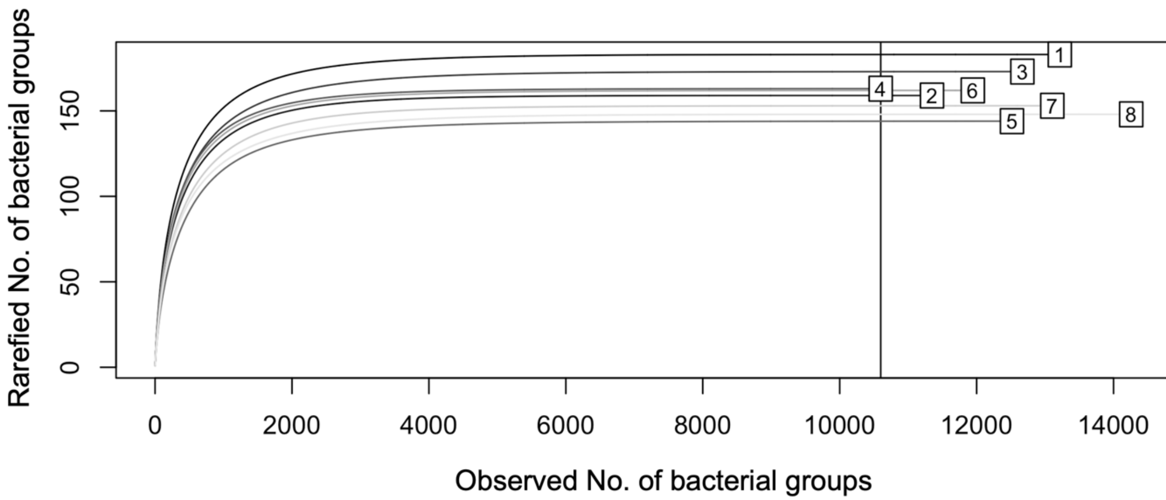


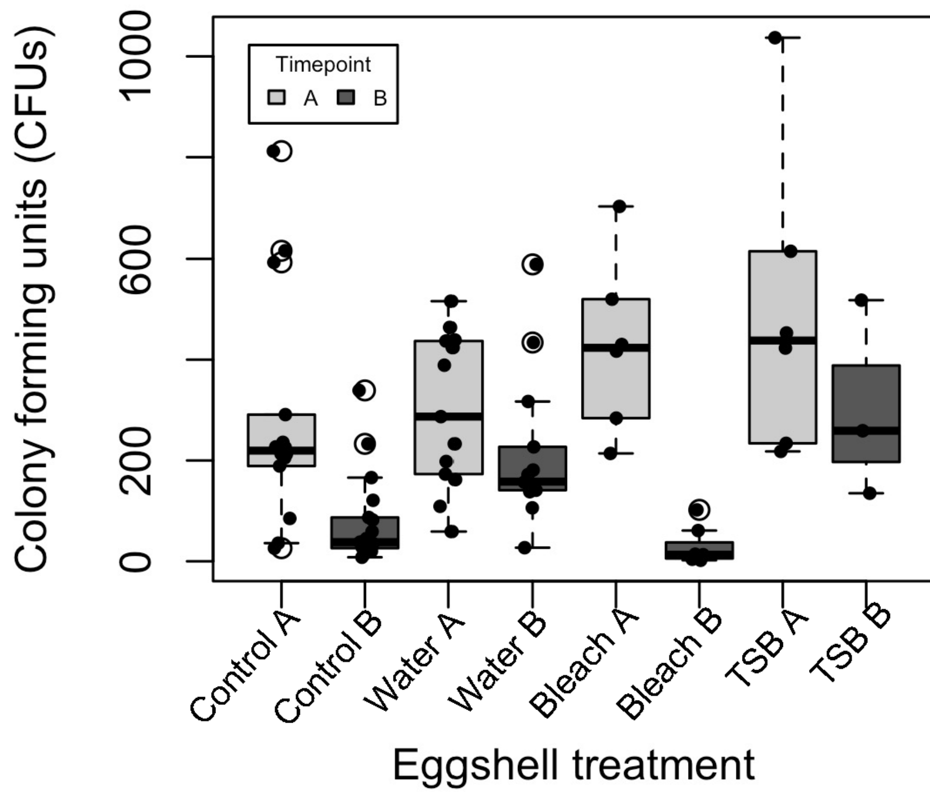
Figure 2.5. Percentage change in colony forming units (CFUs) between treatment groups in eggshell bacterial manipulation experiment (2021 – 2022) experiment. Treatment of eggshells with TSB (tryptic soy broth) has a decreased sample size because 4 of 8 (50%) eggs had bacterial colonies that were too numerous to count. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

Supplementary Table 2.1. Number of brown anole (*A. sagrei*) adults used for experiment 1 and 2 from 2019 to 2022. All adults were collected from Palm Coast, FL

Year	Experiment	# total adults	# females	# males
2019	#1 Eggshell bacterial sampling	N = 36	n = 24	n = 12
2020	#1 Eggshell bacterial sampling	N = 36	n = 24	n = 12
2020	#2 Eggshell bacterial manipulation	N = 78	n = 52	n = 26
2021	#2 Eggshell bacterial manipulation	N = 78	n = 52	n = 26
2022	#2 Eggshell bacterial manipulation	N = 75	n = 50	n = 25



Supplementary Figure 2.1. Rarefaction curve of rarified number of bacterial groups by observed number of bacterial groups. Labels are numerically associated with individual egg*s* in the dataset (i.e., samples 1-4 were sonicated, while samples 5-8 were swabbed).



Supplementary Figure 2.2. Variation in colony forming units (CFUs) across eggshell treatment groups. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents average.

Discussion

Our primary objective was to compare methods for sampling and manipulating the microbiome on eggshells of an oviparous vertebrate. We demonstrated that sonication of entire eggs resulted in higher concentrations of isolated DNA in comparison to swabbing and that bacterial beta diversity differed between sampling methods. Thus, the methods used for sampling could have important consequences on how we characterize the bacterial community on eggshells. We also showed that brief submersion of eggs in 20% bleach reduced bacterial colonies on eggshells surfaces, and TSB increased bacterial colonies. Overall, our study provides a useful guide for experimental approaches designed to examine the eggshell microbiome and its consequences.

We show that sonication significantly reduces egg survival and, of those that survived, hatchlings from sonicated eggs were smaller than those from their control counterparts. The use of entire egg sonication is a novel sampling method for documenting the microbiome of eggs of oviparous vertebrates. Interestingly, water bath sonicators are much less aggressive in comparison to probe sonicators, which should be considered if researchers want to reduce egg mortality. This method could also be employed for eggs of many sizes and shell structures as the parchment shell of *Anolis* species was not agitated during water bath sonication. However, the relatively small size of *Anolis* eggs makes them particularly robust to movement (i.e., the embryos are small). Moving or shifting reptile eggs has previously been shown to negatively affect hatching success in many other reptiles (e.g., turtles (Williamson, Evans, and Reina 2017)), and therefore our technique might have negative consequences on other species. However, eggs of most species are still robust to movement immediately after oviposition when extraembryonic membranes have not yet

attached. Therefore, we recommend sonication as early as possible and dissuade this technique with eggs with advanced embryos if researchers want to maintain high egg survival.

Entire egg sonication revealed similar representation of bacterial OTU absolute and relative abundance as well as Shannon's and Pielou's diversity indices in comparison to swabbing of eggshells. However, sonicated eggs had distinct clustering in terms of beta diversity. Sonication may discharge bacteria that are tightly adhered to the eggshell that swabbing does not contact. A caveat of our study is that the soil in which eggs were laid may have contributed to the presence of some bacterial sequences on the eggshell, which we did account for by collecting a soil sample; however, this would have been similar between our two methods because all soil was obtained from the same source (i.e., potting soil).

In the second part of this study, we sought to manipulate the microbiome on eggshells by first evaluating the efficacy of antimicrobial treatments on egg survival and secondly, attempting to promote or reduce eggshell bacterial growth. We found that the use of ethanol on eggshell surfaces for *A. sagrei* induces very high egg mortality, whereas the use of bleach of different concentrations does not. The manipulation of eggshell microbiota has allowed for the exploration of microbiota-mediated effects on hatchling phenotypes, whereby reducing or promoting bacterial growth has been shown to influence offspring morphology (Jacob et al. 2015). Our techniques used to manipulate eggshell bacteria showed that submersion in TSB significantly increases bacterial load on eggshells, while submersion in water also increases CFU counts (but to a lesser extent than TSB). CFUs declined from before to after treatment, but the magnitude of that decline was less in the TSB treatment comparted to control. This might suggest that TSB promoted more growth of bacteria compared to controls. Additionally, we saw that swabbing eggshells multiple times decreases bacterial load which might support studies that seek to manipulate the eggshell

microbiome through rubbing with cloth or wipes. This was apparent by the overall decrease in CFUs regardless of treatment on the second plate (i.e., timepoint B). Effects on offspring phenotypes through the alteration of eggshell microbiomes by microbial sampling is unknown but may be considered in future studies.

Hatchlings from eggs treated with bleach were heavier and longer than hatchlings from other treatments in 2020 and a similar trend was observed in the 2021-2022 experiment. Because eggs were treated with this broad-spectrum antimicrobial and immediately incubated in moist vermiculite (an industrially-produced substrate), hatchlings exposed to less bacteria throughout development may redirect nutritional resources within the egg to growth rather than immune function. Indeed, negative correlations in growth and immunity in *A. sagrei* has been demonstrated (Cox et al. 2009). In avian reptiles, a negative relationship was observed between investment in reproduction and oxidative damage based on exposure to antimicrobials (Jacob et al. 2015).

Oviparous reptiles serve as excellent models for studying the influence of environmental variation on the microbiome, and whether these microorganisms influence phenotypic traits of offspring. As in other oviparous species, embryos are subject to a broad range of environmental conditions that mediate development and growth (Noble, Stenhouse, and Schwanz 2018; D. C. Deeming 2004; Dennis Charles Deeming, Deeming, and Ferguson 1991). These early-life incubation environments can have lasting impacts on offspring traits that have consequences on adult survival and reproduction (Mitchell, Hall, and Warner 2018). The use of sampling methods that accurately depict eggshell microbiomes without influencing egg survival is an important compromise that researchers must consider, and decisions on which methods to use will depend on research goals. For example, sonication might provide a more accurate characterization of the microbiome but it may negatively affect egg survival and offspring size. While our study model,

Anolis sagrei, is widely used in egg incubation studies because of their ease of captivity and high fecundity (Sanger et al. 2008), other species with larger eggs might respond differently to the ultrasonic waves produced during sonication. Additionally, studies may consider attempting to both positively and negatively alter bacterial load on eggshells through our methods described above. Future studies could explore functional diversity in microbiome community structures on the eggshell, embryos, and early-life hatchlings that may explain differences we observed in hatchling phenotypes.

Development of methods for collecting and manipulating microbiomes will be crucial for future research as microbiome science is incorporated into the fields of evolutionary and conservation biology. Our methods will provide useful guidelines for better understanding the role of bacteria on reptilian development and physiology, with a particular emphasis on studies exploring maternal transfer of microbes. Although many microbiome studies focus on viviparous taxa, the majority of animal species are oviparous (Blackbum, 1999) and are poorly studied in terms of microbial vertical transmission (Murphy et al. 2023). The application of bacterial sampling and manipulation to explore consequences on offspring fitness in oviparous organisms is thus essential to furthering our understanding of the origin and development of microbiomes.

Chapter 3

Maternal antibiotic exposure decreases gut microbiota diversity and alters hatchling size in an oviparous lizard

Manuscript in-preparation for submission to *Physiological and Biochemical Zoology*

Introduction

Microbiota associated with maternal sources (i.e., breast milk, birth canal) are known to shape neonatal phenotypes in humans, particularly during gestation, birth, and the first years of life (Nyangahu and Jaspan 2019; Macpherson, de Agüero, and Ganal-Vonarburg 2017). The microbiome is also vertically transmitted between mother and offspring in other viviparous species (Kohl et al. 2017), implying that some of this variation is maternally-derived. In oviparous vertebrates, microbiota associated with eggshells may develop from various maternal sources (Martínez-García et al. 2016) as well as the nest environment (van Veelen, Salles, and Tieleman 2018). However, little is known regarding how maternally associated microbiota might also influence offspring phenotypes in oviparous taxa.

In a study using a polyphagous fruit fly (*Bactrocera tryoni*), authors used axenic parents to document offspring developmental traits, body composition, and fecundity (Nguyen et al. 2020). Offspring from parents with reduced microbiota had lower percent egg survival and body weight, suggesting that transgenerational effects of commensal microbiota may influence offspring life-history traits (Nguyen et al. 2020). In vertebrates, antibiotics have been used to document the effects of microbiome changes in many taxa (Kokou et al. 2020; Carlson et al. 2015; Klowden and Greenberg 1977). Indeed, manipulation of the nest environment with broad-spectrum

antimicrobials has shown reduced nestling mass and tarsus length (Jacob et al. 2015). Measurements of other hatchling variables, however, may provide insight into embryonic development. For example, embryonic heart rate is often measured to understand the immediate effects of the nest or developmental environment on embryo physiology (Hulbert et al. 2017). Higher heart rates are often considered measurements of stress (Martínez Silvestre 2014), and the microbiome has been linked to modulating and regulating stress in mammals (Rea, Dinan, and Cryan 2016). Additionally, in oviparous taxa, the cloaca is likely an important site for bacterial transmission because both the reproductive and digestive tracts empty into this cavity. Thus, the cloaca may be inoculated with bacteria from maternal food items, which in turn, can influence the eggshell microbiome as the egg passes through the cloaca at oviposition. To examine the influence of maternal diet on the eggshell microbiome, and potentially on offspring phenotypes, we exposed prey items (standard house crickets; *Acheta domesticus*) of captive Cuban brown anoles (*Anolis sagrei*) to a cocktail of antibiotics and fed them to reproducing female anoles. We quantified the microbiota associated with female gut and cloacae in an experimental treatment and control.

The aim of this study was to evaluate the influence of antibiotics on both the prey items and female microbiomes of *A. sagrei*. Based on previous studies of birds (Jacob et al. 2015; Soler et al. 2008), we hypothesized that antibiotic treatment would influence the maternal microbiota over time and that the phenotypes of offspring from antibiotic-exposed mothers would vary compared to those from control mothers. We predicted that: 1) prey items and mothers given a diet exposed to antibiotics would have reduced gut microflora abundance and diversity, 2) this reduction in microbiota diversity would be similar in both the intestinal tract and cloaca of *A. sagrei* mothers, 3) deviation between microbiomes in control and antibiotic-exposed *A. sagrei*

mothers would increase over time, and 4) hatchlings from antibiotic-exposed mothers would be larger than those from control counterparts.

Methods

Animal collection and housing

Seventy-eight *A. sagrei* (n=52 females/26 males) were collected from Palm Coast, FL USA in March 2020 and transported to Auburn University, Auburn, AL, USA. Cages were equipped with cage carpet (ZooMed, Inc) on the floors, bamboo perches, and artificial foliage. Each cage also contained a nesting pot (4-inch diameter, 4-inch-tall) filled with clean, but not autoclaved vermiculite. Cages were illuminated overhead with Reptisun 5.0 UVB bulbs (ZooMed Inc.) and kept on a 12:12 light-dark cycle. Individual cages were cleaned once per week, which involves removing lizards from the cage, and scrubbing down cage walls and perches with a sponge and hot water. At each egg check (see below), vermiculite was changed to maintain as much sanitation as possible. Each cage contained 1 male and 2 females, and these individuals were never moved among cages to prevent cross-contamination.

Experimental design

All adult *A. sagrei* were randomly assigned into one of two treatment groups: those fed crickets gut-loaded with antibiotics (n=26 females) and those with no antibiotic exposure (control; n=26 females). Crickets (standard house cricket: *Acheta domesticus*) were obtained from Premium Crickets, Winder, GA. When they arrived in Auburn, AL at a size of 0.006 to 0.01 cm in length, half of the crickets (N=500) were fed a cocktail of antibiotics (erythromycin, tetracycline, and rifampicin, and of the fungicide sorbic acid; Sigma Aldrich) mixed with a standard cricket diet for

the lab (dried cat food; Purina Cat Complete Chow) for 10 days (Schmid, Lehman, and Lundgren 2014). The standard house cricket (*Acheta domesticus*) is commonly used as a feeder invertebrate for many domestic reptile species (Finke 2003). Additionally, the gastrointestinal tract morphology and digestive processes in *A. domesticus* are similar to that of the cockroach (Cornwell 1968; Ulrich, Buthala, and Klug 1981). Previous use of antibiotics in altering gut microflora has been documented in *Gryllus pennsylvanicus*, an omnivorous field cricket (Schmid, Lehman, and Lundgren 2014). Crickets in the control treatment were only fed the standard diet for the lab and clean water. All other aspects of cricket were similar between the two treatments. For example, all crickets were housed in plastic bins with 5-6 cardboard egg cartons, and the quantity of food and water remained the same. The lifespan of a house cricket is 8-10 weeks; however, to minimize potential effects of time exposed to antibiotics on cricket gut microflora, all crickets were fed to anoles following 10 days after crickets arrived at the lab. Crickets were routinely ordered, and this procedure occurred continuously throughout the experiment to ensure that crickets had about 10 days of exposure to antibiotics before being fed to lizards. Hindguts of crickets from each treatment (N=18; see Table 3.1) were also sampled for 16S rRNA gene-sequencing prior to antibiotic exposure and at the time fed to lizards. Cricket hindguts were obtained surgically under a dissecting microscope using all sterile dissection equipment (e.g., scissors, forceps; Domingo et al. 1998).

All lizards were fed twice a week for a period of 60 days so that lizards in each treatment were maintained on their respective diets over the duration of the study. This approximately eight-week time period has previously been shown in another lizard to be sufficient time for gut transition of bacteria between diets (Kohl et al. 2017). Gut tissues (small and large intestine) were aseptically collected from females (N= 24; n=4/treatment/time point) prior to antibiotic treatment (Day 0), midway through treatment (Day 30), and at the end of treatment (Day 60). Gut samples

were collected via homogenizing gut tissue from the stomach to the cloaca. Cloacal samples (N= 8; n=4/treatment/time point) were also collected from females by gently inserting a swab (Puritan flocked swab) into the cloaca and rotating it for 20 seconds. Cloacal swabs were immediately placed in a sterile 1.5 mL Eppendorf tube and kept at -80°C until DNA extraction. Cloacal swabs collected from females were from the same individuals as those dissected for gut samples. See Table 3.1 for a summary of total samples from each surface or tissue type.

Nesting pots were checked for eggs three times per week by emptying pots into individual, aseptic plastic bins that were separated by treatment using sterile gloves. Soil was searched for eggs by gently rocking soil back and forth in the plastic container. Following each egg check, individual nest pots were replaced with clean vermiculite and hydrated with DI water. The plastic bins were sprayed with 70% ethanol and wiped dry in between subsequent egg checks for different cages. Eggs were weighed and placed in petri dishes layered with moist (-150 pKa) vermiculite at a constant 28 °C.

Low egg production in 2020, greatly limited the sample sizes for measuring treatment effects on eggs and offspring, and therefore the experiment was repeated in March 2021. Adult lizards were collected in March 2021 and housed under the same procedures described above. We followed the same protocols as above throughout the treatment period during this second study. Egg production improved in 2021 compared to the previous year, and therefore offspring phenotypic measurements were from the study in 2021, whereas microbiome samples from adults were collected during both years. Additionally, microbiome samples were collected from mothers at the beginning (Day 0) and end of the study (Day 60); whereas in 2020, samples were collected at the middle (Day 30) and end of the study (Day 60).

At approximately halfway through development in 2021 (2 weeks following oviposition), embryonic heart rate was measured (n=20/treatment) through a non-invasive Buddy egg monitoring system (https://www.avitronics.co.uk/; (Du et al. 2009) This was done by removing eggs from the incubator and placing them into the Buddy system in a separate constant 28 °C incubator (Hall and Warner 2018); heart rate was measured within 20 – 30 s of being placed on the monitor. Removing the egg from the incubator to measure heart rate with the Buddy system does not influence developmental rate or survival of anole embryos (Hall and Warner 2018; Hulbert et al. 2017). If an egg needed to be repositioned to measure heart rate, the number of repositions were recorded and included as a covariate in further analyses. Because temperature within the incubator may have been influenced with the opening and closing of the incubator door, a digital temperature probe was placed inside in the Buddy system to record temperature. Each time heart rate was measured, temperature via this probe was recorded as well. For further details on this design see (Hall and Warner 2018). Eggs were checked daily for hatchlings and when a hatchling was found, snout-vent length (SVL), tail length (TL), mass, and sex were recorded.

Microbial inventories and statistical analysis

All cricket, gut, and cloacae microbiome samples (N=119) had DNA extracted (*Quick*-DNA Fecal/Soil Microbe Miniprep Kit; Zymo Research) and were sent in July 2021 and March 2022 to the Integrated Microbiome Resource (Dalhousie University, Nova Scotia, Canada) and amplified at the V6-V8 regions. All sequences were demultiplexed, which resulted in 222 base-pair reads, and imported into QIIME2 version 2020.2 (Caporaso et al. 2010; Bolyen et al. 2019) where they were denoised and chimeras removed using DADA2 (Callahan et al. 2016). *Sklearn* was used to filter and classify feature tables into operational taxonomic units (OTUs) on a pre-

trained 99 OTU GreenGenes v 13.8 database (DeSantis et al. 2006). Feature tables were filtered and classified into operational taxonomic units (OTUs) using a minimum sequence identity of 97% (He et al. 2015). Any OTUs identified in the blank sample were considered contaminants and thus removed from the following analyses (Salter et al. 2014). Sequences were annotated and rarefaction curves calculated, elucidating sample coverage. Datasets were used to calculate rarefaction curves which revealed high to moderate sample coverage (Supplementary Fig. 3.1 and Supplementary Fig. 3.2). All 16S rRNA sequences will be deposited in the Sequence Read Archive (SRA).

We calculated several measurements of alpha diversity: the Shannon index, Pielou's evenness index, and absolute abundance of OTUs. R Studio software (Version 1.0.153; R Development Core Team 2018) was used to compare the alpha diversity measurements using a linear mixed-effects model with alpha diversity as a dependent variable and treatment, time, and sampling region (i.e., gut vs cloaca) as independent variables. Year was also included as an independent variable in this linear mixed effects model. Because several cloaca and gut samples came from the same females, maternal ID was included as a random effect. Beta diversity was compared among treatment groups using a non-metric multi-dimensional scaling analysis (NMDS) to calculate the level of similarity between bacterial community composition using the Bray-Curtis dissimilarity index in the R *vegan* package (Oksanen et al. 2007). Bacterial community compositions (Bray-Curtis distance metric) was analyzed using an analysis of similarity (ANOSIM, permutations=999).

Egg hatching success was analyzed with generalized linear mixed models with a binomial error distribution and treatment and egg mass as independent variables. Linear mixed models were

used to quantify the effects of maternal antibiotic-exposure on embryonic variables (e.g., incubation length, heart rate) and hatchling morphology (e.g., mass, SVL, TL) using egg mass as a covariate. Cage number was used as a random factor in all models. All scripts will be uploaded to GitHub.

Results

Microbiota associated with prey items and maternal lizards

A total of 10 hindguts from antibiotic-treated crickets and 8 hindguts from controls (N= 18) were received as 36 demultiplexed, base-pair reads totaling 11,735 OTUs. Of these, 9,777 were only classified as Kingdom Bacteria and the remaining 1,958 were unassigned (Supplementary Table 3.1). Interestingly, species richness did not differ between treatment and controls (β = -138.25; *SE* =122.88; *P* = 0.277; Figure 3.1A). Similarly, we found no difference between treatment and control groups in terms of Shannon's diversity index (β = -0.92; *SE* =0.56; *P* = 0.117; Figure 3.1B) and through Bray-Curtis analysis of variance (*R* = -0.057; *P* = 0.794; Figure 3.1C).

A total of 57 maternal gut samples (n = 11 from 2020, n = 46 from 2021) and 44 cloacal swabs (n = 10 from 2020, n = 34 from 2021) were received as 202 demultiplexed, base-pair reads totaling 264,389 OTUs. These OTUs span across 16 different bacterial phyla and 168 bacterial genera. The most predominant phyla for both maternal gut and cloacal samples were Bacteroidota (54% of total OTUs) followed by Firmicutes (36% of total OTUs). The most predominant genera were *Bacteroides* (42% of total OTUs) followed by *Clostridium sensu stricto* 1 (6% of total OTUs). Antibiotic-exposed mothers had 438 fewer OTUs than controls (Figure 3.2A), but this difference was not statistically significant (*SE* = 730.9; *P* = 0.554). However, in terms of Shannon's diversity index, antibiotic-exposed mothers had less bacterial diversity than control mothers (β =

0.96; *SE* = 0.25; *P* = 0.0005; Figure 3.2B). Treatments did not differ between years (i.e., 2020 vs 2021), across time periods (i.e., Day 0, 30, 60), and between regions (i.e., gut or cloacae) using Bray Curtis analysis of similarity (*R* = 0.007; *P* = 0.223; Figure 3.3).

Gut vs. cloacae microbiota

Samples collected from female intestinal tracts had 3,770 more OTUs compared to those collected from cloacae (*SE* = 739.7; *P* < 0.001; Figure 3.2A). Additionally, gut samples revealed higher diversity values than those collected from female cloacae (β = 1.28; *SE* = 0.25; *P* < 0.01; Figure 3.2B). There was a significant difference in terms of Bray Curtis analysis of similarity where cloacal and gut samples clustered separately (*R* = 0.08; *P* = 0.003; Figure 3.3).

Changes in microbiota over time

Absolute abundance of OTUs did not vary over time across sampling regions in females (i.e., gut and cloacae); females sampled on day 30 (β = -989; *SE* = 1,844; *P* = 0.594) and on day 60 (β = -244; *SE* = 843.3; *P* = 0.774) did not differ from those on Day 0 in terms of OTU absolute abundance. However, females sampled on day 60 had a reduced Shannon's diversity index compared to those from day 0 (β = -0.55; *SE* = 0.29; *P* = 0.066), but this was not true for samples collected midway through the experiment at Day 30 (β = -0.45; *SE* = 0.63; *P* = 0.479). Lastly, Bray Curtis analysis of similarity showed significant clustering by timepoint (*R* = 0.24; *P* = $1e^{-4}$; Supplementary Figure 3.3).

Year (i.e., 2020 or 2021) did influence OTU absolute abundance, where females from 2021 had 2,560 more OTUs than those from 2020 (*SE* = 1,322; *P* = 0.057). Additionally, females from 2021 had a higher Shannon's diversity index compared to those from 2020 (β = 1.37; *SE* = 0.45;

P = 0.004). There was a significant difference in clustering by year in terms of Bray Curtis analysis of similarity (R = 0.430; P = 1e^{-4}; Supplementary Figure 3.4).

Treatment effects on phenotypes

Egg production varied among individual females over time (β = 0.12; SE = 0.03; P = 0.0004), and control females produced 2 fewer eggs than antibiotic-exposed females over the duration of the experiment (β = -1.63; SE = 0.60; P = 0.007; Figure 3.4). Female *A. sagrei* from the control group produced slightly smaller eggs than those from exposed to antibiotic-fed crickets (β = -0.01; SE = 0.006; P = 0.054; Figure 3.5A). Embryonic heart rate did not differ between eggs produced from control or treatment females (β = -0.31; SE = 2.21; P = 0.888; Figure 3.5B) and was not affected by egg mass (β = 16.30; SE = 46.20; P = 0.725). However, embryonic heart rate was positively correlated with temperature (β = 4.88; SE = 2.00; P = 0.016). Incubation duration of eggs was not influenced by maternal antibiotic exposure (β = 0.32; SE = 0.23; P = 0.164; Supplementary Figure 3.5) nor by egg mass (β = -3.11; SE = 5.21; P = 0.552). Egg survival did not differ between control or treatment females (β = -0.19; SE = 1.47; P = 0.90); however, larger eggs were more likely hatch than smaller eggs (β = 36.60; SE = 17.50; P = 0.037).

Hatchlings from eggs produced by treatment females did not differ in mass from those produced by control females (β = 0.003; SE = 0.003; P = 0.279; Figure 3.6A); however, hatchlings from control mothers were 0.25 mm shorter in SVL than those produced from treatment mothers (β = -0.25; SE = 0.10; P = 0.017; Figure 3.6B). Neither hatchling tail length (β = 0.18; SE = 0.37; P = 0.633; Figure 3.6C) nor sex was influenced by maternal antibiotic exposure (β = -0.10; SE = 0.30; P = 0.735; Supplementary Figure 3.5).

Table 3.1. Summary of sample sizes and methods for collecting bacterial samples from different tissues and time periods in this experiment. All gut and cloacal samples were collected from female *A. sagrei*. Sample sizes are reported for treatment females (T) given antibiotic-fed crickets and for females given control crickets (C). Total number of samples is N = 119 sent for 16S rRNA gene-sequencing.

Time	# Samples in 2020	# Samples in 2021	Sampling Method
Day 0 (start of experiment)			
Cricket	18 total (T=10, C=8)	---	Whole tissue dissection (hindgut)
Gut	---	15 total (T=7, C=8)	Whole tissue dissection (small and large intestine)
Cloaca	---	15 total (T=8, C=7)	Swab inside the cloaca
Day 30 (halfway)			
Gut	7 total (T=4, C=3)	---	Whole tissue dissection (small and large intestine)
Cloaca	4 total (T=2, C=2)	---	Swab inside the cloaca
Day 60 (end of experiment)			
Gut	4 total (T=2, C=2)	31 total (T=15, C=1)	Whole tissue dissection (small and large intestine)
Cloaca	6 total (T=4, C=2)	19 total (T=7, C=12)	Swab inside the cloaca

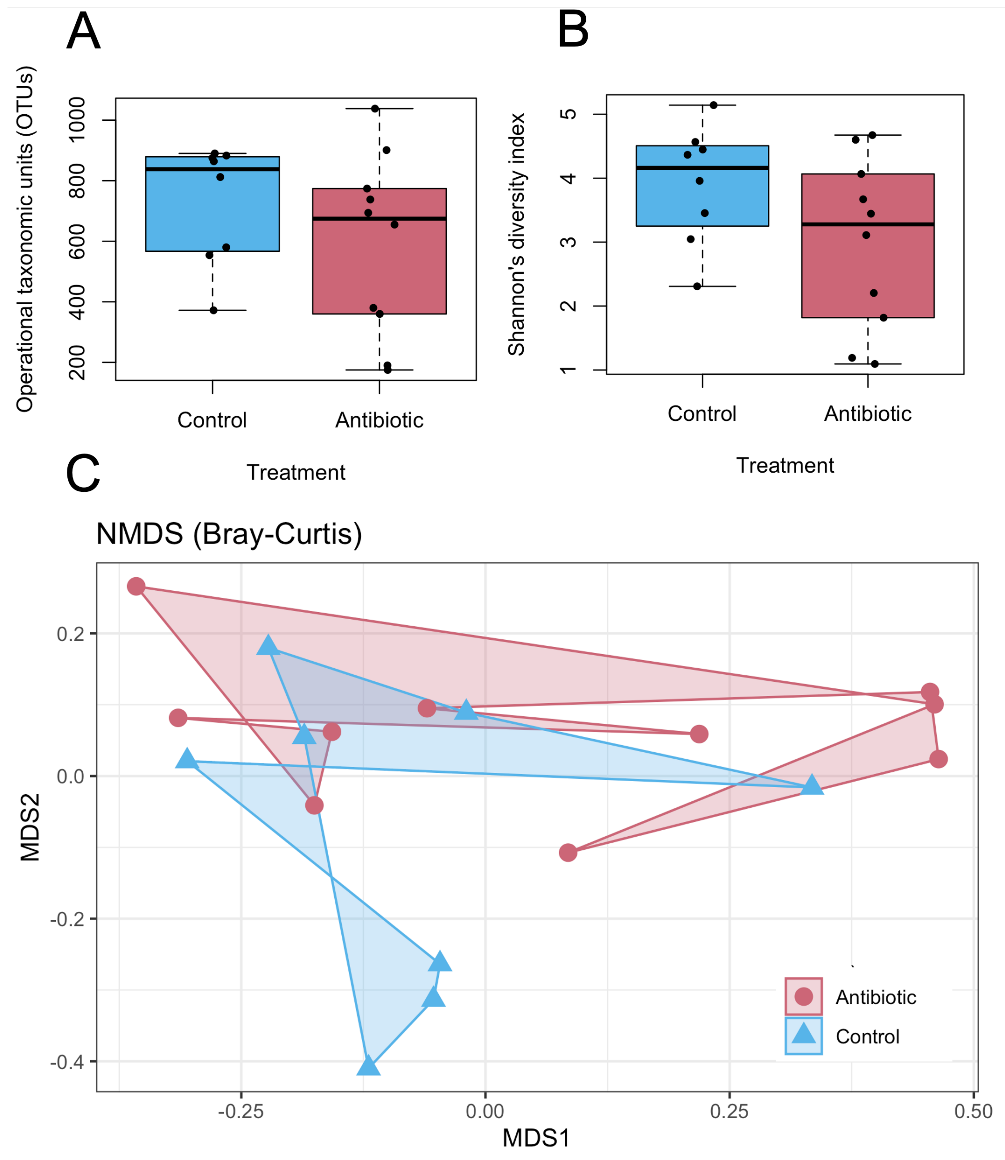


Figure 3.1. Variation in microbial communities and abundance in cricket hindguts across control and antibiotic-treated groups. (A) Differences in microbial operational taxonomic units (OTUs) among treatments. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (B) Differences in Shannon's diversity index among treatment groups. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (C) Non-metric multidimensional scaling (NMDS) plot of all individuals. The red circles in the NMDS

plot are for crickets in the antibiotic group and the blue triangles are for crickets in the control group. Lines denote overlays on NMDS ordination, indicating the directionality and strength of change (line length) for a specific parameter.

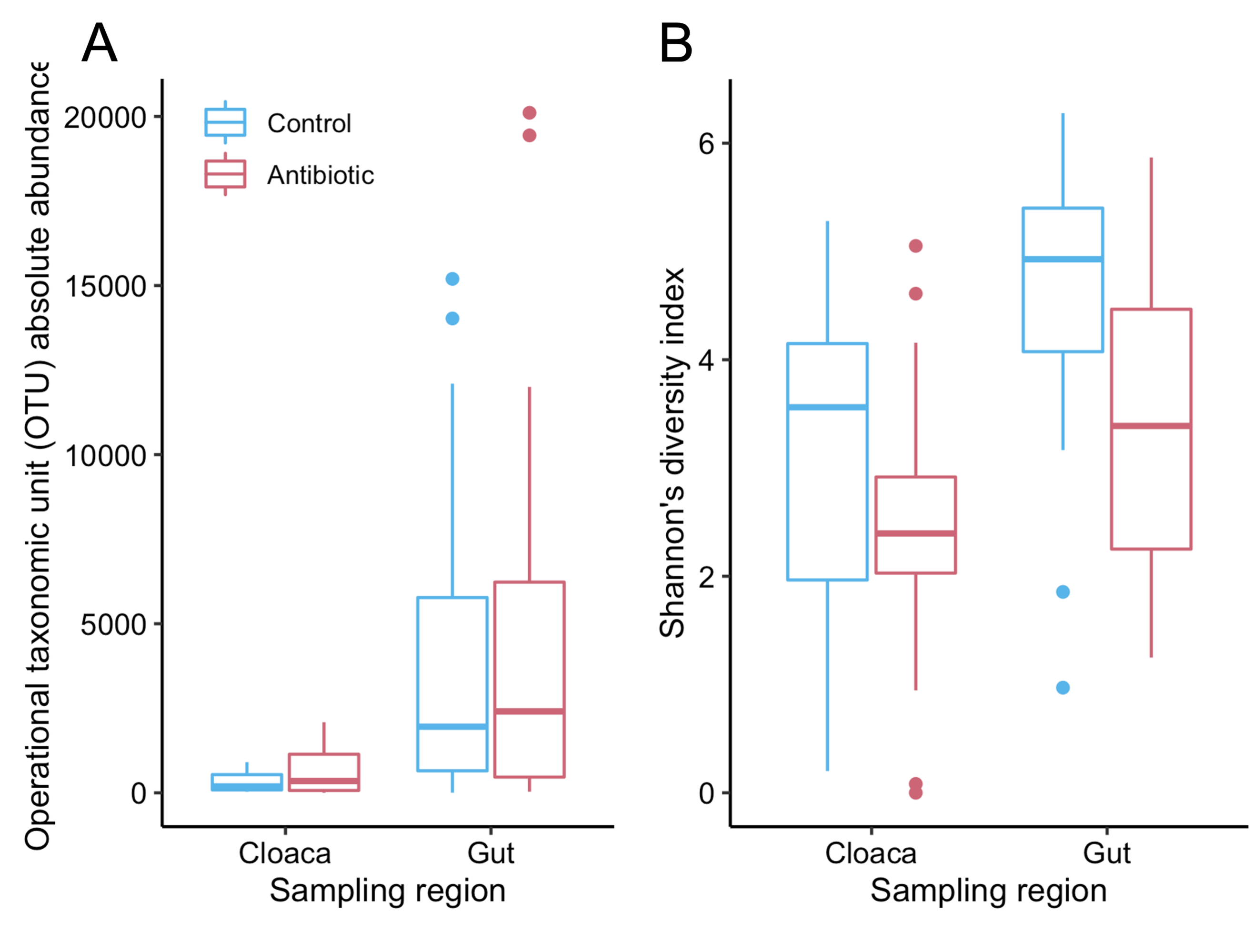


Figure 3.2. Variation in microbial communities and abundance in female *A. sagrei* across control and antibiotic-treated groups. (A) Differences in microbial operational taxonomic units (OTUs) among treatments and by sampling region (i.e., cloaca vs. gut). The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the

average. (B) Differences in Shannon's diversity index scores among treatments and by sampling region (i.e., cloaca vs. gut). The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

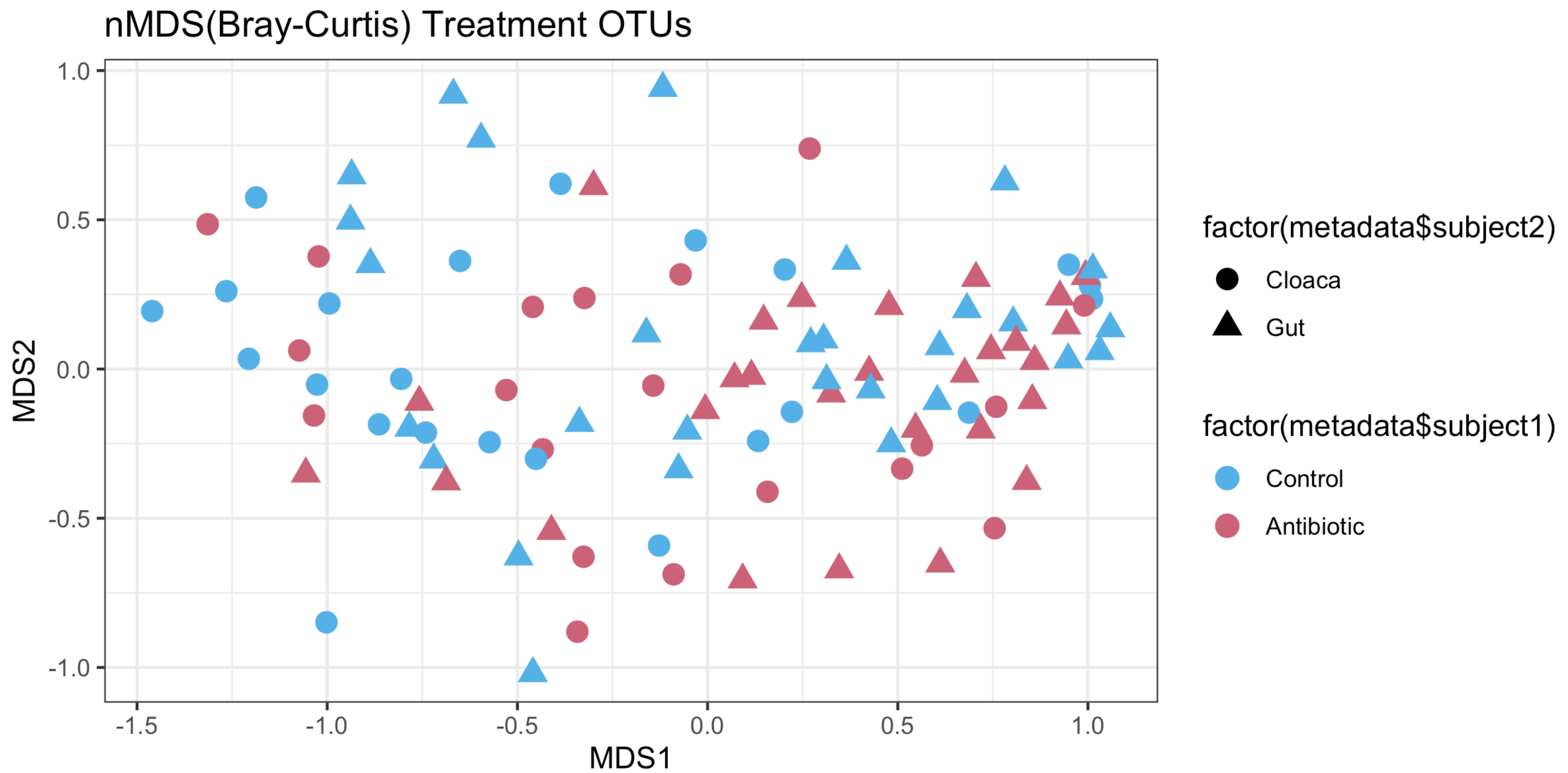


Figure 3.3. Variation in microbial community structure between female *A. sagrei* in control and antibiotic-exposed groups. Non-metric multidimensional scaling (NMDS) plot of all individuals. The red shapes in the NMDS plot are for individuals from the antibiotic group and the blue shapes are for individuals from the control group. The circles represent samples collected from female cloacas, while the triangles represent gut tissue collection.

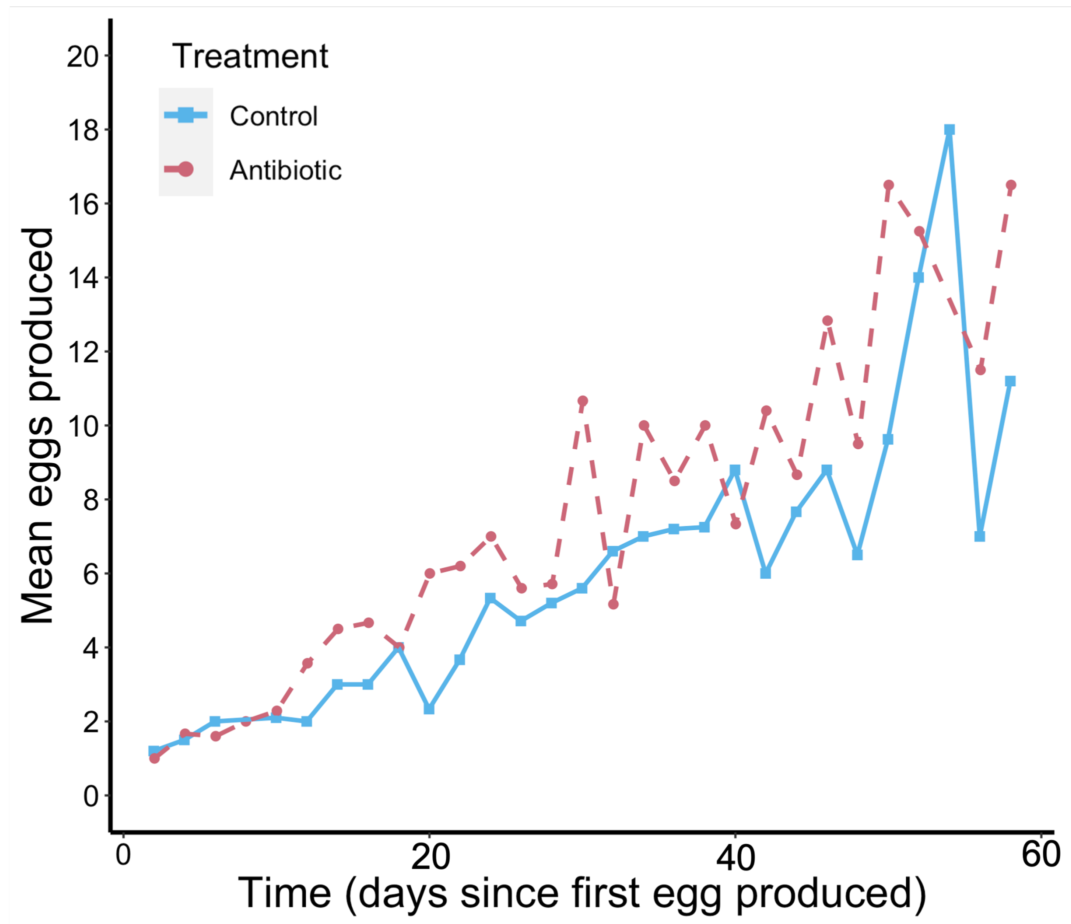


Figure 3.4. Mean eggs produced over time (days since first egg produced by female). Dots on the graph represent the average number of eggs produced every 3 days over a 60-day span from 2021.

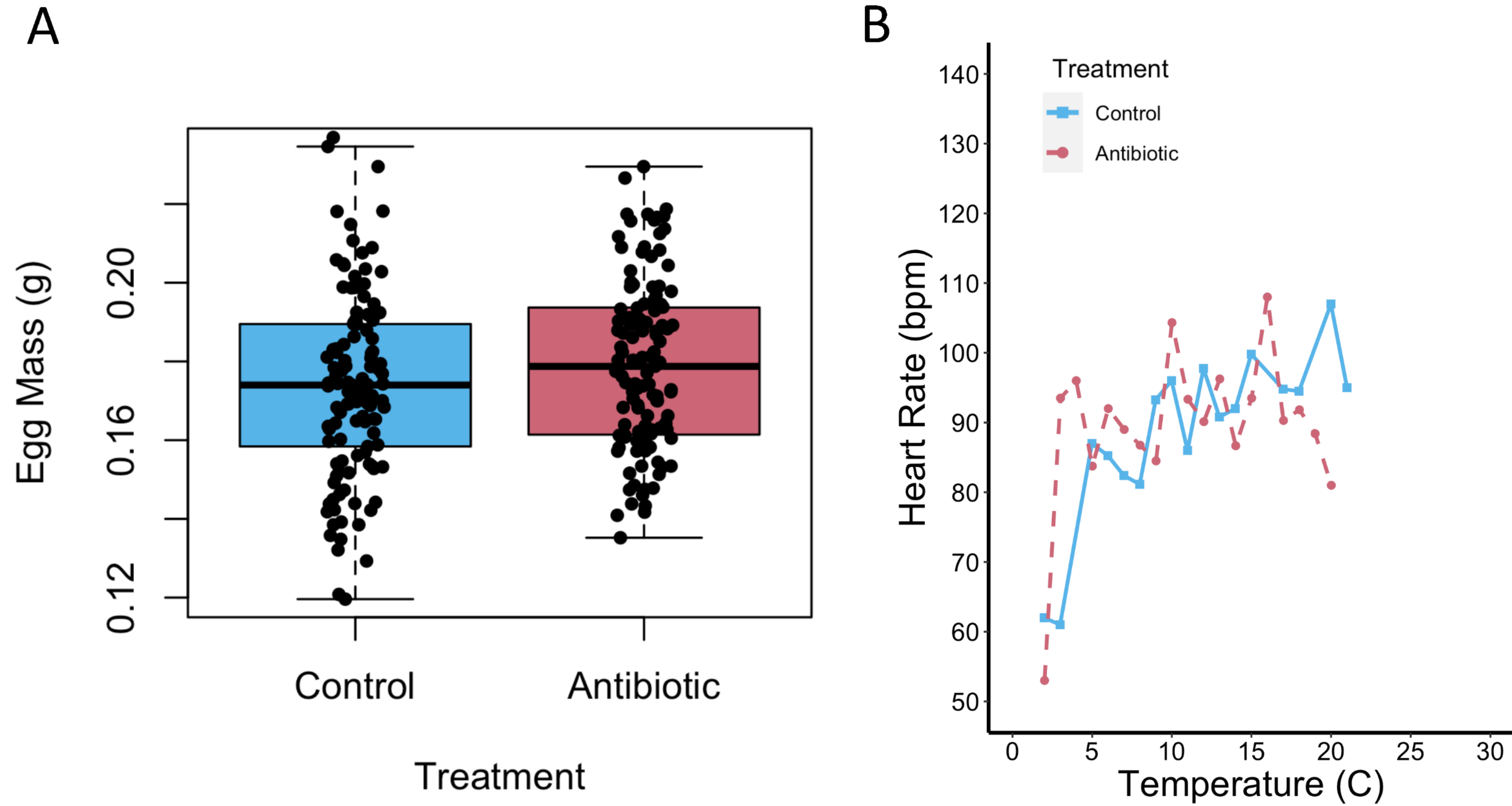


Figure 3.5. Variation in embryonic phenotypes from control and antibiotic-exposed mothers. (A) Differences in egg mass (g) from eggs produced from control and antibiotic-exposed mothers. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. (B) Average heart rate (bpm) of embryos across temperature measured inside the heart rate monitor (Buddy System) from control and antibiotic-exposed mothers.

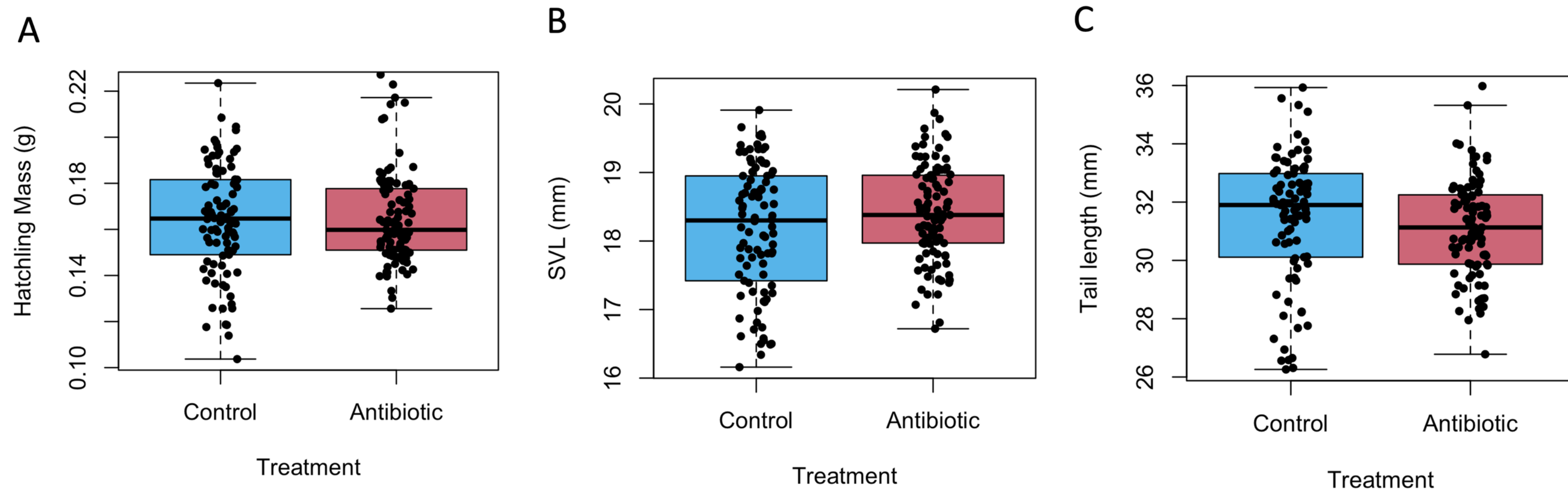
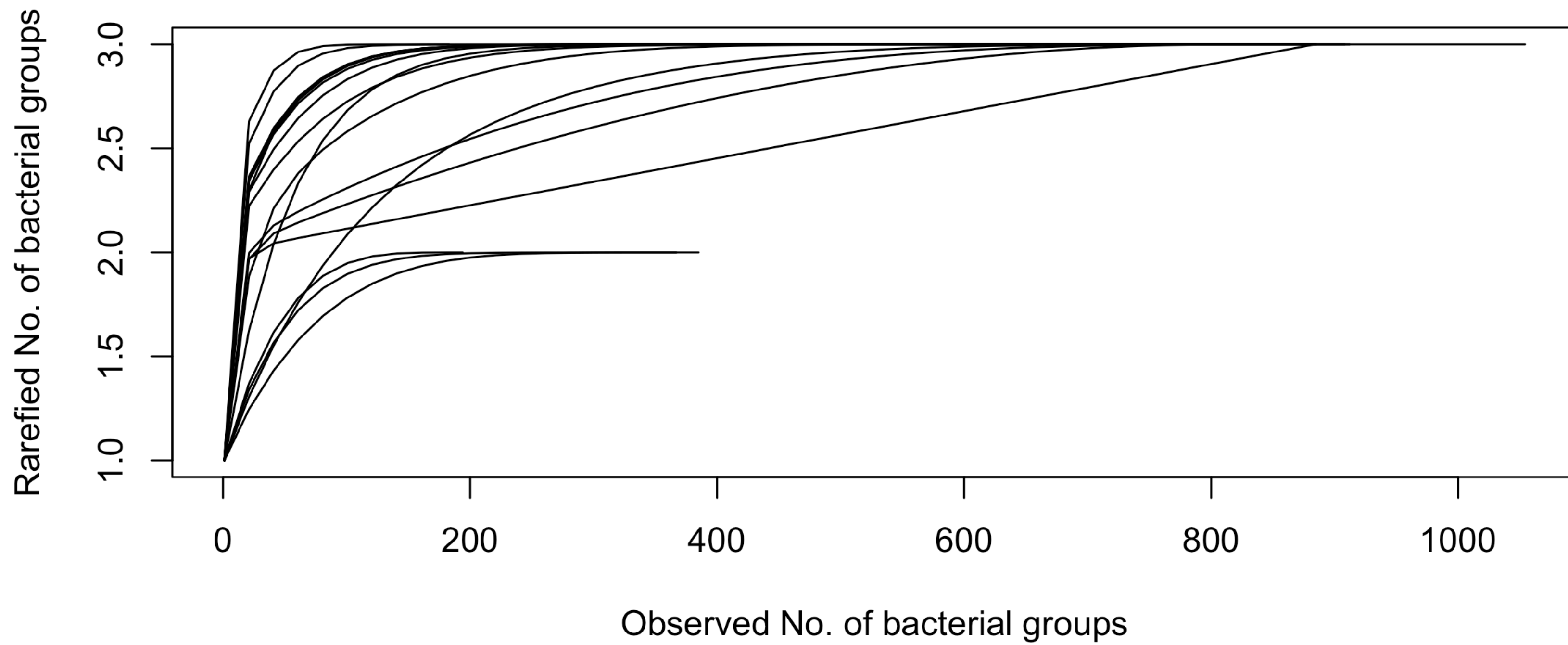


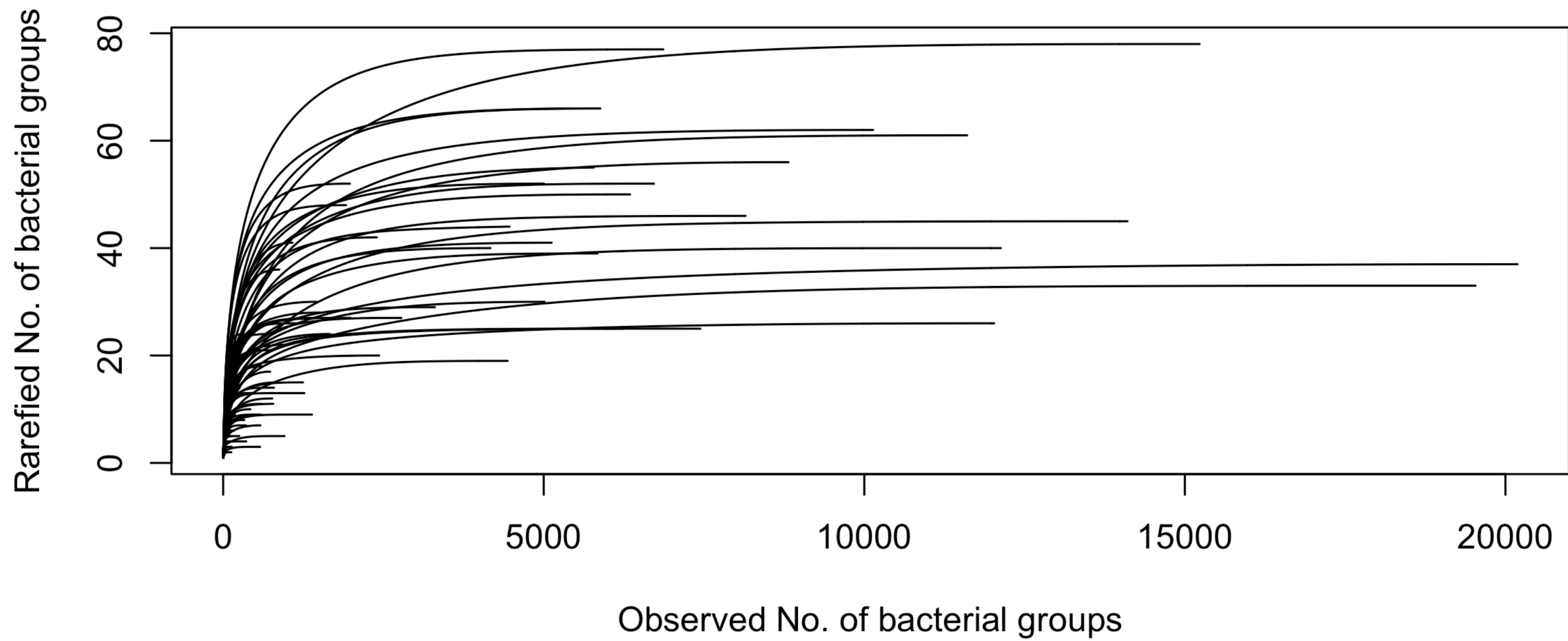
Figure 3.6. Variation in (A) hatchling mass (g), (B) hatchling snout-vent length (SVL; mm), and (C) hatchling tail length (mm) from control and antibiotic-exposed mothers. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average.

Supplementary Table 3.1. Summary of operational taxonomic units (OTUs) sampled from cricket hindguts in control and treatment groups.

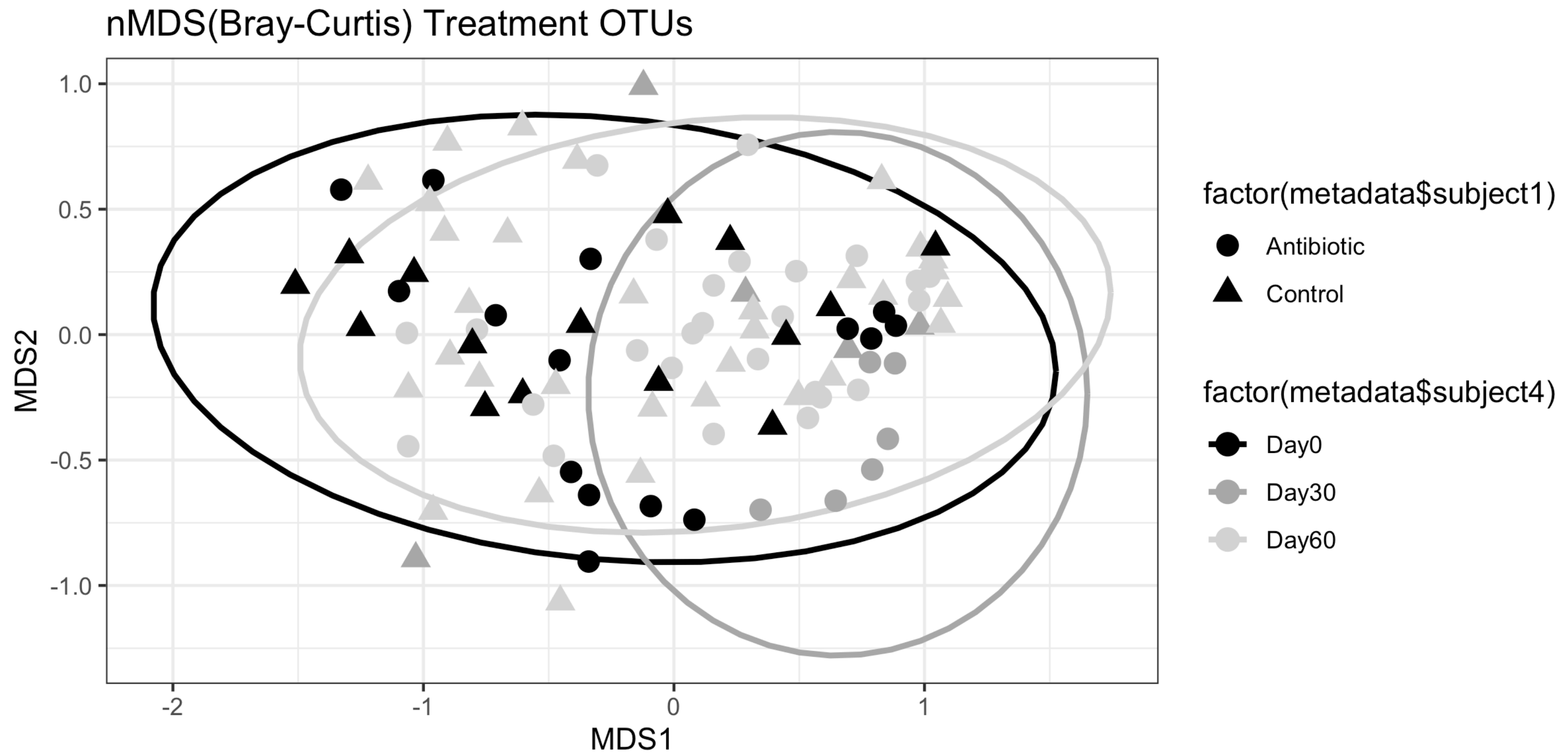
Sample	Treatment	Bacteria	Unassigned
A3	Control	769	114
A5	Control	722	90
A6	Control	764	100
B4	Control	341	213
B5	Control	871	4
B7	Control	505	75
B8	Control	228	144
C4	Control	651	239
A7	Antibiotic	190	0
A8	Antibiotic	380	0
A9	Antibiotic	694	44
B1	Antibiotic	360	0
B3	Antibiotic	58	117
B6	Antibiotic	694	207
B9	Antibiotic	496	159
C1	Antibiotic	387	307
C2	Antibiotic	1017	21
C3	Antibiotic	650	124



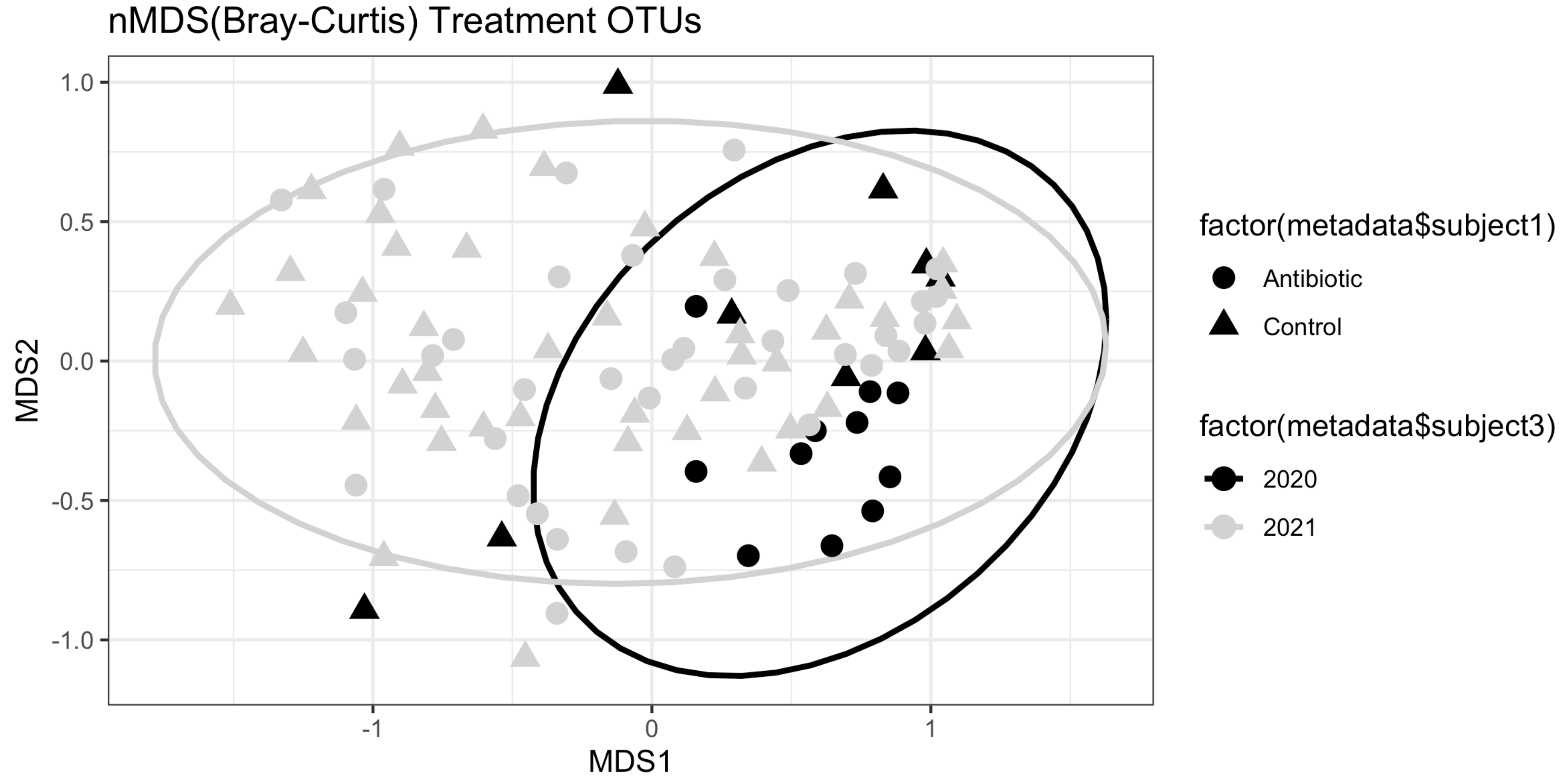
Supplementary Figure 3.1. Rarefaction curve of rarified number of bacterial groups by observed number of bacterial groups. Each line is associated with individually dissected and sequenced hindgut of domestic house crickets (*Acheta domesticus*) in the dataset.



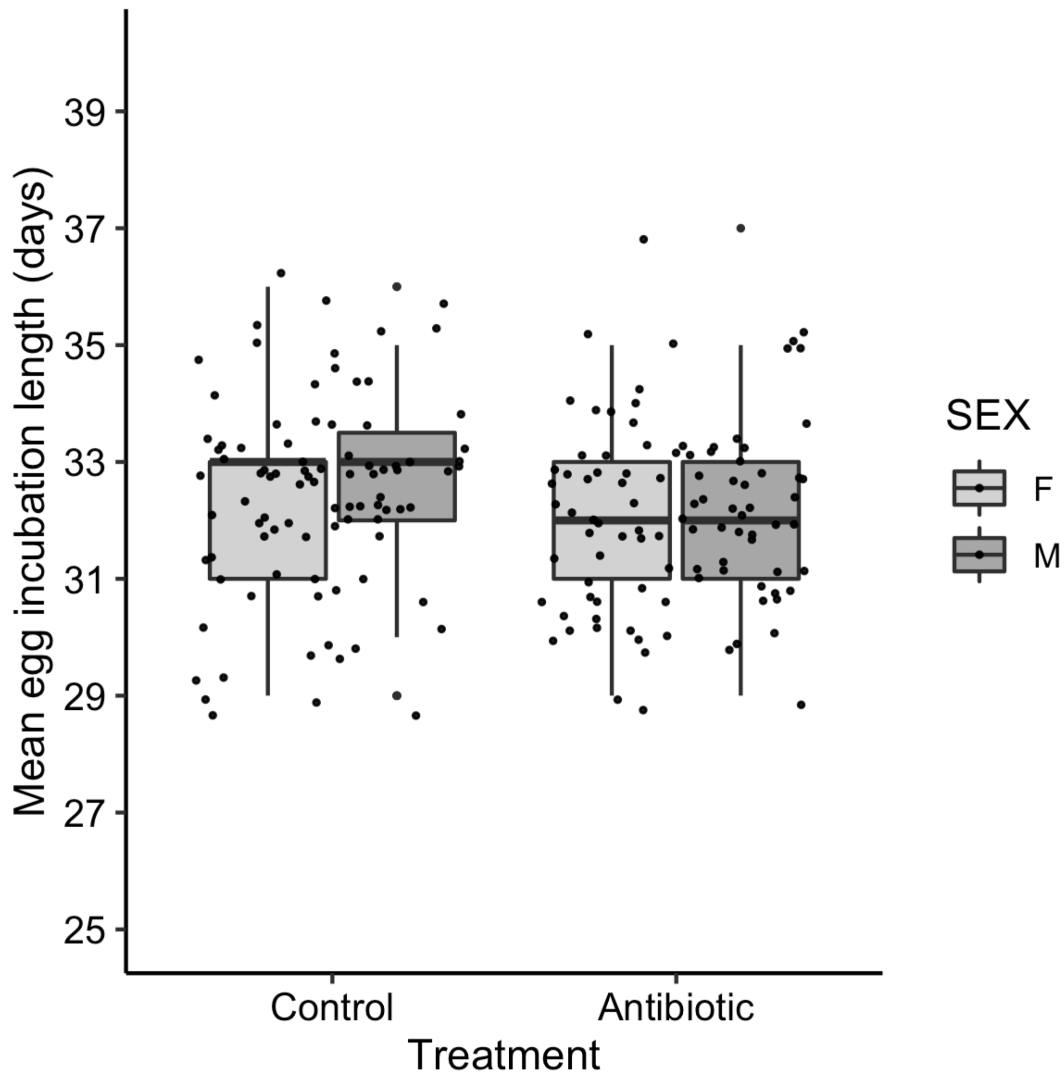
Supplementary Figure 3.2. Rarefaction curve of rarified number of bacterial groups by observed number of bacterial groups. Each line is associated with either gut or cloacae samples from brown anoles (*Anolis sagrei)* in the dataset.



Supplementary Figure 3.3. Variation in microbial community structure in *A. sagrei* gut and cloacae across treatments and time (i.e., Day 0, Day 30, Day 60). Non-metric multidimensional scaling (NMDS) plot of all individuals. The black shapes in the NMDS plot are for samples collected at Day 0, the grey shapes are for samples collected at Day 30, and the light grey shapes are for samples collected at Day 60. The circles represent samples collected from individuals in the antibiotic-exposed group and the triangles represent samples collected from individuals in the control group.



Supplementary Figure 3.4. Variation in microbial community structure in *A. sagrei* gut and cloacae across treatments and year (i.e., 2020 and 2021). Non-metric multidimensional scaling (NMDS) plot of all individuals. The black shapes in the NMDS plot are for samples collected in 2020 and the light grey shapes are for samples collected in 2021. The circles represent samples collected from individuals in the antibiotic-exposed group and the triangles represent samples collected from individuals in the control group.



Supplementary Figure 3.5. Variation in mean egg incubation length by days between hatchlings from control and antibiotic-exposed mothers. The whiskers extending from the boxes represent the 1st and 3rd quartiles ± 1.5 x interquartile range, while the bold line represents the average. The lighter grey represents female hatchlings while the darker grey represents male hatchlings.

Discussion

The goal of this study was to evaluate the influence of diet manipulation, using antibiotics, on the microbiomes of female *A. sagrei*. We predicted that: 1) antibiotics will reduce the bacterial abundance and diversity of prey items, and in turn, reduce the gut microflora abundance and diversity in lizards; 2) this reduction in microbiota diversity would be similar in both the intestinal tract and cloaca of *A. sagrei* mothers; 3) deviation between microbiomes in treatment and control *A. sagrei* mothers would increase over time, and 4) hatchlings from treatment mothers would be larger than those from control counterparts. We demonstrated that treatment mothers had reduced gut microbiota diversity, but interestingly, this was not true for the prey items receiving antibiotics directly. We also show that the gut harbors far more OTUs and a greater diversity index than the cloacae. We demonstrate a distinct difference in microbiota over the course of the experiment and between years. Lastly, we show that egg production and hatchling size varies based on maternal treatment group. Overall, our study provides insight into whether antibiotic manipulation of prey items can influence the gut and cloacal microbiome of female lizards and how that exposure may alter reproduction.

Crickets exposed to antibiotics did not differ from controls in terms of alpha or beta bacterial diversity. Additionally, most of the OTUs revealed through 16S rRNA gene-sequencing were only identified to the kingdom level (i.e., bacteria) while the rest were unassigned. This suggests that 1) *A. domesticus* hindguts harbor previously unsequenced bacteria that are not present in the GreenGenes database, which would 2) explain why no difference between treatment groups was detected. Indeed, bacteria associated with *A. domesticus* has been explored using culture dependent methods and the hindgut had an increased number of cultivable bacteria compared to the midgut (Ulrich, Buthala, and Klug 1981). These crickets are sometimes reared for human

consumption and their whole-body microbiome has been previously documented (Aleknavičius et al. 2022; Milanović et al. 2020); however no study to date has explored only hindgut-associated bacteria in *A. domesticus* using culture independent methods.

Maternal microbiota of *A. sagrei* differed between treatments, where antibiotic exposed individuals had decreased richness and evenness values. The use of antibiotics to reduce gut microbiota diversity is not uncommon and has been used in experimental studies across taxa (Abdelmotaleb A. Elokil et al. 2020; Yukgehnaish et al. 2020; Thomas et al. 2017). However, the application and/or exposure to antibiotics has not been documented in *Anolis* lizards. In other reptiles, the use of antibiotics have been explored following rehabilitation or long-term captivity (Samuelson et al. 2020). Because this study did not use whole genome sequencing (WGS) methods, nor any metabolite exploration, there remains a knowledge gap into the functional variation produced by antibiotics in anoles. This may be of particular importance with the growing spread of antibiotic-resistant genes as a global health concern because *A. sagrei* are an invasive species from Cuba populating much of the Southeastern US (Kolbe et al. 2004). Indeed, wildlife can contribute to the spread of antibiotic resistant genes through horizonal gene transfer among bacteria associated with wildlife microbiomes (Laborda et al. 2022)

The most abundant phyla associated with *A. sagrei* was Bacteroidota, followed by Firmicutes. Similarly, the most abundant genera associated with *A. sagrei* was *Bacteroides*, followed by *Clostridium sensu stricto* 1. In a study exploring bacteria associated with fecal samples in *A. sagrei*, authors documented a large proportion of OTUs belonged to the phyla Firmicutes, followed by Proteobacteria and Bacteroidetes (Ren et al. 2016). The most abundant genera was *Bacteroides* (Ren et al. 2016), similar to the present study. However, the second most abundant genera in the present study (i.e., *Clostridium sensu stricto* 1) was not detected in the prior study.

This genus is a gram-positive spore that is commonly found in human and animal guts (Lopetuso et al. 2013) and has been observed in Italian wall lizards (*Podarcis siculus*; Buglione et al. 2022).

Gut and cloacal microbiota in *A. sagrei* were distinct from one another, suggesting that studies that only collect cloacal swabs may underestimate bacterial diversity within digestive tracts. This difference may reflect an effect of sampling method, or it could be due to real difference in the microbiota between the gut and cloaca. While fecal samples were not collected or sequenced in this study, feces in avian-reptiles has been shown to represent the gut microbiota better than cloacal swabs as well and may be used as an alternative to destructive sampling (Videvall et al. 2017). However, this was not the case in fecal samples from fence lizard (*Sceloporus virgatus*), whereby feces had distinct compositions compared to gastrointestinal tissue, cloacal swabs, and cloacal tissues (Marie E. Bunker, Martin, and Weiss 2021; Colston et al., 2015).

The abundance and diversity of bacteria associated with female *A. sagrei* also differed over time. Indeed, richness and evenness values decreased between Day 0 and Day 60 of the experiment. This suggests that the increased exposure to antibiotics can enhance the change in abundance and diversity metrics associated with microbiomes. This finding parallels that found in experimental studies in other taxa (Clemmons, Voy, and Myer 2019; Gao et al. 2017). However, bacterial richness and evenness values differed between years in the present study. Females collected in 2021 had higher values compared to those from 2020. While methods used in both years were the same, there are a few explanations for why this may be: 1) many more samples were collected from female gut and cloacae in 2021 than in 2020. This dramatic increase in both gut and cloacal representation in 2021 (N = 80) may have revealed a greater range in diversity values that was not apparent with the small (N = 21) sample size from 2020. 2) Decreased egg

production may explain variation between years. Indeed, broiler chickens with diverse microbiomes had high egg-laying performance compared to those with less diverse microbiomes (A. A. Elokil et al. 2020). This explanation, however, would also require a systematic review of bacterial metabolite production between control and antibiotic-exposed *A. sagrei* and would be counterintuitive to the results from our study that show control individuals (with more diverse microbiomes) produced fewer eggs. 3) Lastly, because these were two different years, lizards collected in each year could have come from different environmental conditions that would have affected their microbiome. It should be noted, however, that both years lizards were collected from the same location around the same time of year.

Antibiotic exposed mothers produced more eggs and larger hatchlings than control mothers. Eggs produced from control mothers were also slightly smaller than those from their treatment counterparts. Similar results have been shown in nestling house sparrows (*Passer domesticus*) exposed to antibiotics where treatment significantly increased growth and food conversion efficiency (Kohl et al. 2018). Additionally, conventional chicks reared with penicillin grew more than controls (Coates et al. 1963) and those reared in germ-free environments grow 15-25% faster than controls (Forbes and Park 1959). An important caveat to this study is that perhaps mother anoles were exposed to antibiotics themselves (e.g., antibiotics on the exterior of the cricket), rather than through any changes to bacterial communities associated with prey items. Indeed, this is exemplified through the similarity between control and antibiotic-treated cricket microbiomes.

Results from our study demonstrate that the maternal microbiome may be manipulated using antibiotic-exposed prey items, and that these changes can be observed through maternal reproduction and offspring phenotypes. Additionally, time and sampling regions are important to

consider when evaluating microbiota abundance and diversity. Time and sampling regions may be imperative for studies exploring vertical transmission of microbes, where unlike our study, sampling offspring microbiota is included. Our study serves as an important link between maternal microbiota and offspring phenotypes and exemplifies how future research might expand upon the influence of such changes in oviparous taxa.

Chapter 4

The microbiome as a maternal effect: A systematic review on vertical transmission of microbiota

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Synopsis

The microbiome is an interactive and fluctuating community of microbes that colonize and develop across surfaces, including those associated with organismal hosts. A growing number of studies exploring how microbiomes vary in ecologically relevant contexts have recognized the importance of microbiomes in affecting organismal evolution. Thus, identifying the source and mechanism for microbial colonization in a host will provide insight into adaptation and other evolutionary processes. Vertical transmission of microbiota is hypothesized to be a source of variation in offspring phenotypes with important ecological and evolutionary implications. However, the life-history traits that govern vertical transmission are largely unexplored in the ecological literature. To increase research attention to this knowledge gap, we conducted a systematic review to address the following questions: 1) How often is vertical transmission assessed as a contributor to offspring microbiome colonization and development? 2) Do studies have the capacity to address how maternal transmission of microbes affects the offspring phenotype? 3) How do studies vary based on taxonomy and life history of the study organism, as well as the experimental, molecular, and statistical methods employed? Extensive literature searches reveal that many studies examining vertical transmission of microbiomes fail to collect whole microbiome samples from both maternal and offspring sources, particularly for oviparous vertebrates. Additionally, studies should sample

functional diversity of microbes to provide a better understanding of mechanisms that influence host phenotypes rather than solely taxonomic variation. An ideal microbiome study incorporates host factors, microbe-microbe interactions, and environmental factors. As evolutionary biologists continue to merge microbiome science and ecology, examining vertical transmission of microbes across taxa can provide inferences on causal links between microbiome variation and phenotypic evolution.

Introduction

Most surfaces, including the internal and external membranes of vertebrates, are colonized by microorganisms that form interactive and fluctuating communities, commonly referred to as the "microbiome." The microbiome is a network of bacteria, archaea, fungi, algae, and protists as well as associated structural elements (e.g., DNA/RNA) and metabolite products that create an environment with distinct bio-physio-chemical properties (Berg et al. 2020). Microorganisms are important to the health and function of organ systems in many species (McFall-Ngai et al. 2013) and often exhibit symbiotic relationships with their host (Douglas and Werren 2016). Indeed, deviations away from microbiome community structures that promote health (i.e., dysbiosis) have been shown to reduce host survival (Shreiner, Kao, and Young 2015). Thus, characterizing microbiomes across organismal regions and lifespans, as well as identifying the source of different microorganisms that make up a microbiome, is critical for understanding how variation in microbiota may influence host phenotypes.

Identifying life-history traits, such as reproductive mode or presence of maternal care, that have the potential to influence vertical transmission (i.e., the passing of symbionts or pathogens from mother to offspring) of microbiota may help in guiding research that addresses the process

of microbiome colonization and development in offspring. In viviparous species, for example, neonates obtain critical microbial symbionts via vertical transmission from mothers (Grieneisen et al. 2021; Pelzer et al. 2017; Funkhouser and Bordenstein 2013). Vertical transmission of microbes aids in the development of neonates by stimulating metabolic processes (Mueller et al. 2015) and can influence survival through the acquisition of critical bacterial groups (Korpela et al. 2018). In many oviparous organisms (e.g., many reptiles, including birds), eggshells may present a barrier to vertical transmission to the neonate. Nevertheless, eggshells are readily colonized by bacteria from maternal sources (Li et al. 2022; Martínez-García et al. 2016) as well as the nest environment (van Veelen, Salles, and Tieleman 2018) in ways that have the potential to affect developing embryos and the microbiome of hatchlings. For example, the microbiome associated with eggshells prevents fungal growth and pathogenic bacteria from negatively impacting embryo survival (M. E. Bunker et al. 2021). Thus, sampling at this early stage is critical for understanding how microbiomes may be passed from mothers to offspring and how microbial communities change throughout host growth.

The timing and order of colonization by different microbial taxa on their hosts can have important long-term effects on host physiology and fitness (Wallace, Gohir, and Sloboda 2016). Because neonates are at an early ontogenetic stage with little, if any, prior exposure to microbes, the initial microbes that they receive (potentially transmitted from the mother) could have important impacts on the arrival order and relative abundance of bacteria at subsequent periods of life. This is often referred to as a priority effect (Debray et al. 2022), where the first microbes to which an organism is exposed will colonize some internal or external surface (e.g., gastrointestinal tract) and then influence subsequent colonization of other microbial species (Kapourchali and Cresci 2020). Consequently, early microbes transmitted by mothers might have direct effects on

offspring or indirect effects at other ontogenetic stages of the host. Hence the ontogenetic patterns of co-evolution between host and microbes could have been critical in organismal adaptation to different environments, which potentially originate from vertical transmission. These long-term influences of the maternal microbiome on offspring may resemble the effects that have been documented for many other maternal factors (Mousseau & Fox 1998).

Maternal influence on offspring microbiomes

Our understanding of how interactions among host genomes, the environment, and associated microbiome(s) determine the phenotype of an organism is still developing and has been an important focus of recent research (Oyserman et al. 2021). The relationship between a host and its microbiome is defined in two ways: first, microbiome community structure, or the taxonomic and functional diversity represented in a microbiome that interact with the host genotype and the environment, is a phenotype of the host (Oyserman et al. 2021). This definition implies that microbiomes differ by taxonomic structure and/or function (e.g., metabolite production), and this phenotype is unique to an individual host. That is, the microbiome is an extended phenotype that is an integral part of the host's phenotype. Second, the microbiome may be defined by its impact on the host phenotype (or defined as "microbiome-associated phenotype") rather than a host-specific characterization of the microbiome; this metric is quantified by physiological changes in the host generated by the microbiome (Oyserman et al. 2021). In this context, the origin and initial colonization of the microbiome can also influence host phenotypes. This variety in definitions of host microbe-interactions can influence our understanding of the role that vertical transmission plays in shaping host phenotypes, which has implications for organismal evolution (Osmanovic et al. 2018).

The second definition described above is relevant to the growing area of research on microbiome-mediated plasticity, which refers to how variation in the structure and composition of microbiota shapes the phenotypes of the host organism (Henry et al. 2021; Fischbach 2018). For example, the immune system of many organisms is shaped by their gut microbiome, and must maintain mutualistic homeostasis with microbial communities (Hooper, Littman, and Macpherson 2012). Examples of microbiome-mediated plasticity are most studied within specific life stages (Cresci and Bawden 2015; Blaser et al. 2013), thus we have a poor understanding of the impacts of ontogenetic changes in the microbiome (Caporaso et al. 2011) as well as the potential for maternal microbiota to directly or indirectly impact offspring phenotypes independent of any vertically transmitted microbes. This area of microbiome research warrants attention (Comizzoli et al. 2021; Rowe et al. 2020; Mueller et al. 2015).

Maternal effects are a form of developmental plasticity in which offspring phenotypes are influenced by the mother at embryonic or neonatal stages (Uller 2008). Maternal effects tend to arise when a mother's environment or phenotype, rather than only her own genetic material, influences the phenotypes of her offspring (Wolf and Wade 2009; Wade 1998). Under this definition, vertical transmission of microbes from mothers to offspring is a clear example of a maternal effect. Yet, the microbiome is poorly studied in the context of a maternal effect in ecological and evolutionary research. Indeed, only a few studies demonstrate a causal link between maternal microbiomes and offspring phenotypic variation. For example, mice delivered via cesarean section that are colonized with microbiota from the vaginal fluid of mothers had a different microbiome (taxonomic and functional) than those that were not exposed to vaginal fluid (Jašarević et al. 2018). The researchers also exposed mothers and offspring to various stressors and demonstrate that the microbiota that colonized offspring (i.e., maternal origin) decreased body

weight and growth of offspring when taxonomic and functional diversity was reduced in the sourced maternal microbiome (Jašarević et al. 2018).

The influence of the maternal microbiome on offspring may vary considerably among taxa, particularly those with different modes of reproduction. Exploration of relationships between host genotype, the environment, and associated microbiome can lead to new hypotheses on how these interactions drive evolutionary change (Henry et al. 2021; Oyserman et al. 2021; Osmanovic et al. 2018; Abdul-Aziz, Cooper, and Weyrich 2016). Variation in reproductive strategies and mode (i.e., time point of vertical transmission) is suggested to critically influence microbial colonization in neonates (Comizzoli et al. 2021). For example, microbiota transfer to offspring in viviparous species is more direct (e.g., there is no eggshell barrier) than in oviparous species. Indeed, the eggshell and yolk might hinder maternal transfer of microbiota to the embryo or neonate. In addition, the time gap between oviposition and hatching in oviparous species means that mothers can be absent at time of hatching. In these cases, much of the maternally transferred microbes might later get swamped by microbes in the nest and maternal signature could decline with time. However, to describe these causal mechanisms, studies need to measure the taxonomic and functional diversity of microbes as well as the phenotypic traits of hosts under different environmental conditions.

Mode of vertical transmission

Studies that examine vertical transmission of the microbiome from parents to offspring typically assess the timing and mechanisms of microbial transfer. Microbial transfer can occur before fertilization, which is likely common in species with external fertilization where microbes originating from the mother colonize the external oocyte. Eggs obtained from female Sydney rock

oysters (*Saccostrea glomerata*), for example, share over 20% of bacteria with their day-old larvae and these colonizing microbes may have shaped the development of the larval oyster microbiome through colonization from maternal sources (Unzueta-Martínez et al. 2022). Importantly, ontogenetic stages had distinct microbiomes but harbored a core microbiome, overlapping with maternal sources, that persisted throughout development and were distinguishable from the water column (Unzueta-Martínez et al. 2022).

A second time point when maternal microbes may be acquired by offspring is during embryonic development (i.e., following fertilization). Bacterial phyla have been shown to be present inside eggs from four different bird species (*Poecile atricapillus, Sialia sialis, Passer domesticus, Tachycineta bicolor*) and eastern fence lizards (*Sceloporus undulatus*) shortly after oviposition (Trevelline et al. 2018), and this pattern likely occurs in other taxa. Importantly, comparisons of bacterial communities inside eggs with substrate from lizard nests reveal that microbes present within the egg differ in abundance and taxonomic diversity than those from the surrounding environment (Trevelline et al. 2018). These findings strongly suggest that maternally derived microbes contribute to the diversity within eggs. However, whether microbes are present within the yolk and/or developing embryos across oviparous taxa and when they are transmitted (i.e., prior to vitellogeneis; Nyholm 2020) remains largely understudied, particularly for animals with no agricultural importance. Moreover, some studies characterize the microbial content in yolk of poultry eggs (Ding et al., 2022; Jin, et al., 2022; Lee et al., 2019), but little is explored in free-ranging wild animals. Notably, embryonic development occurs externally to mothers in oviparous species and continues following oviposition. This distinction may guide future questions into how and when vertically transmitted microbes colonize offspring in species with varying reproductive modes.

A third time point when maternal microbes may be acquired by offspring is during oviposition or birth. For example, the microbiome of newborn humans differs between babies from vaginal births versus those from a cesarean sections due to bacteria in vaginal fluid (Mueller et al. 2017; Tamburini et al. 2016). This pattern likely occurs in many other mammals (Hummel et al. 2021; Owens et al. 2021; Kimura et al. 2020), but studies that examine these patterns in wildlife are limited. Nevertheless, in populations of Antarctic fur seals (*Arctocephalus gazella*), mother-offspring pairs shared a greater overlap in bacterial groups or operational taxonomic units (OTUs) than their non-related counterparts and those from different regions (Grosser et al. 2019). In oviparous vertebrates (e.g., reptiles including birds), eggs may be inoculated with bacteria as they pass through the cloaca at oviposition (Trudeau et al., 2020; Lee et al., 2019). The degree to which the eggshell microbes later colonize embryos or newly hatched offspring is poorly studied but is likely to occur most frequently in viviparous species (e.g., many squamates) where the eggshell does not present a barrier to direct transmission to the neonate.

A fourth time point when maternal microbes may be acquired by offspring is following oviposition or birth, and offspring are actively influenced by mothers (e.g., parental care). Female burying beetles (*Nicrophorus vespilloides*) regulate carcass microbiota by applying anal and oral secretions to carcasses that larvae migrate to and feed on, meaning the carcass functions as both larval nutrition and transmission of microbial communities from mother to offspring (Shukla et al. 2018). Many examples exist in mammals, which often exhibit parental care and can mechanically transfer microbiota through lactation or touch (Owens et al. 2021; Klein-Jöbstl et al. 2019; Yeoman et al. 2018). Additionally, eggshells and nestlings of various bird species have been shown to share microbiota with parental counterparts through nest attendance (C.-Y. Chen et al. 2020; Martin-Vivaldi et al. 2018; van Veelen, Salles, and Tieleman 2018; Martínez-García et al. 2016).

Lastly, vertical transmission may also occur from adults to offspring during early neonatal stages but may not involve direct transfer from mothers to their own offspring. This is illustrated by hatchling green iguanas (*Iguana iguana*), where juveniles behaviorally acquire microbes that are necessary for hindgut fermentation of plant matter they consume (Troyer 1984; 1982). Upon hatching, young iguanas will not only feed on nest site material but will also actively feed on adult feces (which may or may not be solely from maternal sources; (Troyer 1984; 1982). This behavior is critical to the development of gut microbiota that facilitate digestive efficiency in these herbivorous lizards (Troyer 1984; 1982). Similar types of adult-to-juvenile transfer of microbes may also be present in highly social species [e.g., many mammals and hymenopteran insects (Sarkar et al. 2020; Tung et al. 2015; Ezenwa et al. 2012)]. The diversity of potential mechanisms for vertical transmission that are described above highlight the complexity of this field and challenges that researchers face in better understanding variation in life history traits that are shaped by interactions between hosts, their associated microbiomes, and the environment.

Overall, maternal transfer of microbiota to offspring is well accepted, but the mechanisms and timing of transmission, as well as their implications for evolutionary adaptation in taxa with different life histories is poorly understood. Moreover, in addition to its potential role in ecology and evolutionary biology, vertical transmission of microbes is important for informing efforts in wildlife conservation (Trevelline et al. 2019). One factor that contributes to our poor understanding of the microbiome as a maternal effect is the lack of a comprehensive synthesis on this topic, which would bring attention to knowledge gaps and help guide future research. Thus, the goal of this review is to identify trends in the literature on vertical transmission of microbes, review key findings, identify knowledge gaps, provide suggestions for future research, and discuss the ecological and evolutionary implications of vertically transmitted microbiomes as a maternal

effect. We argue that microbiota acquired from mothers should be considered a maternal effect. Examining the relationship between microbiota shared between maternal and offspring sources through a multi-faceted approach will provide a robust foundation for future microbiome studies seeking to address knowledge gaps in evolutionary biology. Here, we provide a systematic review, and where possible, conduct analyses to address the following questions:

1) How often is vertical transmission assessed as a contributor to offspring microbiome colonization and development?
2) Do studies have the capacity to address how maternal transmission of microbes affects the offspring phenotype?
3) How do studies vary based on taxonomy and life history of the study organism, as well as the experimental, molecular, and statistical methods employed?

By addressing these three broad questions with a systematic review, and focusing on non-human studies, we hope this paper provides a useful guide that stimulates research on vertical transmission of the microbiome in a wide range of taxa.

Methods

This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (Page et al. 2021).

Study identification

An extensive literature search was performed using three databases, including Web of Science (Core Collection), BIOSIS Citation Index, and Zoological Record, on 22 November 2022, using

the comprehensive keywords: (“microbiome*” OR “microbe*” OR “microbial”) AND (“maternal*” OR “mother” OR “vertical transmission”) AND (“offspring” OR “egg*” OR “neonat*”) NOT (“human” or “child*” OR “infant”). In each of these databases, these keywords were all searched under “Topic.” This setting searches titles, abstracts, author keywords, and KeyWord Plus. We limited our search to non-human animals to address microbiome sampling among wildlife populations.

Documents were filtered to only scientific articles written in English and published between 2010 and 2022, which is when high-throughput sequencing methods were standardized (Rocca et al. 2019). This initial search resulted in 575 studies on Web of Science (Core Collection), 330 studies on BIOSIS Citation Index, and 245 studies on Zoological Record for a total of 1,150 studies. Of these, 368 were duplicates and were thus removed from the dataset. Seven additional articles were identified through citation searching for a total of 789 articles screened. However, 58 of these were reviews or papers that focused on developing statistical models only rather than conducting an empirical study. If a study conducted a meta-analysis and met the following inclusion criteria, it was included in downstream analyses. Thus, a total of 731 articles were filtered using the criteria below (Figure 4.1).

Inclusion and exclusion criteria

Articles were screened from November 2022 to February 2023, and were included in the systematic review if they: (1) examined invertebrates and/or vertebrates that reproduce sexually and/or asexually (except for humans), (2) collected whole microbiome samples (i.e., all or most microbiota present), (3) identified microbiota present (i.e., using 16S rRNA gene-sequencing, shotgun-metagenomic sequencing), and (4) sampled and reported findings, at a minimum, of both

microbial communities derived from mothers and their offspring to assess vertical transmission of microbiota (as defined by transfer or acquisition of microbiota from maternal, rather than environmental, sources).

The exclusion criteria used for filtering studies were: (1) utilized only plants, single-celled organisms, or humans to address research question(s), (2) did not sample the microbiome (i.e., only examined a single species or set of microbes), (3) utilized only metatranscriptomic and/or metaproteomic sequencing and/or did not sequence microbiota (i.e., culture-dependent methods), and (4) did not sample microbiota from both maternal and offspring sources and/or only assessed horizontal transmission and/or environmental input (e.g., soil, diet other than maternal milk) on microbial colonization. If a study only sampled from the amniotic fluid of a developing fetus, this was not considered as maternal sampling in viviparous organisms because the amniotic fluid at gestation is mostly comprised of fetal urine (W. M. Gilbert and Brace 1993). Additionally, studies that only collected from the placenta (in terms of an 'offspring' sample) were excluded. However, in oviparous reptiles, the yolk and surrounding egg compartments besides the developing embryo could be considered as 'maternal' sources as these are deposited by the mother during vitellogenesis (Schwabl 1993).

Study selection

Articles were initially screened based on titles and abstracts to determine fit within our inclusion criteria. A second screening of the full scientific article was conducted following this to determine fit within our inclusion criteria. If the article did not meet the inclusion criteria, it was given a number correlating to which exclusion criteria it fit with first (e.g., examined microbiota transfer in plants, thus was excluded due to exclusion criteria 1).

Data extraction and analysis

A total of 106 studies were used for downstream analyses (see below). Data were manually extracted from text when reported and checked for accuracy. Information on study taxa, such as taxonomic classification and reproductive mode (e.g., oviparous, viviparous) were extracted from articles. Study design metrics, such as sequencing method (e.g., 16S region, metagenomic), experimental vs. observational designs, whether functional diversity of microbes was explored (e.g., through metatranscriptomics), whether phenotypic variables of hosts were measured (e.g., mass, growth rates, immune parameters), and sample region (e.g., cloacal, egg, fecal) from maternal and offspring sources, were also recorded. If a study did not use 16S rRNA gene sequencing and/or metagenomic sequencing, they were labeled as not meeting inclusion criteria 3 and removed from analyses. For functional predictions, if the authors used metagenomic (e.g., shotgun metagenomic sequencing of microbiota), metatranscriptomic (e.g., RNA-seq), or compared targeted sequencing to known databases [e.g., National Center for Biotechnology Information (NCBI), BugBase (Ward et al. 2017)], they were recorded as exploring microbial functional diversity in the article. If a study collected cecal samples without specifying if samples were tissue or cecal contents, the sample region was labeled as 'gut.'

Denotation of statistical methods used in articles was examined to quantify variation in analyses across microbiome studies. We recorded whether a study analyzed amplicon sequence variants (ASVs) or operational taxonomic units (OTUs), used Shannon's diversity index (Shannon 1948), Bray-Curtis dissimilarity index (Bray and Curtis 1957), or weighted Unifrac distances (Lozupone et al. 2011), and reported results using a principal coordinate analysis (PCoA; Lozupone et al. 2011) and permutational multivariate analysis of variance (PERMANOVA;

Anderson 2017). These metrics were selected because they are commonly used as alpha and beta diversity metrics in microbiome studies (Berg et al. 2020; Allali et al. 2017; Kim et al. 2017). Lastly, if the authors reported any overlap between maternal and offspring sources, such as relative abundances of OTUS/ASVs between mother and offspring pairs (most often reported as bar graphs), they were considered to have reported ‘overlap’. A study that did not report ‘overlap’ is one that 1) did not analyze or visually display abundances of microbial taxa (i.e., absolute, or relative abundance) and/or 2) did not describe variations in maternal/offspring taxa (most commonly in studies that did not solely focus on vertical transmission).

Using these data, we quantified publication rates over time as well as variation in study factors between and within articles using RStudio software version 2022.12.0 (R Development Core Team 2018). Figures were generated using ggplot2 (Wickham 2011).

Results

From 2010-2022, 782 articles were identified from a literature search of Web of Science (Core Collection, BIOSIS, and Zoological Record). An additional 7 articles were identified from citations in other articles resulting in a total of 789 papers screened. The number of publications over this period increased by 7.3% on average each year (Figure 4.2A).

Of the 789 articles, 58 were removed because they did not collect empirical data (i.e., were reviews or focused on developing statistical models). Thus, a total of 731 were filtered using the inclusion criteria. A total of 625 studies failed to meet the inclusion criteria (85.5% of total studies; see Methods above). Of these, 131 papers (21.0% of total) utilized only plants, single-celled organisms, or humans to address research questions, thus failing to meet inclusion criteria 1 (Figure 4.2B). If a study used an invertebrate or vertebrate group but did not sample the

microbiome (i.e., only examined a single species or set of microbes), they were excluded based on inclusion criteria 2. Of these, 317 papers were excluded (50.7% of total) based on inclusion criteria 2 (Figure 4.2B). If an article met the first two criteria, but did not sequence the whole microbiome (e.g., utilized only qPCR), they were excluded based on inclusion criteria 3. Of these, 35 papers were excluded (5.6% of total) based on inclusion criteria 3 (Figure 4.2B). Lastly, if an article met all three of these original criteria but did not sample microbiota from both maternal and offspring sources, they were excluded based on inclusion criteria 4. Of these, 142 papers were excluded (22.7% of total) based on inclusion criteria 4 (Figure 4.2B).

Of the original 731 articles filtered, 106 studies met all inclusion criteria and were included in the systematic review (14.5% of total studies). Of these 106, about a quarter (27.3%, n = 29) used invertebrates for their study. The vertebrates used in studies spanned across major classes, including 62.3% mammals (n = 66), 2.8% fish (n = 3), 5.7% birds (n = 6), and 1.9% non-avian reptiles (n = 2; Figure 4.3). No studies on amphibians in the current literature met all the inclusion criteria. Most (64.2%, n = 68) of these study organisms are viviparous (i.e., live-bearing, largely because most studies were on mammals), with only 23.6% (n = 25) of studies using organisms representing oviparous (egg-laying) species and 12.3% (n = 13) that used other modes for reproduction such as broadcast spawning, fragmentation, or other forms of asexual reproduction (Figure 4.3).

Less than a half of the 106 studies (46.2%, n = 49) measured variables related to offspring phenotype and fewer (40.6%, n = 43) measured microbiome functional diversity. Most of the 106 studies (66.0%, n = 70), however, used an experimental approach to address research questions (Figure 4.3). The most common anatomical regions that were sampled for microbes from mothers was fecal contents (37.7%, n = 40) followed by a combination of tissue types (32.1% of studies, n

= 34; e.g., feces, gut). Like that of mothers, the most common anatomical regions that were sampled for microbes from offspring was from fecal contents (35.0%, n = 37) followed by a combination of tissue types (24.5% of studies, n = 26; e.g., feces, gut). Additionally, the majority of studies used 16S rRNA gene-sequencing on the Illumina Miseq (Caporaso et al. 2012) for microbiome analyses (79.2%, n = 84).

A substantial amount of variation exists in bioinformatics pipelines for microbiome data, where 61.3% (n = 65) of studies used OTUs while the remaining 38.7% (n = 41) either used ASVs or did not report their variant method. Additionally, 68.9% (n = 73) reported Shannon's diversity index as an alpha diversity metric while others used Chao1 (Chao 1984) or Faith's phylogenetic index (Faith 1992). Bray-Curtis indices and weighted UniFrac distances for beta-diversity metrics were used in 48.1% (n = 51) and 37.7% (n = 40) of the studies, respectively. To ordinate data for beta-diversity analyses, 56.6% (n = 60) of studies used a principal coordinate analysis (PCoA) while others used a principal component analysis (PCA) or Non-metric multidimensional scaling analysis (NMDS). To quantify beta-diversity analyses, 49.1% (n = 52) of studies used a PERMANOVA while others used an Analysis of Similarities (ANOSIM) or an Analysis of Compositions (ANCOM). Lastly, 85.8% (n = 91) of studies reported any sort of overlap between maternal and offspring sources.

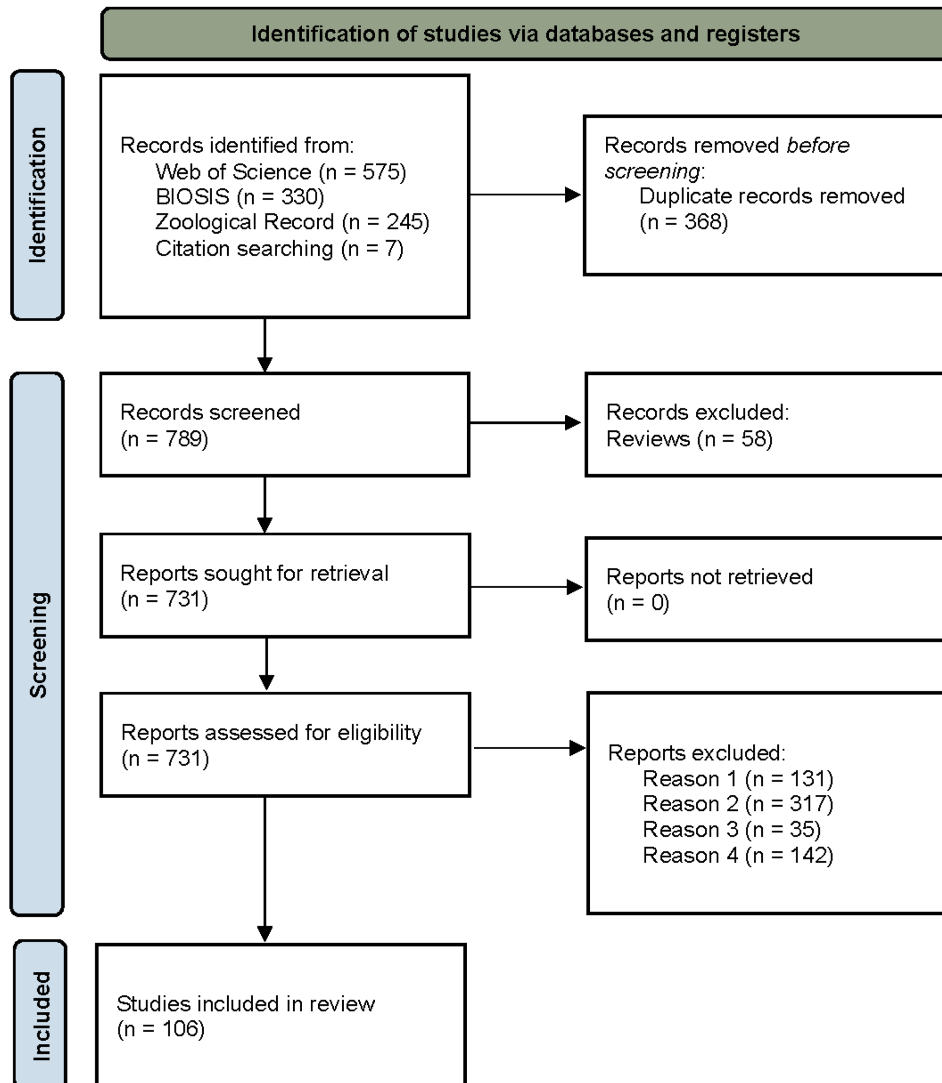

Figure 4.1. Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) flow diagram of the study selection process for this systematic review on vertical transmission of microbes across taxa. 'Studies were excluded from analysis if they did not meet the following criteria: (1) utilized only plants, single-celled organisms, or humans to address research question(s), (2) did not sample whole microbiome, (3) utilized only metatranscriptomic and/or metaproteomic sequencing and/or did not sequence microbiota (i.e., culture-dependent methods), and (4) did not sample microbiota from both maternal and offspring sources.

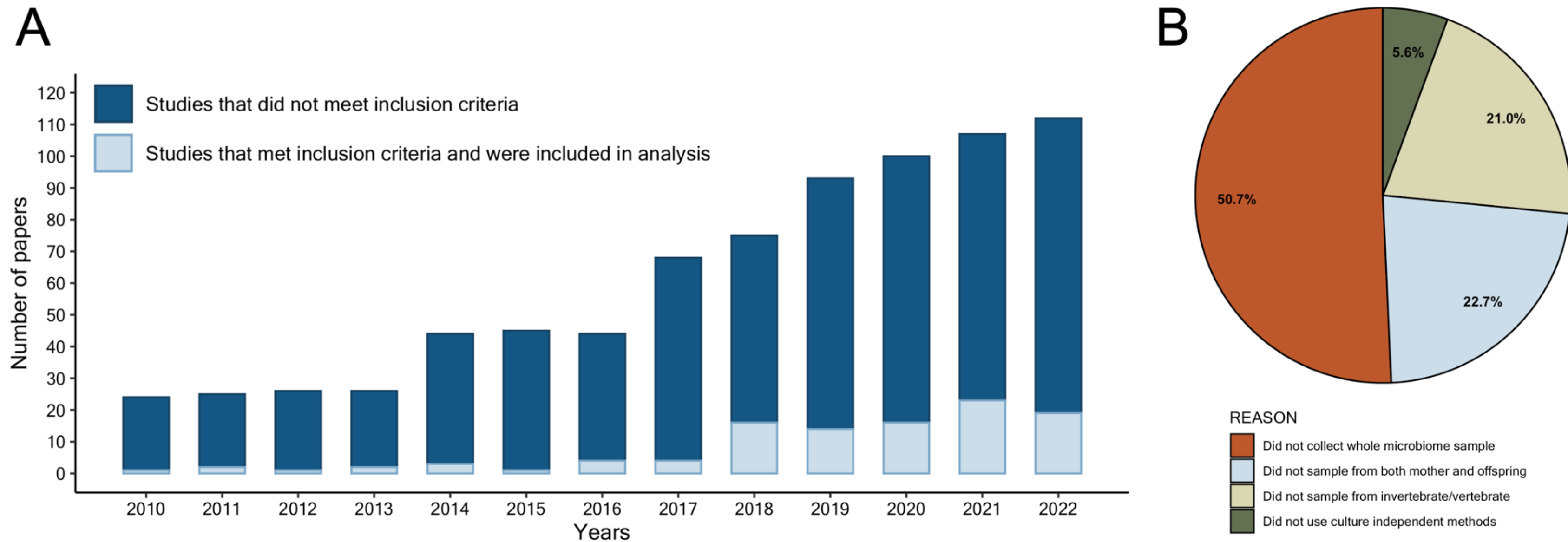

Figure 4.2. (A) Increase in the number of publications over time. Dark blue bars denote publications that did not fit inclusion criteria, while light blue bars are studies that met all inclusion criteria and were included in downstream analyses. (B) Percentages of publications that failed to meet inclusion criteria based on the listed reasons. For studies that did not collect whole microbiome samples (orange, 50.7%), n = 317. For studies that did not sample from both mother and offspring (light blue, 22.7%), n = 142. For studies that did not sample from invertebrate/vertebrate (tan, 21.0%), n = 131. For studies that did not use culture independent methods or only used metabolomics/transcriptomics (green, 5.6%), n = 35.

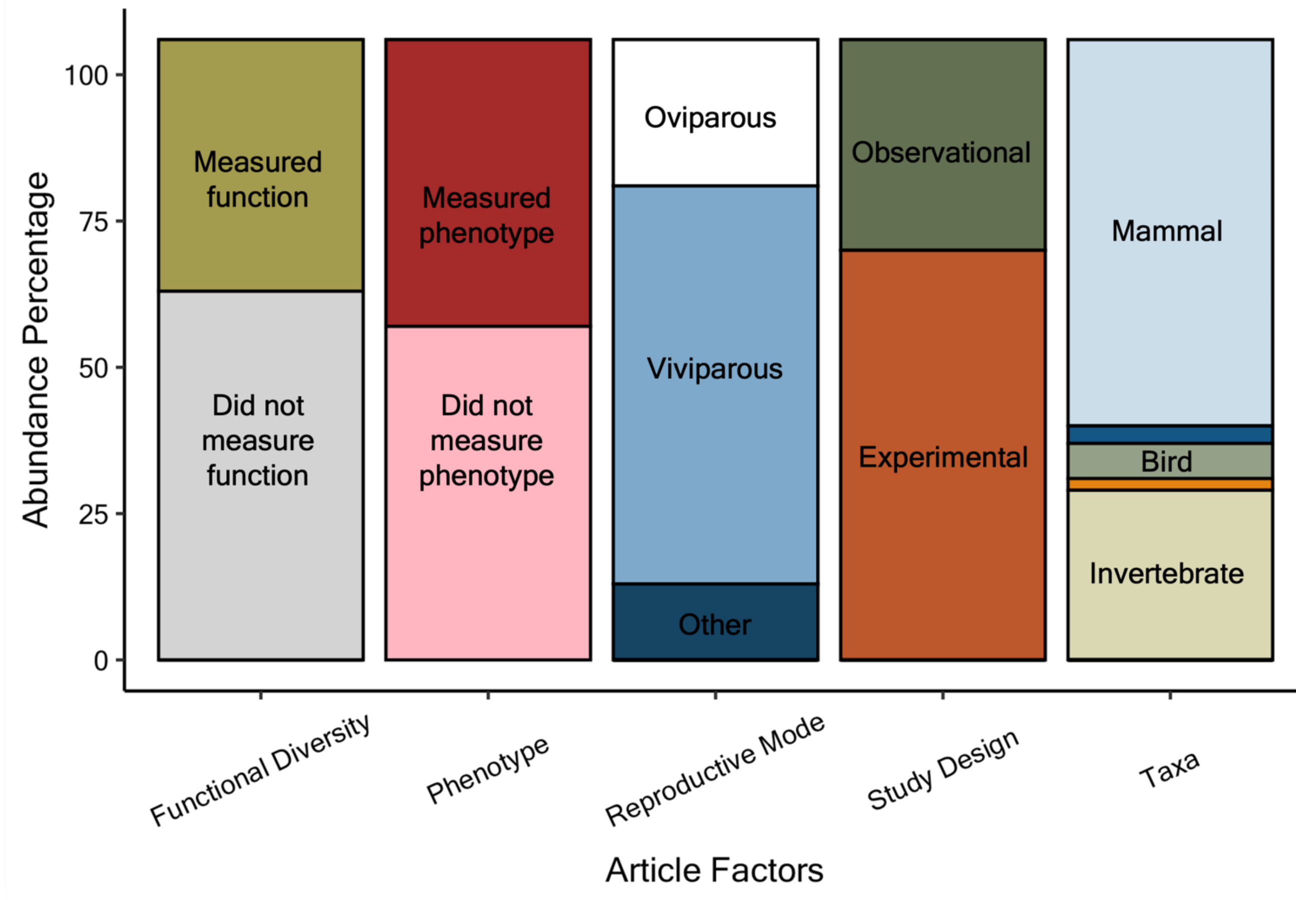

Figure 4.3. Percent abundance for article factors (left to right): whether or not a study measured microbiome functional diversity, whether or not a study measured phenotypes of offspring, whether the study organism was oviparous (egg-laying) or viviparous (live-bearing), whether or not the study design was observational or experimental, and taxonomic classification of the organism used in a study [dark blue bar (second from top) = fish, light orange bar (second from bottom) = reptile].

Discussion

Vertical transmission of microbes from mothers to offspring likely plays an important role in generating variation in phenotypes and fitness among individuals. While many studies demonstrate convincing evidence for this maternal effect (Kapourchali and Cresci 2020; Mueller et al. 2015), its prevalence and consequences in natural systems are still poorly understood. The main goals of our study are to identify reasons why knowledge in this field is lacking and to provide recommendations for future research that will fill in knowledge gaps. We address these goals with a systematic review of the literature aimed at answering three primary questions. First, how often is vertical transmission assessed as a contributor to offspring microbiome colonization and development across ecological literature? We observed a steady annual increase in the number of publications on vertical transmission over time. This positive trend may reflect reductions in sequencing costs associated with microbiome studies within the last decade (Bharti and Grimm 2021) and will likely keep the field moving in a productive direction. Indeed, even if future studies fall outside of our inclusion criteria, such work will likely stimulate future research that provides a better understanding of vertical transmission. Of the papers that did not meet the inclusion criteria, few (5.6%) were excluded for absence of culture-independent methods. Next-generation sequencing can provide a much more accurate representation of microbiomes compared to culture-dependent methods (Rani et al. 2009). However, 16S rRNA gene-sequencing largely overestimates prokaryotes and is unable to account for eukaryotic microbes present compared to whole genome sequencing (WGS; (Sun et al. 2013). Many identified papers (21.0%), even with search terms excluding humans, still resulted in articles including human studies and a few that focused on plants. The goal of this study was to assess ecological literature to provide a robust understanding of knowledge gaps related to non-human animals (Trevelline, Fontaine, et al. 2019). The second

largest group excluded from the review (22.7%) were articles that did not sample mother/offspring pairs. Maternal microbiota sampling is important for 1) comparison to offspring microbiota, 2) confirming offspring microbiota were not environmentally acquired, and 3) comparative analyses across literature. The largest majority failed the inclusion criteria for not collecting whole microbiome samples; many articles focused on a single, vertically transmitted symbiont. This is useful because individual symbionts can be easily traced through ontogeny, however whole microbiome sampling is needed to understand interactions between host genomes, microbe-microbe interactions, and the external environment (Douglas and Werren 2016).

Of the 106 papers that met our criteria, the majority (90%) were from mammalian and invertebrate sources. The mammalian studies included a majority of laboratory (e.g., mice and rats) and agricultural animals (e.g., pigs and cattle), while invertebrate taxa spanned across insects, sponges, and corals. Fish, reptiles, and birds made up very few papers identified, and not one study in amphibians met all the criteria. Of the taxa represented, 64.2% are viviparous, signifying that the mode and consequences of vertical transmission for oviparous vertebrates and those using other modes of reproduction (i.e., asexual) is highly understudied. Because most research focused on mammals, it is not surprising that most studies were on viviparous species. However, given the repeated origins of viviparity in reptiles (Pyron and Burbrink 2014), these organisms might be good models to explore the implications of maternal transfer of microbiota in the evolution of viviparity.

Second, we asked if studies have the capacity to address how maternal transmission of microbes affects the offspring phenotype? We showed that offspring phenotypes were rarely measured in most studies (46.2% of studies). Those studies that did measure phenotypic variables demonstrated that there is an association between host phenotype and microbiota taxonomic and/or

functional diversity (a few include: Bunker et al. 2021; Ma et al. 2014; Bansal et al. 2011). Studies must address causation rather than just correlation between microbiome(s) and host phenotypes (Klassen 2018; Hanage 2014). For microbiome studies, this is often examined through up or downregulated gene pathways or metabolomics. However, only 40.6% of studies examined functional diversity in microbiomes. One reason behind this may be that many microbiome studies are designed as observational rather than experimental, which would not allow for delimiting the interaction between microbial diversity from mother/offspring sources and offspring phenotypes. Experimental approaches that manipulate maternal microbiomes and examine those treatment effects on offspring will be the most robust for addressing question #2. The current gaps in the literature hinder a comprehensive understanding of the effects of microbiomes on organismal evolution for at least a couple critical reasons. First, development and colonization of microbiomes in offspring are likely influenced by host life-history traits, but many microbiome studies lack phenotypic measurements of hosts (Klassen 2018). Second, methods vary among studies, which in turn, can affect results and interpretations. Therefore, research would benefit from standardizing sampling methods to allow for comparison of vertical transmission of microbiota across taxa (see below).

The structure of the host's microbiome is influenced by numerous factors, including its own genotype and gene expression, as well general health, age, and life history traits (Abdul-Aziz, Cooper, and Weyrich 2016). Additionally, interactions between microbiota themselves, including any functional hubs, critical groups, and/or metabolites produced by microbes, can influence microbiome community structure (Tamburini et al. 2016; Mueller et al. 2015). Lastly, environmental factors that both influence host factors and microbe-microbe interactions can shape functional and taxonomic diversity of microbes (Oyserman et al. 2021; Bernardo-Cravo et al.

2020). Indeed, microbial effects shaped by environmental variation can favor microbial transmission (Bruijning, et al., 2022). These environmental factors incorporate climate, prey availability, and other important host and environmental factors, such as maternal effects. Altogether, a multi-faceted approach to microbiome studies will provide a robust foundation for future microbiome studies seeking to address evolutionary knowledge gaps (Figure 4.4; adapted from Dastogeer et al. 2020).

Conducting a robust and meaningful microbiome meta-analysis

Our third research question sought to address differences across studies based on taxonomy and life history of the study organism, as well as the experimental, molecular, and statistical methods employed. Our literature survey revealed substantial variation in study designs and microbiome bioinformatic pipelines regarding the microbiome as a maternal effect. This variation results in many inconsistencies among studies, and therefore comparison of results from different studies are sometimes difficult to interpret (Debelius et al. 2016). Consequently, this variation may hinder a meaningful meta-analytic approach in identifying overarching patterns of vertical transfer of microbiota. First, some microbiome meta-analyses directly analyze microbial genome sequences themselves. However, this route is very time-consuming and does not incorporate mechanistic or phenotypic variables of hosts. Without re-analyzing all microbial genomic sequences, studies and reviews (including those seeking to perform meta-analyses) would benefit from a streamlined pipeline for microbiome results. Indeed, simply using OTUs vs. ASVs can influence microbiome results (Caruso et al. 2019). This also includes incorporating and reporting multiple common alpha and beta diversity metrics (such as Shannon’s diversity and/or weighted UniFrac distances) from both mother and offspring sources. Reporting multiple variables for both alpha and beta diversity

would allow for comparisons across studies. Of the papers analyzed in this study, there were too few with overlapping analyses (i.e., without having to reanalyze all sequencing data) to conduct a strong and meaningful meta-analysis. Second, sharing bioinformatics pipelines (including all code and scripts) on a public platform for others to access will benefit researchers, facilitate communication among research groups, and minimize redundancy in research. A common repository will help to streamline this process and would be very useful in advancing the field. Third, experimental designs that consistently manipulate experimental variables (i.e., presence/absence of maternal microbes) across studies would allow for comparisons between studies. For vertical transmission research this could accomplished by removing maternal input or measuring both maternal and offspring sources over time.

Programs and resources exist to streamline microbiome pipelines into a common form. A common tool used for examining shared factors between two sources (e.g., maternal and offspring) is VennDiagram (H. Chen and Boutros 2011), which gives exact numbers of OTUs or ASVs shared between two sources. Additionally, the tool SourceTracker (Knights et al. 2011) can predict the source of a microbiome; however, this tool relies on sampling from multiple sources including maternal and environmental to predict offspring microbiome origin. FEAST (Shenhav et al. 2019) is also a method used for quantifying source contributions. For additional information on sequencing platforms and bioinformatics pipelines of microbiome data, see Berg et al. (2020), Bharti et al. (2019), Allali et al. (2017), and Kim et al. (2017).

Recommendations for future microbiome studies

Our systematic review revealed several important knowledge gaps in microbiome studies on vertical transmission. First, disproportionate representation exists among animal classes. If more

studies focused on taxa such as various invertebrates, fish, amphibians, birds, and non-avian reptiles, it would enable phylogenetic methods to be applied to transmission modes/microbiota data across large classes. Greater taxonomic representation will also aid in formulating new questions on how microbiota shape host phenotypes and are transmitted from maternal sources. Secondly, more studies should target sampling from both maternal and offspring sources to provide robust evidence for transmission among the two, potentially allowing for identification of the time point when this occurred. Third, many studies lack functional diversity profiles of microbes. If provided for both maternal and offspring sources, functional diversity profiles could elucidate the specific metabolic pathways and metabolites gained from vertical transmission of microbes. Fourth, many microbiome studies could benefit from experimental manipulation in their research designs. If this is logistically difficult, a solution could be standardizing or manipulating offspring developmental environments to remove maternal influence and then documenting of any changes to microbiome community structures. Lastly, as pointed out earlier, many studies do not measure offspring and/or host phenotypes, and therefore the potential consequences of this maternal effect cannot be assessed. This can greatly limit our ability to draw ecologically meaningful conclusions about vertical transmission of microbes and hinder our understanding of the role of this particular maternal effect in evolutionary adaptation. We recommend that more researchers quantify offspring phenotypes (particularly morphological, behavioral, or physiological traits that are likely associated with fitness) and make more effort in examining how these offspring traits are associated with their own microbiome, as well as that of their mother. Such measurements will help us better understand the ecological and evolutionary importance of microbiome as a maternal effect.

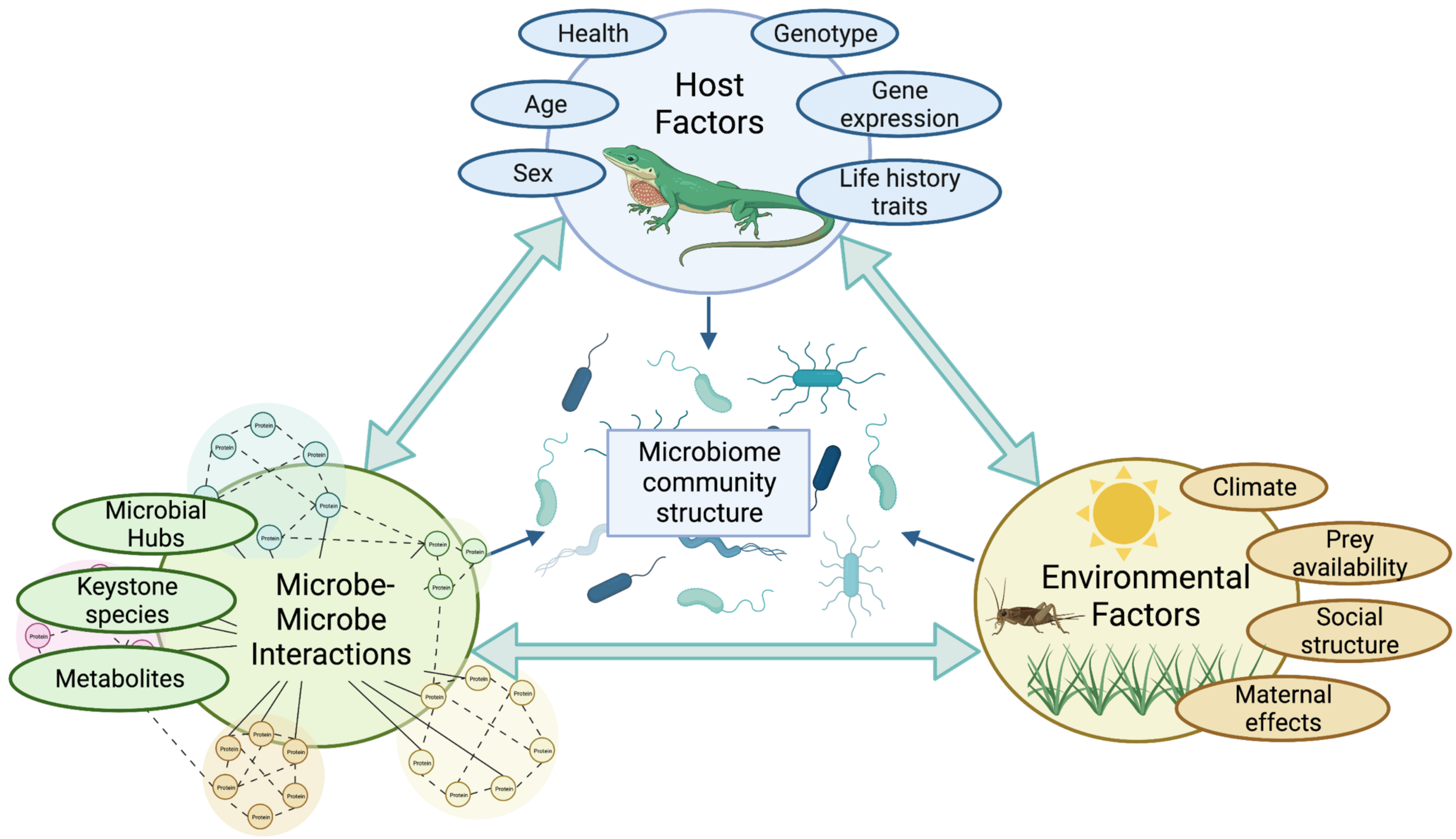

Figure 4.4. An ideal microbiome study is one that incorporates host factors, microbe-microbe interactions, and environmental variables. Adapted from Dastogeer et al. 2020. Created with Biorender.com.

Conclusion

General summary

Microbiome research, through culture-dependent and culture-independent techniques, has propelled the fields of ecology, developmental, and evolutionary biology within the last few decades. Many more studies are published each year that document the influence microbiota have, through functional and taxonomic diversity, on host phenotypes. In my first chapter I show that an exogenous hormone (i.e., estradiol-17ß or E2) reduces bacterial taxonomic diversity in the GI tract of a top-tier predator. Indeed, mass was correlated with alpha diversity metrics as well as plasma E2 concentrations. Perhaps future studies could expand on this work by exploring the functional significance of microbiota associated with E2-exposed individuals.

Because my study organism (i.e., the American alligator, *Alligator mississippiensis*) in my first chapter is a long-lived animal, sexual maturity does occur until many years following hatching. Thus, to explore how microbiota may influence transgenerational life-history traits, I documented eggshell microbiota and maternal effects using the brown anole (*Anolis sagrei*). I developed a novel methods for sampling and manipulating the microbiome of reptilian eggshells using laboratory equipment common to many labs. These methods may be useful to researchers studying microbiota associated with eggs and developing embryos across taxa.

Manipulation of host-microbe interactions (i.e., through the creation of germ-free animals, antibiotic use) is used to measure changes on host phenotypes. In my third chapter, I manipulated maternal diets and saw reduced gut and cloacae bacterial diversity associated with mothers. Egg production was increased as well as egg mass and hatchling length.

For my final chapter, I reflected on what I've learned over the course of my dissertation and where I think the fields of microbiome science and evolutionary biology are trending.

Oviparous organisms, including reptiles, are vastly underrepresented in the literature and to successfully describe causative effects of microbiota, mechanistic variables (i.e., functional diversity, metabolite production) should be measured.

Final thoughts

I began my dissertation with a narrow mindset about what I wanted to study and what I thought a scientist was. Instead, I learned that there are complex interactions between hosts and their microbiota that can shape and influence life-history traits, sometimes across generations. The field of microbiome science is still in its infancy, with many avenues of research and questions to be explored. A scientist with an equally complex toolset to address these questions will provide foundational knowledge into host-microbe interactions. I would like to close with a quote from one of my favorite scientists who describes the benefits of this complexity:

“They talk about science as being a pursuit of the best three jobs on Earth. It's kind of like the adventurer, the artist, the detective… in that you never have all the pieces of the puzzle, use your own creativity, and put together ideas people haven't before."

-Dr. Louis Guillette

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