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The Microbiome of Southwestern Rattlesnakes

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THE MICROBIOME OF SOUTHWESTERN RATTLESNAKES

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
Biological Sciences

by
Erin Elizabeth Stiers
August 2020

Accepted by:
Dr. Christopher L. Parkinson, Committee Chair
Dr. Anna Seekatz
Dr. Barbara Campbell

Abstract

The gut microbiota encompasses the microbial life present in animal digestive tracts, collectively termed the microbiome. These microbial communities are highly adapted to their environment and host, providing beneficial functions not encoded by the host genome. However, there is a lack of gut microbiome studies on wild, non-model organisms; because of the importance of microbiomes in host evolution, it is critical to understand how environment and host alike shape indigenous microbes in wild animal populations. Rattlesnakes (*Crotalus* and *Sistrurus*) provide a useful system to study microbiota differences due to their unique digestive process and locally adapted venoms, which function in prey capture/digestion and predator defense. Here, we use 16S rRNA gene sequencing to investigate factors that influence the microbiota of snakes (n=21) over time from five species in the genus *Crotalus* (the Mojave (*C. scutulatus*), Western-Diamondback (*C. atrox*), Prairie (*C. viridis*), Tiger (*C. tigris*), and Black-Tailed (*C. molossus*) Rattlesnakes). We compared the gut microbiota between species that possess different venom types to investigate whether venom type is playing a role in microbial selection. We also tracked changes in the gut microbiota over time from the wild to captivity and in response to digestion. Across species, the most abundant phyla were Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria, similar to previous reptile gut microbiome studies. Using beta diversity metrics, we observed that snakes harbored a gut microbiota that was more similar to themselves and their species than to geographic location. However, we observed 62 differentially abundant Operational Taxonomic Units (OTUs) between snakes with different venom types.

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Snakes also displayed higher levels of variation in the wild compared to during captivity, losing a substantial portion of OTUs (43%) post-captivity. This loss was sustained in captivity, where snakes gained new OTUs (42%). Post-feeding, we also observed a peak in species diversity. In conclusion, we found that the gut microbiome of southwestern rattlesnakes is distinguishable by different venom types, is more diverse in the wild than in captivity, and is influenced by digestion.

Dedication

This thesis is dedicated to the strong women in my life, past and present, who have supported me in every way; especially to Susan and Doris who were not able to see me graduate.

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Throughout my Master's experience I have had the pleasure of working with some incredible people who have helped shaped me in research and in life. I would first like to thank my advisor, Dr. Christopher L. Parkinson, for supporting me in pursuit of a project I was passionate about, and for pushing me to make sure my goals were realized. I would also like to thank Dr. Anna Seekatz, who helped guide my research in a way I would not have been able to accomplish without her. And to my thesis committee, Dr. Barbara Campbell and Dr. Anna Seekatz for being unduly understanding and supportive as I have written my thesis. Much of my work was made possible through some incredibly supportive lab mates and post-doctoral researchers who have helped me edit, brainstorm, and develop my research. Dr. Mark Margres, Dr. Jason Strickland, Dr. Andrew Mason, Erich Hofmann, Rhett Rautsaw, and Tristan Schraemer were incredibly central to my time at Clemson. You all could always provide a reason to laugh, for better or worse. Additionally, I would like to thank Dr. Rooksie Noorai who, along with being an exceptional researcher, always made me feel welcome at Clemson.

Personally, I would like to thank my family who supported me from afar and listened to me talk about science more than anyone wants to hear. And to the friends I made at Clemson without whom, I would have been much worse off. Kat Zapfe for being the best, kindest roommate I've ever had, Hayley Hassler who always managed to brighten my days, and Rainnee Stevens for being quite possibly one of the funniest and most caring people I've met. Finally, I would like to thank the most supportive partner in the world, Tom, I could not have done this without you.

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Chapter 1

The Gut Microbiome in Non-model Systems

Bacterial life is the oldest on earth, with some geochemical estimates placing the first traces of bacteria at three and a half billion years ago (Schopf and Packer 1987). Often able to take advantage of limited resources, bacteria can multiply to pass on and exchange genetic information at a rapid rate. This swift exchange of genetic information means bacteria evolve quickly and are able to interact with their respective environments in various ways. Bacteria can inhabit almost any environment on earth, from deep sea thermal vents (Russell 1984) to earth's upper atmosphere (Griffin 2004). Due to the ubiquity and diversity of bacterial species, it is unsurprising that microbial life has inhabited not only human skin, but the digestive, respiratory, and excretory systems (Costello et al. 2009).

Bacteria have been studied in the human gut for over 100 years, but because many of the organisms inhabiting the human digestive tract are anaerobic, the limitations of culture-based bacterial identification have hindered our understanding of bacterial community structure. The advent of high throughput sequencing has allowed for the description of all organisms inhabiting and interacting with the human body, known as the human microbiota (Gill et al. 2006). Together, these organisms and the environment they inhabit are collectively referred to as the microbiome, and can be thought of as organs themselves (Baquero and Nombela 2012). The microbes that compose an individual's microbiome are symbionts that affect a range of processes from digestion to regulation of the immune system (Carballa, Regueiro, and Lema 2015; Kau et al. 2011).

In humans, the study of gut microbiome differences across individuals has led to the discovery of its influence on diseases including irritable bowel syndrome and autism (Belzer and Vos 2012; Cryan and O'Mahony 2011).

Sequencing the microbiome can be used to study a range of questions related to host-microbe evolution. The microbiome is species and individual specific, as every organism cultivates a microbiome that is uniquely suited to handle the environment and digestive needs specific to their habitat, diet, and physiology (Contijoch et al. 2019; Garud et al. 2019; Muegge et al. 2011). Bik *et al.*, (2016) showed that marine mammals, dolphins and sea lions, not only had a microbiome that was unique to each other, but they were both uniquely distinct from terrestrial mammal microbiomes and the water microbial samples in which they were swimming. Ley *et al.* (2008) looked across the mammalian phylogeny and found that animals adapted for a specific diet had microbiomes that were more closely related to one another, despite differences in host taxonomy. These studies indicate that host phylogeny, environment, and diet all play a role in shaping the microbiome of diverse animal life.

The study of microbiomes in animals are specifically helpful when asking questions about digestion within a system. Human microbiome research has demonstrated that upwards of 95% of bacterial diversity occurs within the gastrointestinal tract, or the gut (Lozupone et al. 2012; Thursby and Juge 2017). The gut microbiome is primarily composed of commensal and mutualistic bacteria that influence the host in a variety of functions, including nutrient acquisition, immune regulation, proper digestive processing, and resistance against external pathogens (Buffie et al. 2015; Fujimura et al.

2014; Lawrence and Hyde 2017). Microbes in the gut are beneficial and act as a barrier between food toxicants and digestive lining, so the microbiome subsequently plays an important role in protecting host cells from the external environment (Ashida et al., 2012; Söderholm & Perdue, 2001). Maintenance of proper gut functioning is largely influenced by which microbes colonize the gut of a host, as these microbes are crucial to feeding the protective mucosal barrier (Merga, Campbell, and Rhodes 2014; Schroeder 2019) and are key to breaking down substrates that are indigestible by the digestive lining alone (Karasov, Martínez del Rio, and Caviedes-Vidal 2011). The substrates that need to be broken down by the gut are largely dependent on the dietary items a host is consuming, and microbiome variation between hosts with different diet patterns has been demonstrated across different animal populations (Contijoch et al. 2019; Ley et al. 2008). For example, in freshwater fish living in the same habitat, the microbiome was distinct between herbivorous, omnivorous, and carnivorous fish (Liu et al. 2016); cellulose-degrading bacteria were dominant in the guts of herbivorous fish and protease-producing bacteria were dominant in carnivorous fish.

Compositional microbiome data can be informative as to the host-microbiome interactions that influence trait adaptation (Brucker and Bordenstein 2012; Shapira 2016). The evolutionary potential of adaptive traits is a function of many ecological and evolutionary pressures, including strength and direction of selection, genetic variation, and physiological constraints. The microbiome is at the intersection of these pressures. Due to the swiftly evolving nature of microbes, the microbiome can respond rapidly to changes in environmental conditions where genetic variability of the host genome is

limited (Shapira 2016). Thus, compositional and functional changes to the microbiome often precede, and may even mediate, adaptive evolution and speciation (Garud et al. 2019). A dramatic example of the gut microbiomes role in adaptive evolution is in high altitude mammals where convergent evolution of the gut microbiome has led to similar functions of the gut rumen in both high altitude yaks and Tibetan sheep, that is distinct from the gut microbiome of their low-altitude counterparts (Zhigang Zhang et al. 2016).

When characterizing host-specific microbiome profiles in systems with highly divergent adaptive traits, especially those related to food acquisition, microbiome sequencing can lend insight into the ecological and evolutionary pressures of the host. Gut microbiomes have been shown to influence digestion and adaptation but have rarely been studied in wild, non-model vertebrates (Behar, Yuval, and Jurkevitch 2008; Colston and Jackson 2016; Shapira 2016; Vatanen et al. 2019). More research needs to be done to characterize the microbiome in non-model systems to achieve a better understanding of the composition and function of the gut microbiome across the tree of life.

Chapter 2

The Gut Microbiome of Southwestern Rattlesnakes as Revealed by 16S rRNA Sequencing

2.1 Introduction

Snakes, which are understudied in the field of microbiomes, fit well within the frontiers of microbiome discovery because of the unique physiology of snake digestion (Pough and Groves 1983). Snakes undergo more pronounced physiological shifts during digestion than most vertebrates (Castoe et al. 2013), marked by consumption of sizeable prey and extended periods of digestive torpor. To conserve energy during the weeks and months of starvation or brumation, the digestive system becomes inactive and physically atrophied (Castoe et al. 2013). After long periods of digestive inactivity, snakes consume prey of considerable size by swallowing their prey whole (Cundall and Greene, 2000; Lee, Bell, & Caldwell, 1999). Without the aid of physical mastication available to most vertebrates, snakes rely entirely on chemical and bacterial breakdown of their meal (Cundall & Greene, 2000). Snakes that consume large, bony vertebrates not only experience organ size shifting during digestion, but their guts must be able to breakdown fully intact collagenous matrices (Rodríguez-Robles, Bell, and Greene 1999). The microbiome across most snake species, therefore, has to be adapted to dramatic shifts in

organ conformation and secretion, periods of limited nutrient acquisition, and the presence of dietary toxicants.

In addition to typical snake physiological changes during digestion, some snakes use venom for prey capture. Snake venoms are potent cocktails of digestive enzymes and polypeptides which aid in digestion and potentially defense plus have high variability both inter- and intra-specifically (Casewell, Huttley, and Wüster 2012; Margres et al. 2017). Some of these venoms, which can vary considerably by diet and location (Barlow et al. 2009), are thought to aid in digestion by means of tissue-degrading venom peptides (Mackessy 2010; Thomas and Pough 1979). While venom evolution in snakes is often used as a model for testing adaptive trait processes in vertebrates, it remains largely unexplored in the context of host-microbiome interactions (Ul-Hasan et al. 2019). Recent research has shown that the gut microbiome in vertebrates can influence traits that drive adaptation (Brucker and Bordenstein 2012; Zhigang Zhang et al. 2016). One example is in Siu-Ting *et al.* (2019) that compared the role of gut microbiota in modulating toxin sequestration in poison frogs as compared to the microbiota in non-poison frogs. Researchers, however, have yet to explore the influence of the gut microbiome in a species undergoing rapid selection on a phenotype relating to toxin production.

Rattlesnakes (*Crotalus spp.* and *Sistrurus spp.*)*,* are an example of venomous snakes that exhibit inter- and intraspecific venom variation across expansive geographic distributions (Glenn et al. 1983), undergo digestive torpor consistent with most large-bodied snakes, (Tattersall et at., 2004), and possess a well-studied venom phenotype (Bjarnason and Tu 1978; Gibbs and Mackessy 2009; Mackessy 2010; Massey et al. 2012;

Strickland et al. 2018). There are approximately fifty species of rattlesnake in the world and twenty of these species are distributed throughout the United States, many of them living sympatrically (Uetz, Freed, & Hošek 2020). Rattlesnake venoms within the American Southwest generally fall into one of two categories based on the constituent venom peptides and their phenotypic expression (Mackessy, 2010). The first category, called hemorrhagic Type B venoms, are largely composed of snake venom metalloproteinases (SVMPs) that thin blood and cause tissue necrosis (Gutiérrez et al. 2016). Species such as the Western Diamondback (*C. atrox),* Black-Tailed (*C. molossus*), and Prairie (*C. viridis*) rattlesnakes all have hemorrhagic venom profiles which are hypothesized to aid in digestion by means of the SVMPs which act as tissue-degrading venom peptides (Mackessy 2010; Thomas and Pough 1979). Missing the additional digestive function of SVMPs, Type A venoms such as those possessed by Tiger Rattlesnakes (*C. tigris*), result in a neurological shutdown of their prey due to neurotoxic PLA2s in their venom. The Mojave Rattlesnake (*C. scutulatus*) is a species where local adaptation has led to individuals with a hemorrhagic, neurotoxic, or a mix of both venom phenotypes (Glenn et al. 1983; Strickland et al. 2018; Wilkinson et al. 1991; Zancolli et al. 2019). Inter-individual variation in *C. scutulatus* venoms, presents an interesting case for differentiating venom and species effects on the gut microbiome.

The unique digestive physiology of rattlesnakes provides an opportunity to understand the variation that can occur in host microbiomes that undergo significant changes through time. To understand these changes, we first characterized the wild gut microbiome of twenty-one rattlesnakes from the southwestern U.S. with 16S rRNA gene

sequencing. The gut microbiome of the same individuals was also sequenced post-captivity and during feeding in captivity. We used the wild microbiome samples as the baseline to determine the differences in the gut microbiome caused by 1) species 2) location 3) venom type 4) captivity and 5) digestion. Between individuals, we predicted that species, location, and venom type would all play a role in affecting the composition of the gut microbiome. We predicted that captivity would decrease the diversity and abundance of bacteria in all rattlesnakes and that an increase in compositional changes would occur during digestion.

2.2 Methods

Specimen Collection

Adult rattlesnakes were collected in southeastern Arizona, southwestern New Mexico, and western Texas in July 2018. Upon capture all animals were immediately palpated along the length of their body to control for active digestion. Only those with no discernable prey masses were kept. Final collection included twelve Mojave Rattlesnakes (*C. scutulatus*), four Western Diamondback Rattlesnakes (*C. atrox*), three Tiger Rattlesnakes (*C. tigris*), one Black-Tailed Rattlesnake (*C. molossus*), and one Prairie Rattlesnake (*C. viridis*); Table 2.1, Fig. 2.1. Five animals in total dropped out of the study at various times due to poor body condition, inability to feed, and two were released post-capture.
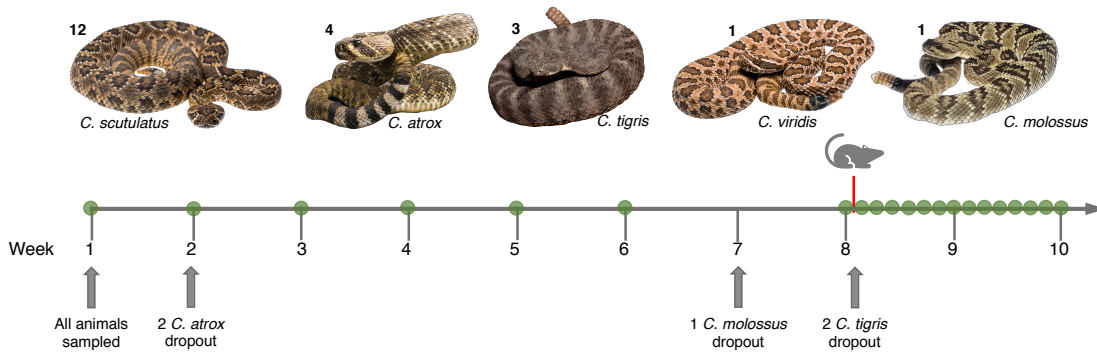
Figure 2.1: Sampling scheme. The number to the top left of each snake represents the number of individuals collected for each species. The green dots on the timeline indicate microbiome sample collection. Snakes were fed after 8 weeks in captivity, as indicated by the mouse, and were sampled right before feeding and 24 hours post feeding. Two *C. atrox* were released post capture, one *C. molossus* dropped out due to poor body condition, and two *C. tigris* did not feed on the correct date; no samples were taken from these individuals after the point of dropout.

Sample ID	Species	Venom Type	Age	County	State	GPS
CLP2727	*C. scutulatus*	A	Adult	Brewster	TX	30.313070, -103.116630
CLP2728	*C. scutulatus*	A	Adult	Brewster	TX	30.275310, -103.174450
CLP2729	*C. scutulatus*	A	Subadult	Brewster	TX	30.281840, -103.162420
CLP2730	*C. scutulatus*	A	Adult	Pecos	TX	30.138115, -102.583988
CLP2746	*C. scutulatus*	A	Adult	Hidalgo	NM	32.040450, -109.023220
CLP2747	*C. scutulatus*	A	Adult	Hidalgo	NM	32.044080, -109.019880
CLP2748	*C. scutulatus*	A	Adult	Hidalgo	NM	31.932800, -109.035860
CLP2764	*C. scutulatus*	A	Adult	Graham	AZ	32.589777, -109.908096
CLP2741	*C. tigris*	A	Adult	Santa Cruz	AZ	31.389800, -111.092250
CLP2742	*C. tigris*	A	Adult	Santa Cruz	AZ	31.389250, -111.093550
CLP2752	*C. tigris*	A	Adult	Santa Cruz	AZ	31.394744, -111.090466
CLP2734	*C. scutulatus*	B	Adult	Pinal	AZ	32.763280 -111.498330
CLP2736	*C. scutulatus*	B	Subadult	Pinal	AZ	32.772760, -111.316950
CLP2737	*C. scutulatus*	B	Subadult	Pinal	AZ	32.764530, -111.326400
CLP2738	*C. scutulatus*	B	Adult	Pinal	AZ	32.764530, -111.326400
CLP2735	*C. atrox*	B	Adult	Pinal	AZ	32.824850, -111.256000
CLP2739	*C. atrox*	B	Adult	Pinal	AZ	32.816710, -111.264570
CLP2740	*C. atrox*	B	Adult	Pinal	AZ	32.763280, -111.498330
CLP2765	*C. atrox*	B	Adult	Hidalgo	NM	31.983180, -109.035880
CLP2750	*C. molossus*	B	Juvenile	Cochise	NM	31.883267, -109.206087
CLP2751	*C. viridis*	B	Adult	Hidalgo	AZ	31.974547, -108.822581

Table 2.1 Sample table of all individuals used in this work.

Microbiome Sample Collection

Swabs were taken from the cloaca of each individual to serve as the representative

microbiome for that individual (Colston, Noonan, and Jackson 2015). Initial samples

were taken within 12 hours of capture to represent the "wild" microbiome. Additional

samples were taken at distinct time points, post-capture, to assess captive and digestive

effects (Fig. 2.1). To sample the microbiome, all investigators wore gloves to avoid

inadvertent contamination and snakes were restrained in plastic tubing leaving the cloaca

exposed. Before swabbing, an alcohol pad was used to wipe the external cloaca to remove foreign or transient microbes. A sterile polyester-tipped applicator was inserted approximately 3-5 mm into the cloacal vent and the applicator was rotated for 2-5 s. The applicator was cut at the tip with sterile scissors and placed into a sterile 1.5ml micro-centrifuge tube before being flash frozen and stored at -80 C. Initial samples were taken in the field, and captive experiments were performed at Clemson University using the same methodology. Table 2.1 shows all animals from which microbiome samples were collected. An overview of the sampling numbers per species and timeline of gut microbiome sample collection can be seen in Figure 2.1. Each animal was sampled every week post captivity for 6 weeks and was not fed during this time, although water was available. After eight weeks of captivity the snakes were sampled once more and then were fed either one or two specific-pathogen-free (SPF) mice each, depending on body size of the snake, to determine how the microbiome changes in response to digestion. Snakes that consumed the mice were then sampled every day for two weeks.

Venom microbiome samples were taken via manual extraction from five individuals; two *C. scutulatus* with venom Type A, and two *C. scutulatus* and one *C. atrox* with venom Type B. From one of the *C.* scutulatus, three samples were collected at twelve, fourteen, and sixteen weeks post capture. Again, snakes were restrained in plastic tubing but were allowed to move through the tube until their heads were exposed. Their heads were placed at the edge of the sterile cup and their fangs manually exposed, preventing them from contacting the side of the cup. The venom glands were manually expressed causing venom to drip out. To employ a clean catch system, the first drop of

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venom from each fang was allowed to fall to the bottom of the cup and the second drop was collected from the side of the cup using a 200-microliter pipette and sterile tips. The venom was expelled into an empty sterile 1.5ml microcentrifuge tube and placed directly into liquid nitrogen.

DNA Extraction and 16S rRNA gene Sequencing

DNA was extracted from all samples using the MagAttract PowerMicrobiome DNA/RNA Kit (Qiagen, 27500-4-EP) adapted to manual extraction in a 96-well plate using manufacturer's protocol with the following modifications. Briefly, the frozen swabs were added directly to the PowerBead DNA Plates containing MBL solution, β-mercaptoethanol, and phenol:chloroform:isoamyl alcohol (25:21:1; pH 6.5-8). Sample preparation, cell lysis by bead beating with a TissueLyser II (Qiagen), and inhibitor removal with Solution IRS (Qiagen) were performed following manufacturer instructions. After inhibitor removal, each Collection Plate contained 850 μl of supernatant. Further explanation of the DNA extraction protocol used in this study can be found in Appendix A.

The isolated DNA was quantified using the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Q32850) on the Qubit 3.0 Flurometer (ThermoFisher Scientific) following the manufacturer's protocol. Samples were normalized to concentrations between 1-5 ng/μl. Each plate included a positive control (mouse feces), negative controls of both water and sterile swabs, and Zymo mock community samples.

The mouse feces used as the positive control was collected from the same colony as the mice fed to all of the snakes in this study.

The isolated DNA was sent to the University of Michigan Microbial Systems Molecular Biology Laboratory for PCR amplification, library preparation, and 16S rRNA gene-based sequencing using previously described methods (Kozich et al. 2013). Briefly, the V4 region of the 16S rRNA gene was amplified using dual-index primers following the procedures described in Kozich et al. 2013. 309 samples amplified with standard PCR (Seekatz et al. 2015), whereas 40 of the remaining samples were amplified using touchdown PCR. The touchdown PCR cycle consisted of 2 min at 95°C, followed by 20 cycles (with a temperature decrease of 0.3) of 95°C for 20 s, 60°C for 15 s, and 72°C for 5 min, followed in turn by 20 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min and a final 72°C for 10 min. Final PCR products were normalized using the SequelPrep Normalization Plate kit (Life Technologies, Cat# A10510-01) following manufacturer's protocol, and pooled per 96-well plate. The Kapa Biosystems Library Quantification kit for Illumina Platforms (KapaBiosystems, Cat# KK4824). The Agilent Bioanalyzer High Sensitivity DNA Analysis kit (Cat# 5067-4626) was used to determine the concentration of the pooled library and amplicon size in preparation for MiSeq Illumina sequencing with the MiSeq Reagent Kit V2 (500 cycles, Cat# MS-102-2003) as specified in the Kozich et al protocol to generate paired-end sequences of the PCR products (Kozich et al. 2013). A 4% PhiX spike was added to generate diversity in the loaded library. The library was sequenced on the Illumina MiSeq using a paired end 500-cycle V2 flow cell.

Venom Typing by Reverse-phased High-Performance Liquid Chromatography

(RP-HPLC)

Given the known dichotomy of venom phenotypes in *C. scutulatus*, venom

samples were collected in order to determine venom type (A or B) of each individual.

Venom samples were placed in 1.5ml microcentrifuge tubes and vacuum dried. To

remove debris, the dried venom was resuspended in water and centrifuged. Next, the

Qubit Protein Assay Kit (ThermoFisher Scientific, Q33212) was used to measure protein

content in venom samples and reverse-phased High-Performance Liquid Chromatography

(RP-HPLC) was performed following Margres et al. (2014) with 100 µg of venom at the

Florida State University Department of Biological Science Analytical Lab using a

Beckman System Gold HPLC (Beckman Coulter). The assayed venoms were evaluated

for the presence of metalloproteinases and phospholipases to designate the venoms as

Type B and Type A venoms, respectively (Strickland et al. 2018).

Data Processing

Raw 250bp sequence reads were directly uploaded to the BaseSpace Sequence

Hub (Illumina) from the Illumina Miseq after being run at the Michigan Host

Microbiome Initiative. BCL to FASTQ conversion and demultiplexing were performed

automatically in BaseSpace to generate a forward and reverse FASTQ file for each

sample. The FASTQ files were downloaded and stored on the Clemson University

Palmetto Computing Cluster. Sequences were processed in *mothur* (v 1.43.1) according

to the MiSeq SOP (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Briefly,

high-quality sequences were trimmed and filtered in *mothur*, then aligned to the SILVA

ribosomal RNA gene database (v.132) (Quast et al. 2013) followed by chimera removal

using UCHIME (Edgar et al. 2011) in *mothur*. Sequences were clustered into operational

taxonomic units (OTUs) at 97% identity using the *mothur* opticlust algorithm (Westcott

and Schloss 2017). The RDP (Ribosomal Database Project) reference taxonomy classifier

was used to classify representative OTUs and sequences directly for genus-level analyses

(Wang et al. 2007). Samples with less than 2000 sequences were removed (n=4)

manually.

Microbiome Analysis

Summary statistics including Yue and Clayton dissimilarity (Θ_{YC}), Shannon's

index, shared OTUs (sharedsobs), and shared sequences were calculated in *mothur*. To

assess community structure of all samples, beta diversity indices were calculated using

Bray Curtis dissimilarity and Θ_{YC}, and alpha diversity indices using Shannon's index.

Bray-Curtis dissimilarity was calculated in the *R* package phyloseq (McMurdie and

Holmes 2013) and was used to create nonmetric dimensional scaling (NMDS)

ordinations. NMDS ordinations were plotted in *R* using ggplot2 (Ginestet 2011) and plyr

(Wickham 2011) to compare samples based on venom type, species, and location. For all

microbiome analyses, snakes collected in Arizona and New Mexico were treated as one

location and were compared to the Texas samples. The Bray-Curtis dissimilarity metric

was used to account for both OTU presence/absence and abundance. Dissimilarity in

community structure was calculated with the Θ_{YC} dissimilarity metric and was used to

visualize beta diversity over time as well as box plots. Boxplots were created in *R* using

both Θ_{YC} distances and shared OTUs for both gut and venom comparisons. The four

venom comparisons were 1) all venom samples to each other, and each venom sample to its 2) wild, 3) captive, and 4) diet samples. The three gut comparisons were intra-individual comparisons between 1) pre- and post-capture and inter-individual comparisons of individuals 2) pre-captive and of individuals 3) post-capture. Beta diversity over time was plotted twice in *R*; once comparing pre-digestion samples of a snake to its respective wild sample, and again comparing all samples of a snake to its subsequent sample. To identify changes in alpha diversity, Shannon's index for each sample was calculated over time and plotted with ggplot2 (Ginestet 2011). To visualize variation of shared taxonomic features, streamplots of the 60 most abundant OTUs (by relative abundance) were created in R using the package plyr (Wickham 2011).

For statistical testing of the dissimilarity of microbial communities, Permutational multivariate analysis of variance (PERMANOVA), analysis of variance (ANOVA), Kruskal-Wallis, and Wilcoxon were performed in *R*. To determine whether or not the center of the NMDS ordinations were statistically different with venom type, location, and species, PERMANOVA was performed using the adonis function in the vegan package in *R* (Oksanen et al. 2013), and to assess dispersion of the NMDS ordinations Levene's test was performed using the the betadisper function in vegan. ANOVA was performed to determine the difference in alpha diversity when comparing all days. For beta diversity measures, the Kruskal-Wallis rank sum test was used to compare all groupings. Pairwise Wilcoxon rank sum tests were used for pairwise comparisons between all beta and alpha diversity metrics.

To compare the composition of samples using OTU presence/absence, OTUs were filtered in *mothur*, using a 0.0001 abundance cutoff of OTUs; these cutoffs excluded low abundant OTUs and samples with low read counts, respectively. OTU data was converted to presence/absence and the data frame was filtered to calculate the total number of OTUs for wild samples only. An OTU was considered "shared" if it was found in at least 70% of samples and "unique" if it was only ever found in a single individual snake. In addition, OTUs were considered specific to each "species", "venom" type, and "location" if they were seen in at least 50% of the individuals in each category and never seen in another group; with the exception of the Texas location which required a 75% cutoff because there were only 4 individuals from this location. Using these cutoffs, no duplicate counts were possible. OTUs were considered "other" if they were not shared between >=70% of all samples nor >=50% in one species, venom type, or location but were also not unique to an individual. To determine the proportion of OTUs lost in each snake from the wild sample and the proportion gained in captivity, the total number of OTUs observed across all time points was calculated in R for each individual. Those OTUs that were in the first wild sample but never again seen in a captive sample for that individual were considered unique to the wild. OTUs that were seen in captivity and not in the wild for a single individual were considered unique to captivity. The OTUs that were in both the wild and the captive samples for a single individual were considered shared OTUs. The mean for each category across the samples was calculated in *R*. To show the percentage of OTUs explained by each category, the relative abundance

obtained before converting to presence/absence was calculated for OTUs unique to the wild, captivity, and shared at both time points.

Linear Discriminant Analysis Effect Size (*LEfSe*) (Segata et al. 2011) was used to determine differential abundance of OTUs between samples from different venom types. An alpha value of 0.05 was used for the Kruskal-Wallis test among venom types as well as for the pairwise Wilcoxon test between species. Pairwise comparison among species were performed among species with the same name and the stricter all-against-all strategy for multi-class analysis was used. *LEfSe* was run twice; once with the complete gut microbiome data and once with only *C. scutulatus* samples from Arizona/New Mexico. The differentially abundant OTUs were plotted for both of these runs using the Galaxy server. A heatmap was generated using heatmap.2 in the package gplots (Warnes 2012) in *R* to plot differentially abundant OTUs. The function heatmap.2 was also used to plot the OTUS in the venom microbiome samples that were present at ≥1% abundance.

Animal Use and Care

Handling and collection of animals was permitted by Arizona Game and Fish Department (#SP622613), New Mexico Department of Game and Fish (SCP# 3697), and Texas Parks and Wildlife Department (#SPR-0713-098). Sampling methodologies and captive housing at Clemson University were approved by Clemson IACUC (AUP #2017-067). All procedures and housing follow standard protocols for non-traditional species care (O'Rourke DP, Cox JD 2018)

2.3 Results

Sequence Generation of Gut Microbiota Samples

To investigate whether location, venom type, and species affect the gut microbiota of rattlesnakes, we collected cloacal swabs from twenty-one rattlesnakes belonging to five species in different geographical areas, detailed in Table 2.1. Upon capture, snakes were immediately swabbed to sample a pre-capture ('wild') timepoint, sampled weekly in captivity for 6 weeks without feeding ('pre-feeding'), and daily following feeding ('post-feeding') (Figure 2.1). Sequences were successfully generated from 353 samples collected throughout this study for microbiome analysis.

The Gut Microbiome of Southwestern Rattlesnakes is Influenced by Individuality and Host Species

To investigate the factors influencing community structure on all gut samples, we employed Non-metric Multi-Dimensional Scaling (NMDS) on the Bray-Curtis dissimilarity index (Fig. 2.2). Although clear patterns of clustering are not evident in Figure 2.2, PERMANOVA tests for differing centroids between groups were significant for species, venom type, location (Supp. Fig A5), and between individual (Supp. Fig. A6) snakes; all $P < 0.001$. The function betadisper was used determine the homogeneity of dispersion for group clusters and was only significant for species ($P<0.01$) and between individual snakes ($P <0.01$). Thus, variation in the overall composition of the microbiome was most strongly correlated with differences at the species and individual levels.

To determine the taxonomy that was likely to be driving the differences observed between species, genus-level stream plots were made (Fig. 2.2). At the genus level, the

microbiota of all five rattlesnake species appeared to remain relatively stable over time (Fig. 2.2). The most abundant bacterial genera observed across all samples included *Gammaproteobacteria, Bacteroides,* unclassified *Bacteroidetes, Achromobacter,* and *Salmonella*, belonging to the phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Fusobacteria (Fig. 2.3)*.* An OTU in the genus *Salmonella* which is a known cause of zoonotic infections, was found at an average relative abundance of 7.5% in all samples. Interestingly, several taxa at lower abundances were observed in the initial wild samples collected prior to captivity. Alpha diversity of the microbiota samples, as determined by Shannon diversity, also appeared to be increased in the 'wild' timepoint compared to most captive timepoints (Fig. 2.4).
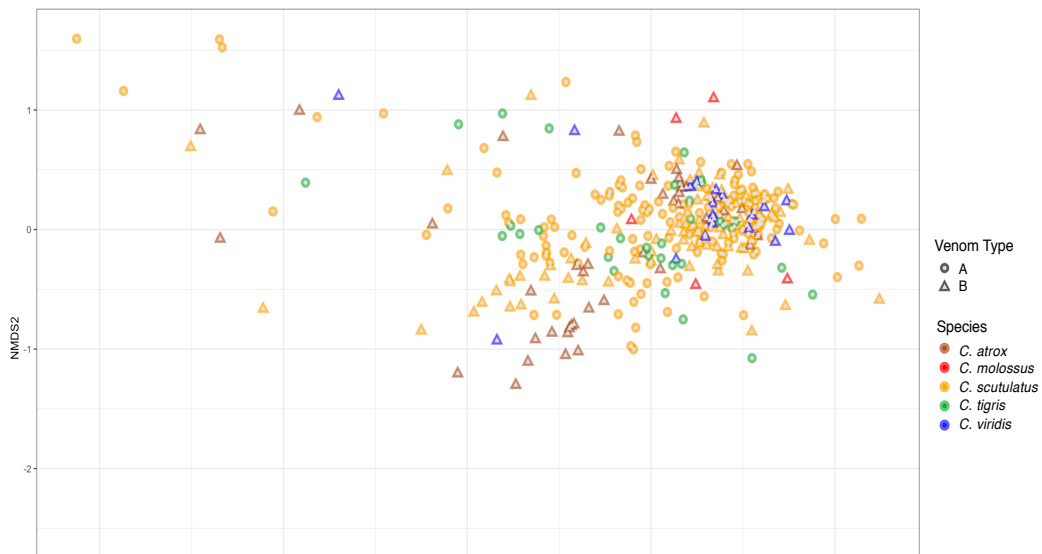
Figure 2.2: The snake microbiota is individualized and driven by host species. Non-metric Multi-dimensional Scaling of the Bray-Curtis dissimilarity metric. Each point represents a single sample, colored by species and venom type (legend). PERMANOVA tests indicate significantly different centroids for venom type and species ($P < 0.001$); dispersion was only significant for species ($P < 0.01$, Levene's test) and not for venom type.
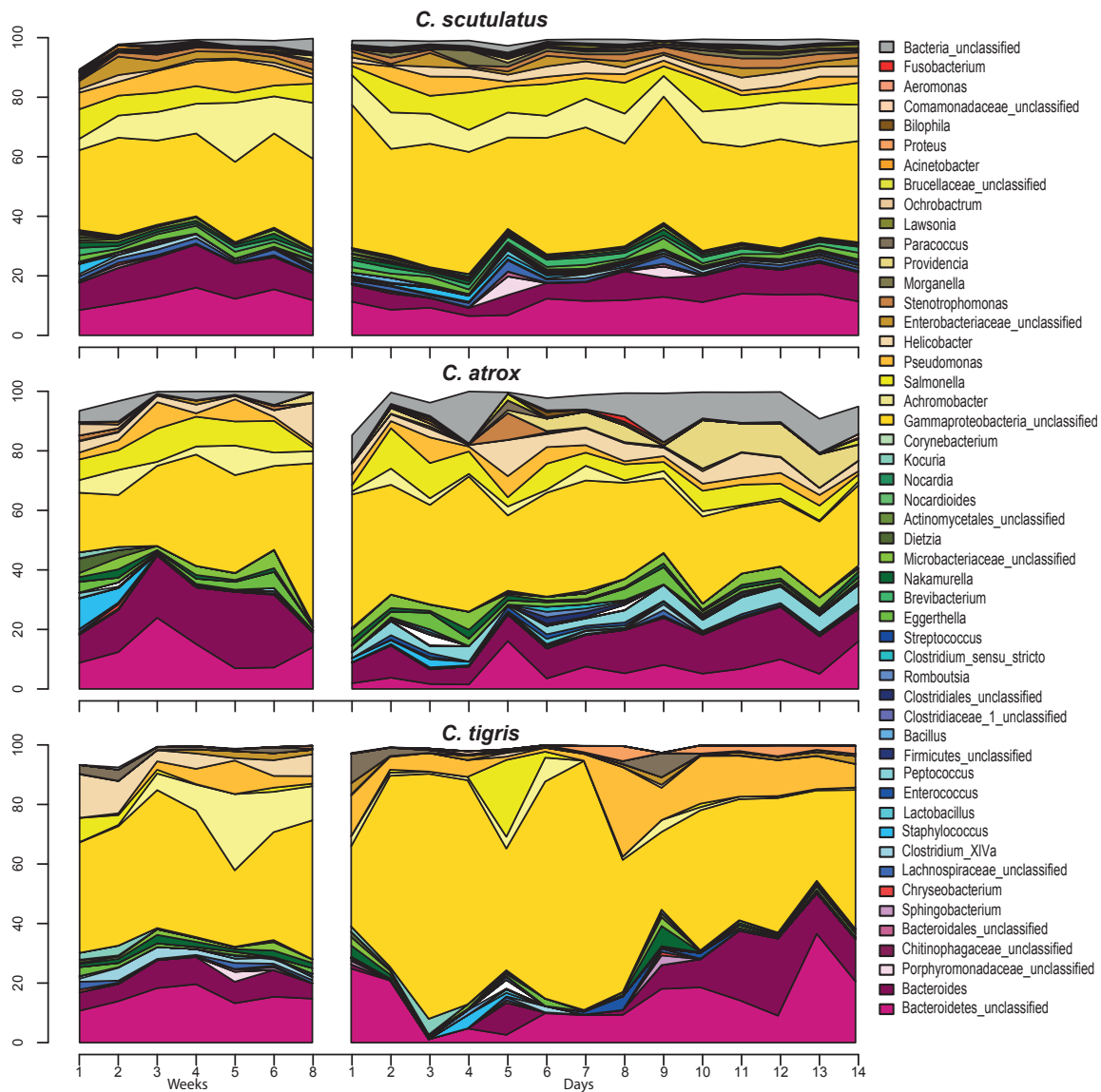
Figure 2.3: The snake gut microbiota is stable over time. Genus-level stream plots of the top 50 most abundant bacterial genera as seen in three species over the course of ten weeks of sampling, measured by relative abundance. *C. viridis* and *C. molossus* were not included because of low sample size both pre- and post-digestion. Colors schemes correspond to phylum; pinks are Bacteroidetes, blues are Firmicutes, greens are Actinobacteria, yellows are Proteobacteria, and red is Fusobacteria. The gap in the timeline represents when feeding took place, followed by daily sampling after pre-feeding weekly sampling.

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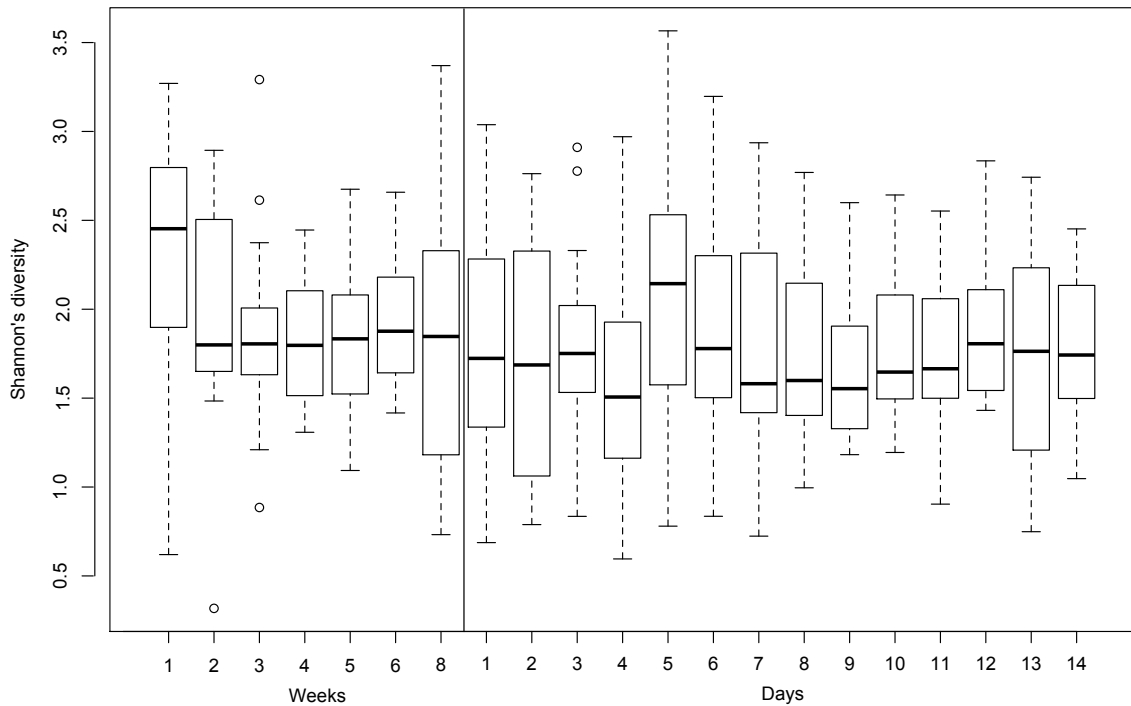
Figure 2.4 The snake gut microbiota decreases in diversity post-captivity. Alpha diversity over time for all individuals as measured by Shannon's diversity (weekly sampling pre-feeding; daily sampling post-feeding). An ANOVA test indicated significant differences between all days (p < 0.01). No pairwise tests were significant (Wilcoxon).

Venom Type Influences Differentiation in the Gut Microbiota at the OTU level

Representative RP-HPLC profiles were created to highlight the presence of hemorrhagic metalloproteases in Type B venoms that are thought to aid in digestion (Fig. 2.5). To investigate whether or not these venom type differences distinguish the gut microbiota, LEfSe was used to identify whether specific OTUs were significantly divergent in snakes classified as venom type A or B. We observed 62 differentially abundant OTUs, with 42 OTUs found to be more abundant in Type B venom animals than in Type A venom animals (Fig. 2.6). Of the OTUs more differentially abundant in

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each venom type, most of the differentially abundant OTUs were classified as

Proteobacteria (37%) and Firmicutes (24%) but Firmicutes and unclassified bacteria

(11%) were more abundant in snakes with type B venom. In type A venomous snakes,

gut microbiota that was differentially abundant was most notable in the genera *Nocardia,*

Sphinogbacterium, Ochrobacterium, and *Paracoccus* (Supp. Fig. A9)*.* More genera (42)

were differentially abundant in Type B venoms, including *Corynebacterium,*

Clostridium_XI, Clostridium_sensu_stricto, Vagococcus, Anaerovorax, and

Sandarakinorhabdus, Bilophila, Lawsonia, Edwardsiella, Proteus, Providencia. We also

used LEfSe to identify differential OTUs in the venom types in only *C. scutulatus* from

Arizona/New Mexico; twenty-five of the OTUs found with the whole dataset were also

found to be differentially overrepresented by venom type (Fig. 2.6). Thus, venom type

was responsible for microbiota differences regardless of species and location effects.
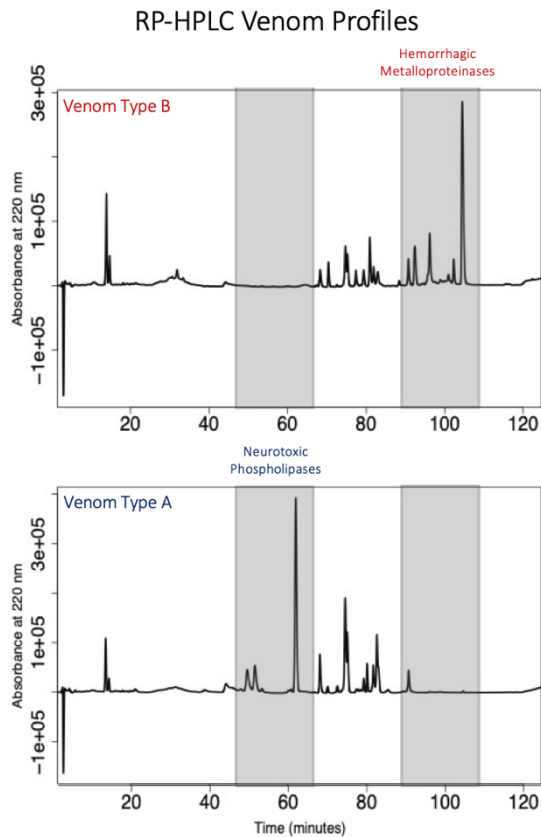
Figure 2.5: Components of Type A and B venoms. RP-HPLC showing venom protein content in Type B (top) and Type A (bottom) venom of *C. scutulatus.* One representative was chosen from each venom class as an example. The left gray marker denotes the presence of Mojave Toxin in Type A venom and absence in Type B venom while the second marker shows the presence of metalloproteinases in Type B venom and their absence in Type A venom.
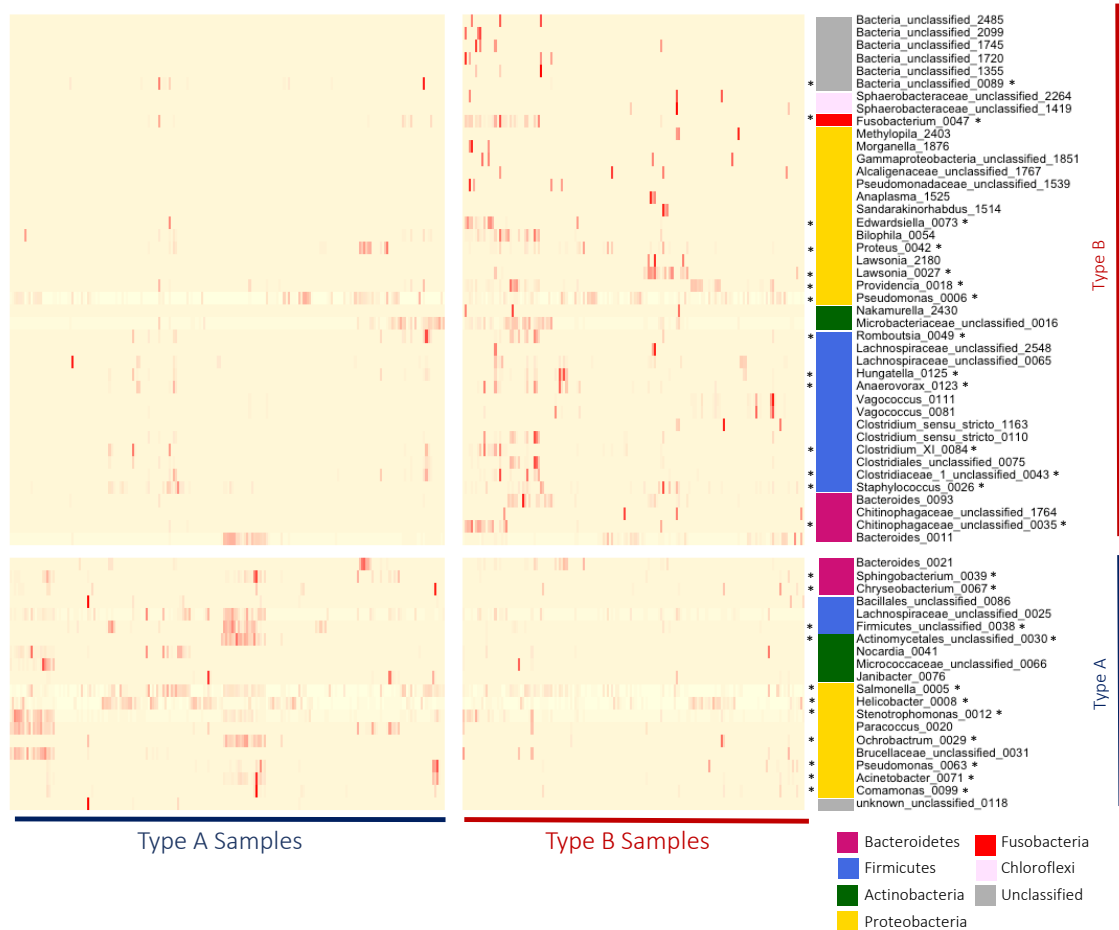
Figure 2.6: Venom type distinguishes the snake microbiota. Heatmap showing the relative abundance of discriminative OTUs with Linear Discriminative Analysis (LDA) scores >2.0 when comparing the gut microbiomes of venom Type A and B individuals (individual samples on x-axis). LDA Effect scores were calculated in LEfSe. 42 OTUs (top OTU panel, y-axis) were identified as OTUs over-represented in Type B individuals and 20 OTUs (bottom OTU panel, y-axis) were identified as being over-represented in Type A venom individuals. The phylum designation for each OTU is shown as represented by the color legend. OTUs with an asterisk were found to be differentially over-abundant in the same venom type when LEfSe was run on *C. scutulatus* found in the same location as when LEfSe was run on the full data set.

Captivity Narrows the Diversity of the Rattlesnake Gut

Temporal alpha diversity measures (Fig. 2.4) and genus level stream plots (Fig.

2.3) suggested that a change occurred from the 'wild' samples to the samples in captivity.

To determine the extent to which change was occurring, we calculated Bray-Curtis

dissimilarity, and visualized by NMDS, between the 'wild' samples and the last pre-

feeding, 'captive' samples. Interestingly, we found that captive samples were

significantly different than wild samples (P<0.001, PERMANOVA; P<0.01, Levene),

and appeared to converge on similar scaling space (Fig. 2.7B), indicating community

structure may be more similar between captive individuals than it is between wild

individuals.

To determine whether or not captive samples had a similar community structure,

shared OTUs and Θ_{YC} distances were calculated between samples from snakes post-

captivity (inter-post) and pre-captivity (inter-pre), and within each snake pre- and post-

captivity (intra-pre-post). Shared OTUs appeared greatest between the pre-captive, 'wild'

samples (inter-pre), likely due to an overall increased OTU diversity in wild populations.

(Fig. 2.7A; top). Θ_{YC} distances showed a significant difference ($P < 0.01$, Wilcoxon rank-

sum test) between the dissimilarity of 'captive' samples compared to one another (inter-

post) and the dissimilarity of 'wild' samples compared to one another (inter-pre) (Fig.

2.7A; bottom). 'Inter-post' samples were more similar to one another than 'inter-pre'

samples (Fig. 2.7A; bottom), confirming the convergence of microbiota composition

from the wild to the last captive time point (Fig. 2.7B). Additionally, Wilcoxon

significance was demonstrated between Θ_{YC} distances of 'intra-pre-post' and 'inter-pre'

comparisons ($P < 0.01$), but not between 'inter-pre-post' and 'inter-post' (Fig. 2.7A;

bottom). Thus, dissimilarity in community structure of 'captive' snakes compared to one

another is similar to the dissimilarity in community structure of each snake compared to

themselves.
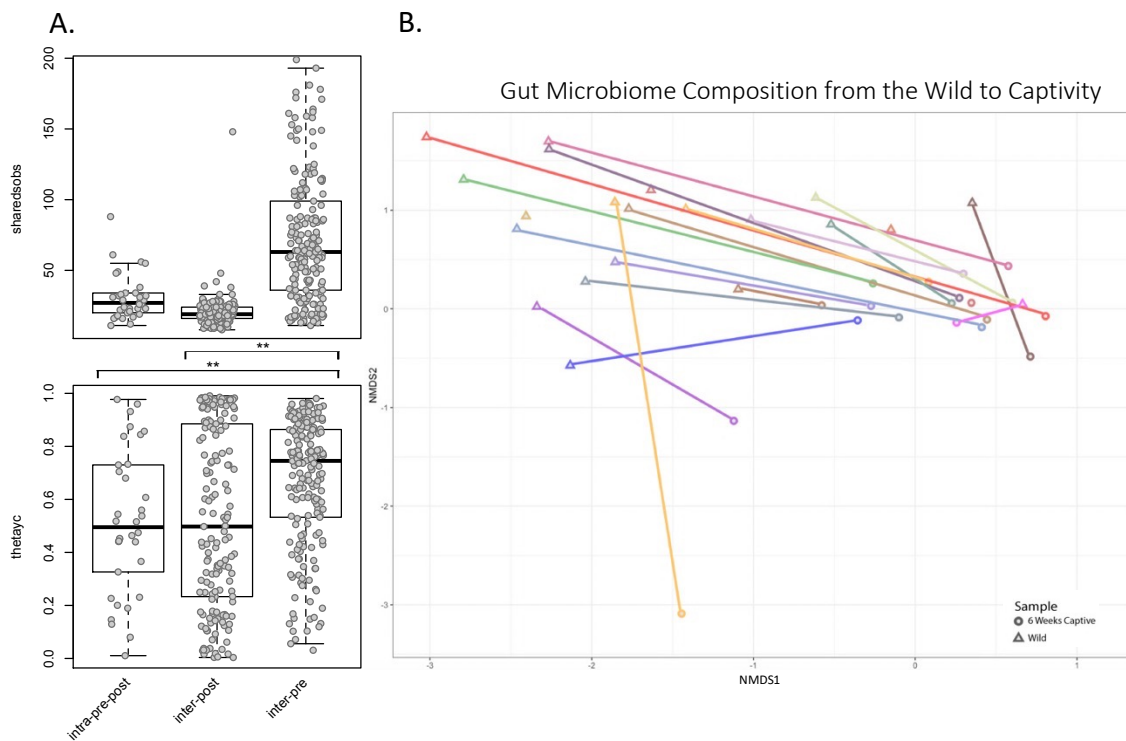
Figure 2.7 The snake gut microbiota is shaped by captivity. A) Box plots showing one individual compared to themselves pre- and post-captivity (intra-pre-post), all combinations of post-captivity samples compared to each other (inter-post), and all combinations of pre-captivity samples compared to each other (inter-pre). The top plot shows number of shared OTUs between the samples tested. The 'wild' samples compared to each other have the greatest number OTUs that are shared (inter-pre). The bottom plot shows Θ_{YC} dissimilarity between the samples (***P < 0.01*, Wilcoxon rank-sum test). B) NMDS showing only the wild sample and the last sample in captivity before feeding for

each individual (*P*<0.001, PERMANOVA; *P*<0.01, Levene's test). The colors represent each individual snake.

To determine when the community structure was changing in captivity, beta-diversity was calculated over time using Θ_{YC} distances. Samples from each snake for all weeks post-captivity were compared to their respective 'wild' sample. All captive weeks were significantly different from the wild samples ($P < 0.001$, Wilcoxon test; Fig. 2.8). The mean Θ_{YC} distances for *C. scutulatus, C. tigris,* and *C. atrox,* the species for which there was more than one sample, were the least similar to their wild sample at four weeks into captivity ($\Theta_{YC} = 0.55$, 0.57, and 0.71). At six weeks post captivity, *C. scutulatus, C. tigris, C. atrox,* and *C. viridis* on average, were all more dissimilar ($\Theta_{YC} = 0.54$, 0.28, 0.51, and 0.86) to their wild sample than they were at one-week post captivity ($\Theta_{YC} = 0.41$, 0.16, 0.51, and 0.86) ($P < 0.1$, Wilcoxon rank-sum test; Fig. 2.8). These data suggest a significant difference in microbiota community structure of snakes in captivity to their respective wild microbiota community, regardless of the time in captivity.
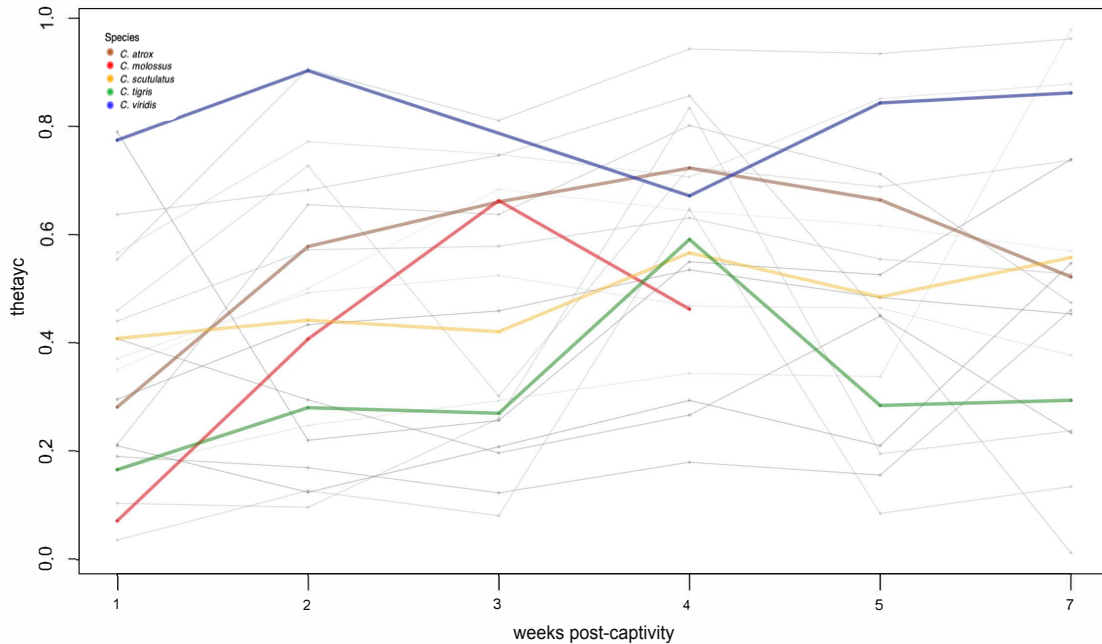
Figure 2.8 Beta diversity over time plotted for each individual post captivity with a colored line for species mean. Θ_{YC} dissimilarity was calculated for each sample compared to their respective wild sample. All weeks were significantly different from the wild samples ($P < 0.001$, Wilcoxon test).

To identify specific OTUs that could be attributed to the observed changes in similarity, we compared the number of OTUs in the 'wild' and 'captive' samples for each snake using presence/absence measures. A mean of 43.0% of all observed OTUs appeared in wild samples but were never observed in subsequent captivity; however, these OTUs only explained a mean of 4.5% of the observed relative abundance of the microbiota, suggesting that a bulk of the lost OTUs represent potentially rare and/or lower abundant OTUs (Fig. 2.9). In contrast, a mean of 42.1% of the OTUs observed in a snake were observed only during captivity, and never in their wild timepoint; on average,

these OTUs accounted for 16.7% of the relative abundance in the snakes. The shared

OTUs account for only a small proportion (only 15.2%) of the OTUs when looking at

number of OTUs present; however, these few OTUs make up about 78.7% of each snake

when looking at the relative abundance (Fig 2.9). To identify the taxonomy of the OTUs

being lost and gained, OTUs belonging to the 'wild' and 'captive' samples were

classified taxonomically. The OTUs lost from the wild to captivity represent sixteen

different phyla, whereas the OTUs that are gained in captivity represent eight phyla (Fig.

2.9)
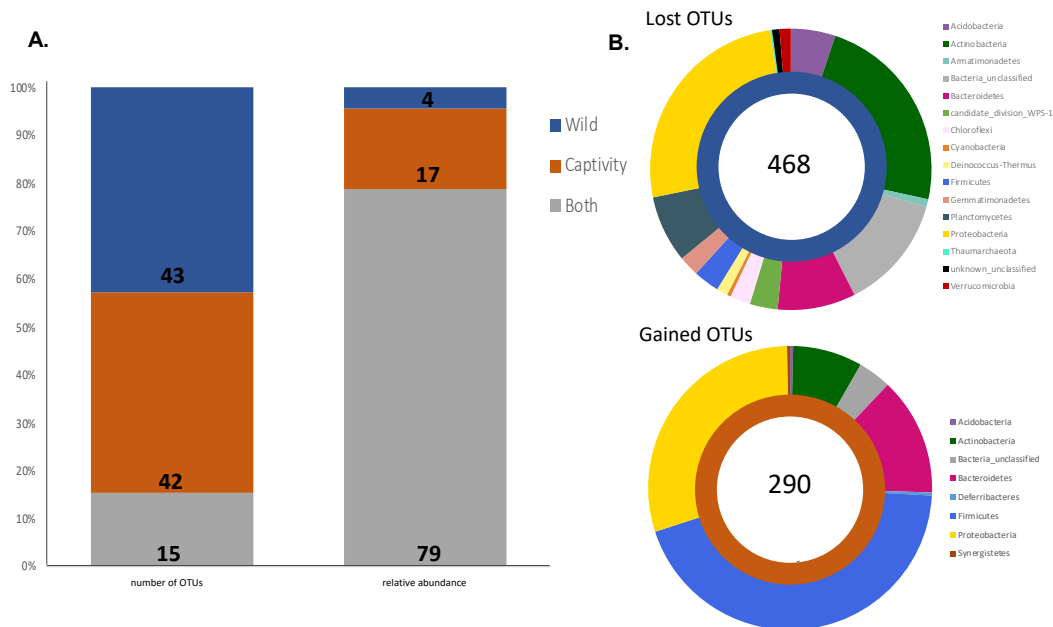
Figure 2.9 Captivity shapes the 'wild' snake microbiota. A) Total number of OTUs
(left) and relative abundance represented by the OTUs (right) observed in wild, captive,
or in both sample types. OTUs were classified as wild and lost after captivity (wild),
gained in captivity and never in the wild (captivity), and those that are shared in both the
wild and captive samples of an individual (both). B) Phylum-level classification of the
relative abundance of OTUs that are lost in captivity (top, n=468 OTUs total) and gained
in captivity (bottom, n=290 OTUs total).

Microbiota Changes are Greatest Five Days Post-Feeding

Once fed, snakes were sampled daily for 14 days to assess potential rapid changes in microbiota due to digestion. Temporal beta diversity, comparing each sample to the previous sample was calculated using Θ_{YC} distances. Additionally, we compared Θ_{YC} distances from post-feeding comparisons to those observed before feeding. The alpha diversity observed in days 4, 5, and 6 in Figure 2.4 was observed as shifts in dissimilarity in days 4:5 and 5:6 in Figure 2.10. We observed an overall increase in microbiota dissimilarity for days 4 and 5 and days 5 and 6 as compared to the pre-feeding sample (*P<0.05,* Wilcoxon rank-sum test) for *C. viridis*, *C. scutulatus, C. atrox,* and *C. tigris* (Θ_{YC}=0.74, 0.59, 0.58, and 0.40), suggesting changes to the microbiota due to feeding on these days. For all species (*C. viridis*, *C. scutulatus*, *C. atrox*, and *C. tigris*) dissimilarity was significantly smaller between days 11 and 12 (Θ_{YC}=0.2, 0.16, 0.02, and 0.01, *P<0.05,* Wilcoxon rank-sum test) than dissimilarity seen before feeding (Θ_{YC}=0.9, 0.10, 0.25, and 0.32). Comparisons approximately two weeks after feeding, seen in Figure 2.10 as 12:13, 13:14 and 14:15 are not significantly different from the Θ_{YC} dissimilarity seen before feeding. Thus, changes in microbiota composition during digestion appear to be greatest approximately five days after feeding and return back to the levels seen pre-feeding after approximately two weeks in post-feeding.
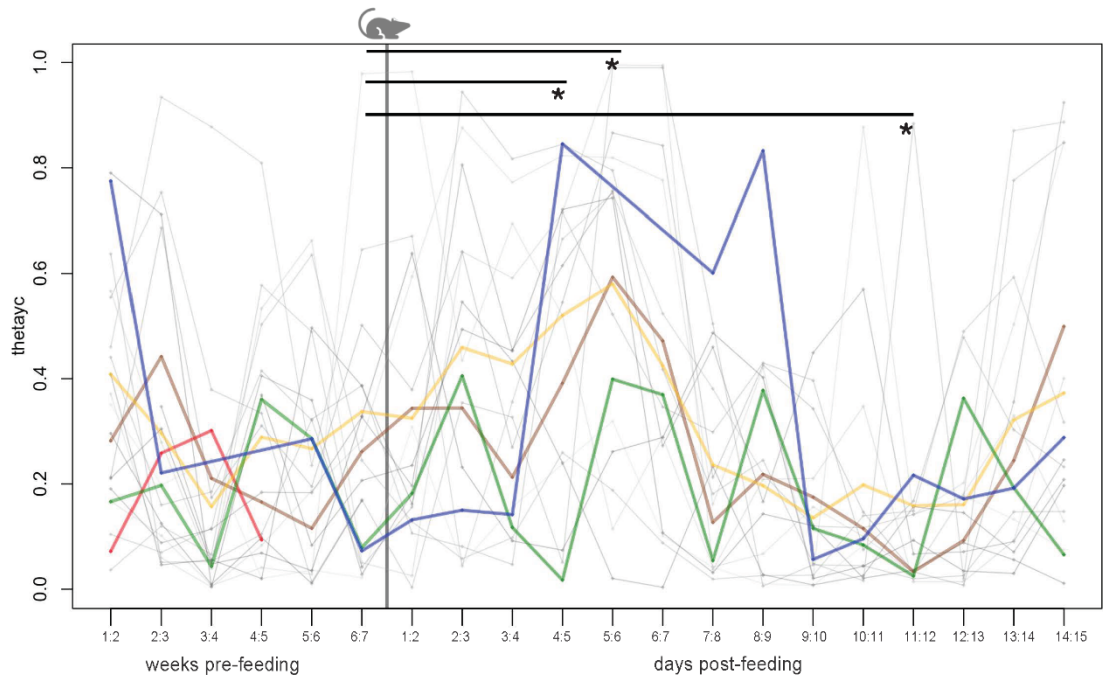
Figure 2.10 Feeding influences the snake gut microbiota. Beta diversity (Θ_{YC} *dissimilarity)* comparing each sample to that of its subsequent sample (x-axis indicates decreasing dissimilarity). The mouse indicates when feeding took place and marks the change from weekly to daily sampling. Colors are representative of the mean for each species. All Θ_{YC} distances were compared to the 6:7 (pre-feeding) Θ_{YC} distance and those that were significantly different were labeled at the top of the plot ('*' $P < 0.05$).

The Venom Microbiome is Similar but Distinct from the Gut Microbiome

We also investigated whether venom itself harbored a microbiota, and how this related to the gut microbiota. We sequenced the venom of five individuals, CLP2730, CLP2734 (we sampled this individual 3 times), CLP2735, CLP2738, and CLP2764 with an average number of sequences being 22,716.14. To identify the OTUs within the venom samples, OTUs with a relative abundance of at least 1% in any venom sample were chosen. The 36 OTUs abundant in the venom samples are similar to the most abundant phyla seen in all the gut microbiome samples, with Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria dominating the most abundant bacteria (Fig 2.11). Three of the OTUs in these phyla belong to the same genera and include *Bacteroides, Staphylococcus, Streptococcus.* Repeat OTUs that remain unclassified within their family are those within *Porphyromonadaceae* and *Chitinophagaceae.* Presence/absence OTU filtering of the venom microbiome samples revealed that of the 78 total OTUs present in more than one venom sample, 18 of those OTUs were rarely seen in gut microbiome samples. Five of the OTUs from the 18 venom-specific OTUs were present in venom samples at >1% abundance and was denoted with an asterisk on the heatmap (Fig 2.11). The venom microbiome specific OTUs consisted of the genus *Porphyromonas* belonging to the phylum Bacteroidetes, two from the genera *Streptococcus* belonging to the phylum Firmicutes, and *Tepidimonas* and *Acidovorax* belonging to the phylum Proteobacteria. There are two OTUs (0167 & 0433) from the genus *Tepidomonas* in our microbiome dataset, although one is of low abundance (not shown in Fig. 2.11). OTU0167 was present in five venom samples and only two gut

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samples, whereas OTU0433 was present in two venom samples and only one gut sample.

OTU 0197 is the only OTU from *Acidovorax* present in our microbiome data set and is

only present in a single gut sample.

Figure 2.11 Heatmap of the most abundant OTUs in the venom samples. The asterisk marks OTUs that are in at least two of the venom samples and are in less than 20 of the 349 total gut samples. Three samples are from the snake CLP2734.
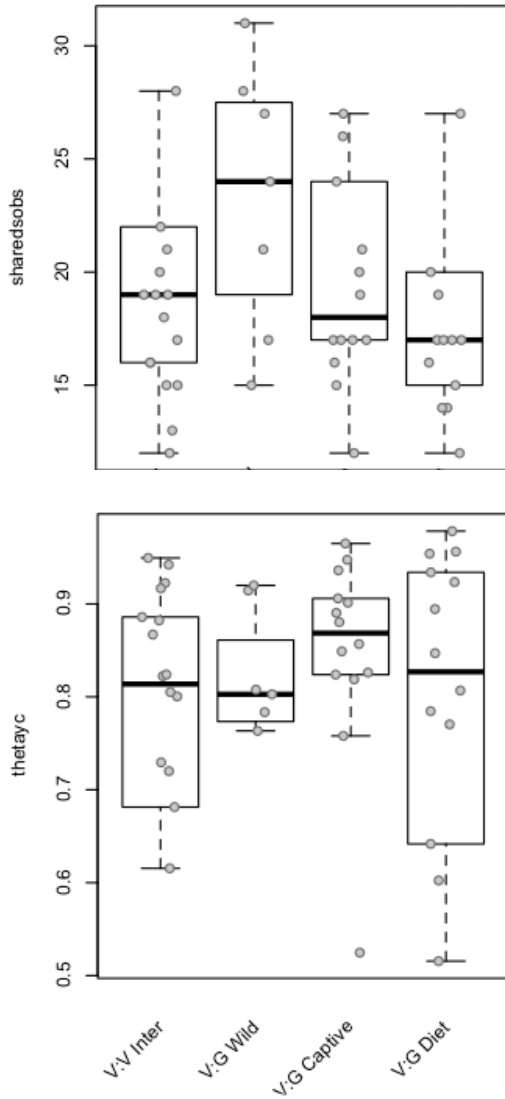
Figure 2.12 The venom microbiome is compositionally similar to the gut microbiome. Box plots showing comparisons between venom samples (V:V Inter) and between each venom sample and two of its respective wild gut sample (V:G Wild), two of its captive gut samples (V:G Captive), and two of its digestion gut samples (V:G Diet). The shared OTU number is on the top and the bottom plot is Θ_{YC} dissimilarity. Groups were not significantly different.

To determine how venom microbiota differed from the gut microbiota, we compared the two types of samples using Θ_{YC} dissimilarity. The comparison among venom samples (V:V Inter) did not include the comparisons between the three samples from the same individual (Fig. 2.12). The number of shared OTUs between samples appears to be highest when comparing the venom samples to their respective wild gut sample (V:G Wild), though this is likely influenced by there being a greater number of OTUs in most wild samples than in the captive ones (Fig. 2.12). The venom microbiomes appear to be most dissimilar to their captive sample than to their wild and digestion samples, although Wilcoxon rank sum tests were not significant between any of the pair. Thus, although there appears to be some variation in dissimilarity, we cannot say whether or not the venom samples are significantly similar to the wild, captive, or digestion samples.

2.4 Discussion

This is the first study of snake microbiota that characterizes different species, locations and compares intraspecifically, a dynamic phenotypic trait (venom type) across individuals. The results of this study suggest that the gut microbiome of rattlesnakes is highly individualized but is influenced by host phylogeny (species) and venom type. We observed a strong correlation between microbiota composition and species, as well as differential abundance of OTUs based on the venom type of the snake, regardless of species.

One of the goals of this study was to determine what bacterial taxa were present in the gut microbiome of five species of rattlesnakes (*Crotalus*) and whether or not this finding was consistent with that of other snake species previously studied in the wild. The major phyla, by relative abundance in all samples, were Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria respectively. This finding is consistent with that reported in a metagenomic study of the timber rattlesnake (McLaughlin, Cochran, and Dowd 2015), a 16S rRNA gene sequencing study of the cottonmouth (Colston, Noonan, and Jackson 2015) and from four species of snake in China (B. Zhang et al. 2019). Colston, Noonan, and Jackson (2015) also compared different regions in the digestive tract and our results appear to be more closely related, in composition, to the cloaca samples in their study where Proteobacteria was the dominant phylum, as opposed to the intestinal samples that were dominated by Bacteroidetes (~45%). The metagenomic study of the timber rattlesnake showed similar composition results to Colston, Noonan, and Jackson (2015) and this study, but interestingly found the pathogen *Salmonella enterica,* a human

pathogen that can also lead to infections in reptile hosts. In this study, we found an OTU in the genus *Salmonella* that had an average relative abundance of 7.5% in all samples, which could be the species *S. enterica*. If this is indeed *S. enterica*, such a high percentage could mean that the snakes are possibly susceptible to infection and is important to note for the purpose of containing zoonotic disease.

Studies evaluating the microbiome of other reptiles in the wild show similar, but distinct profiles from the snake microbiomes. The wild avian microbiome reported in Grond *et al.* (2018) is also dominated by Firmicutes (~45%), Bacteroidetes (~12%), Actinobacteria (~8%), and Proteobacteria (~25%); however, wild birds reported a much higher average relative abundance of Firmicutes and a lower level of Bacteroidetes than seen here (8% and 26%, respectively). Crocodile lizards have demonstrated a similar profile to the avian and snake microbiome, although lower levels of Firmicutes and Actinobacteria were reported compared to our study (Jiang et al. 2017). Our data, in combination with these previous studies, indicate that snakes have a similar but distinct gut microbiome from other reptiles and adds additional evidence suggesting Proteobacteria play a much bigger role in reptiles than in other vertebrates (Grond et al. 2018).

To the authors knowledge, this is the first study to compare differences in the gut microbiome between two species with the same venom type or the same species with different venom types, although some studies have looked at differences between the microbiomes of venomous and non-venomous animals (Krishnankutty et al. 2018; Qin et al. 2019). Qin et al. (2019) found that venom-secretion snakes had shared gut microbiota

features as compared to non-venom-secretion snakes, implication venom as playing a role in selection and/or maintenance of specific microbes in the snake gut. Although venom variation within a population of Mojave rattlesnakes is driven by local selection, the microbes present within these snakes and their potential for mediating interaction between the phenotypic variation of venom and digestive performance have been largely unexplored until now. Significant microbiota composition differences exist between venom types in the species of *Crotalus* we examined. An OTU from the genus *Sphingobacterium,* phylum Bacteroidetes, was differentially abundant in venom Type A snakes and was never seen in a venom Type B snakes in the wild, regardless of geographical location of the host. This OTU had a relative abundance of 0.13% relative abundance, the 39th most abundant OTU in this data set. Notably, species in the genus *Sphingobacterium* are known for having a high concentration of sphingolipids which are known to play a significant role in providing bioactive metabolites to hosts (Gault, Obeid, and Hannun 2010). Another notable OTU in the phylum Bacteroidetes that was differentially abundant in Type A venom animals was from the genus *Chryseobacterium*, with an average relative abundance of 0.053% in all samples. Some species of *Chryseobacterium* have been found to have the ability to degrade collagenous matrices such as feathers because of their production of metalloproteinase enzymes (Pandey et al. 2019; Venter, Osthoff, and Litthauer 1999); metalloproteinases are the major component missing in Type A venoms that are thought to aid in digestion for Type B venom individuals. The differential abundance of the OTU from the genus *Fusobacteria* in Type B animals is notable as *Fusobacteria* is treated as a pathogen when found in humans

(Aliyu et al. 2004). More information is needed to determine whether or not these differentially expressed bacteria are functionally replacing an aspect of digestion that is missing in animals that have either metalloproteinase (Type B) or phospholipase (Type A) venom activity. However, the evidence does suggest that there is a difference in the microbiomes of snakes with these venom types, further suggesting a possible link between venom type and dietary specialization (Barlow et al. 2009; Daltry, Wüster, and Thorpe 2003; Holding et al. 2018)

Captivity and Digestion as Modulators of Microbiome Diversity

Here we found a decrease in diversity of the gut microbiome after captivity. We tracked the same individual snakes from the wild into captivity. Beta-diversity calculations show that the rate of microbiome change from the initial wild sample is highest in the first 4 weeks post captivity, after which point it stabilizes. Previous studies, most of them focusing on mammals, have looked at the effects of captivity on the gut microbiome (Clayton et al. 2016; Gibson et al. 2019; McKenzie et al. 2017; Tong et al. 2019). However, most of these studies have compared captive and wild animals of the same species. Unlike in Kohl & Dearing (2014) which noted only a small change in diversity as the desert woodrat entered captivity, we found that microbiome composition and to some extent microbiota diversity was affected when entering captivity. Our findings were consistent with the findings in a paper by Kohl, Skopec, & Dearing (2014) that suggested a greater loss in diversity upon captivity among species that are dietary specialists than among species that are generalists. The OTUs lost and gained by the snakes in captivity show losses that appeared to be from a diverse range of phyla,

whereas the OTUs gained in captivity were limited to only a few phyla. This indicates a narrowing of bacterial diversity in the gut microbiome once snakes enter captivity. The potentially rare OTUs lost in captivity may represent passing environmental microbes but could also play a role in community diversity that affects overall snake health. The lack of beneficial microbes can be an indicator of the overall health of an individual, and the loss of microbial communities in captivity often leads to malnutrition and disease (McKenzie et al. 2017; Tong et al. 2019).

Many of the most abundant OTUs are shared between the gut and venom microbiome samples and the venom microbiome at the phylum level looks similar to that of the gut microbiome. However, some of the highly abundant venom microbiota OTUs were rarely seen in gut microbiome samples. *Porphyromonas* is commonly seen in the salivary microbiome of humans, dogs, and other animals hosts (Fournier et al. 2001; Summanen, Lawson, and Finegold 2009). *Acidovorax* is a genus marked by acid degradation properties that has species commonly found to be present in the microbiome of tumor growth and cancer in humans, especially oral and lung cancers (Dulal and Keku 2014; Sanapareddy et al. 2012; Zhen Zhang et al. 2019) and many species are plant pathogens (Adhikari et al. 2017), but are not often recovered in healthy animal microbiomes. Similarly, *Tepidimonas* is not a common microbiome isolate outside of the context of disease states such as lung cancers (Greathouse et al. 2018). These results indicate a venom-specific microbiota in rattlesnakes that is distinct not only from their gut microbiota but are distinct from normal, healthy animal microbiota.

We found that the microbiota changed significantly from four to six days post-feeding and that there was less change in the microbiota after twelve days post-feeding. Tattersall *et al.* (2004) looked at the thermogenesis of rattlesnakes during digestion and found that after 168 hours (seven days), snake body temperature returned to the same as that of the pre-feeding time, indicating the conclusion of digestion. Significant changes in microbiota community structure likely occurred during the final passage of digestive material through the cloaca so it is notable that Tattersall *et al.* (2004) saw the conclusion of the thermogenesis associated with digestion just after we saw a spike in the differences of microbiota community structure. These results indicate that rattlesnakes finish with the major energy expenditure of digestion at approximately one-week post-feeding, and the changes occurring in the microbiota that are responsible for aiding in digestion are back to pre-feeding levels at approximately two-week's time.

Concluding Remarks

The gut microbiome in rattlesnakes is marked by high inter-individuality in the wild followed by a decrease in bacterial diversity and inter-individuality once in captivity. Across all treatments, i.e. wild, captivity, and digestion, the gut microbiome was different between animals with both hemorrhagic Type B venoms and neurotoxic Type A venom types. One explanation for a difference in the microbiomes between animals with different venoms is that the tissue-degrading venom peptides called snake venom metalloproteinases, which constitute a large portion of Type B venoms and are lacking in Type A venoms, are thought to aid in digestion by means of tissue-degrading venom peptides (Mackessy 2010; Thomas and Pough 1979). A functional replacement of

bacteria for the tissue-degrading properties may be occurring in Type A venoms that lack metalloproteinases. More research needs to be done to determine whether or not a functional replacement is driving the difference in the gut microbiomes of these venom phenotypes.

Chapter 3

Concluding Remarks

3.1 Implications

The work presented here demonstrates a significant advance in knowledge of an understudied group in the field of microbiome research. No species in this study has a published sequenced microbiota, and there are only two published microbiotas from the genus *Crotalus* (McLaughlin, Cochran, and Dowd 2015; Allender et al. 2018). Importantly, this study provides a foundational basis for investigating a functional replacement of lost venom components with microbiota. This study also adds to the work of other research that has looked at the effects of captivity on the gut microbiome (Clayton et al. 2016; Gibson et al. 2019; Kohl, Skopec, and Dearing 2014; McKenzie et al. 2017; Tong et al. 2019) but is the first to look at the microbiome of a non-mammalian organism entering into captivity.

3.2 Shortcomings

Most of the shortcomings of this study are due to sampling numbers and the methodology of sampling. A larger sample size would always be beneficial statistically; specifically, more samples from Type B *C. scutulatus* individuals would have strengthened interpretation of the observed differences. Additionally, sampling more type B animals from a different geographical location than Arizona, the only currently represented location for type B animals, would have removed potential species-level

effects in differentiating the microbiota by venom expression. A major shortcoming of this study was in not having longitudinal replicates for wild sampling. It would have been especially helpful to have an equal number of snakes from each location, regardless of venom type, for better testing of environmental differences in the gut microbiota. More than one wild sample for each individual would have allowed us to investigate the stability of the wild microbiota itself and confirm whether lost OTUs represented spurious species from the environment or the presence of rare taxa that are part of the snake microbiota naturally. Finally, more representation from some of the host species, such as the individual *C. tigris* sampled in this study, would have allowed more investigation into species-level differences in the microbiota.

Our results demonstrate preliminary data for the presence of a snake venom microbiota. Nevertheless, more research is needed to establish whether or not these microbiota observed were from the venom gland. Sampling more than five individuals would have strengthened these data, as well as sampling a more even number of Type A and Type B individuals to investigate differences between these two types. All of the venom samples were sampled in captivity; because of the change in diversity observed in the wild to compare to captivity in the gut microbiota, a venom sampling in the wild could be different. Additionally, a swab from the oral cavity of each snake could have aided differentiation of oral versus venom microbiota. Nevertheless, given the repeat patterns observed in the venom samples, I am confident that the samples are distinct from the gut microbiota itself. Further comparison of oral, gut, and venom would aid differentiation and identification of a venom microbiota.

Lastly, although 16S rRNA gene sequencing was appropriate in this study to assess the microbiome community structure of a large number of individuals, this type of analysis is limited to microbiota community analysis. Without species-level identification, gene, and transcriptomic expression data, functional predictions as to what is driving community structure remain unknown.

3.3 Future Directions

An interesting direction to take would be to focus on the functional characteristics of the microbes which are differentially expressed in the gut of snakes with differing venom type. Metagenomic sequencing of the gut microbiota of multiple individuals from each venom type in both the wild and in captivity would allow for strain-level differentiation, as well as provide a list of potential functions from the genomes of these microbes that are related to venom. Sequencing the metagenomes of venom in the venom gland of the same individuals to determine whether or not the same bacterial species were colonizing the gut and the venom gland would also allow for identification of species that are present in both environments. Furthermore, meta-transcriptomics testing during digestion could reveal which bacterial functions are expressed during digestion. These could be compared to the lists of differentially expressed species identified in the metagenomic study. Species that are both found to be expressed in the transcriptome during digestion and were differentially abundant in the metagenome are likely functionally replacing an aspect of the venom that is missing in the venom type that animal possesses.

Another potential area of research in which this study could expand is in venom gland microbiome research. There is a dearth of information on venom gland microbiomes (Ul-Hasan et al. 2019), even though there are many examples of microbes with similar characteristics to venom toxins (Marino-Puertas, Goulas, and Gomis-Rüth 2017; Sitkiewicz, Stockbauer, and Musser 2007). For example, many microbes can secrete toxins that lead to tissue necrosis similar to that seen by hemorrhagic venom toxins (Ovington 2003). Additionally, there is a possibility that some bacteria may benefit from the effect different venom types have on a wound (Saravia-Otten et al. 2007), such as activation of the kinin system by bradykinin potentiating peptides (BPPs), which some bacteria can exploit to support their proliferation (Loof, Deicke, and Medina 2014). Understanding what microbes are in venom glands could uncover commensalism between bacteria and venom, and a possible functional replacement of lost venom components with bacteria.

There are many of future areas of research in which to study in regard to microbiota interactions with venom components, as little research is being done in this field. Investigation of the similarity of the virulence pathways (specifically immune and nociceptor regulation) of venom peptides and bacterial toxins would lead to a better understanding of how commensalism may play a role in venom-microbe interactions, whether the microbes are in the gut or in the venom itself.

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Appendix A

Supporting Information for Chapter 2: The Gut Microbiome of Southwestern Rattlesnakes as Revealed by 16S rRNA Sequencing

A.1 Supplementary DNA Extraction Methods

After inhibitor removal, each Collection Plate contained 850 µl of supernatant. For each 96-well plate, a solution of 2 ml ClearMag Beads was added to 85 ml of ClearMag Binding solution and vortexed. From this bead/binding solution, 875 µl was added to each well of the 2 ml collection plate containing the supernatant collected after inhibitor removal. The collection plate was then shaken at 500 rpm at room temperature for 10 min followed by incubation on a magnet for 10 min. Liquid was then discarded, leaving the beads. The plate was then removed from the magnet and 500 µl of ClearMag Wash solution was added to each well before being placed back on the magnet for a 10 min incubation. With the plate still on the magnet, liquid was again discarded leaving the beads. The wash and removal steps were repeated twice more. Once completed, the plate was removed from the magnet and 100 µl of Elution Buffer was added to each well. The plate was shaken for 25 min at 500 rpm and placed on the magnet for another 10 min incubation. While on the magnet, 100 µl was pipetted from each collection plate to a storage plate. The isolated DNA was quantified using the Qubit dsDNA BR Assay Kit

(ThermoFisher Scientific, Q32850) on the Qubit 3.0 Flurometer (ThermoFisher Scientific) following the manufacturer's protocol.

A.2 Additional description of OTUs seen in wild samples

To more specifically investigate the microbiota of snakes in their natural habitat, we focused on the initial 'wild' timepoint collected from each snake. In comparing the total number of OTUS observed in all of the snakes, we observed that 28.2% of all the observed OTUs were unique to single individual snakes (Fig. A1). OTUs that were shared between individual snakes only accounted for 3.7% of all these OTUs (Fig. A1). Approximately 3% of total OTUs in the microbiome of snakes in the wild were unique to geographical location, whereas 1% of OTUs where specific to either species or venom type. The majority of OTUs in the wild samples (63%) did not meet the strict cutoff for group-level designation; however, they represent OTUs that are shared between some snakes regardless of location, species, or venom type.
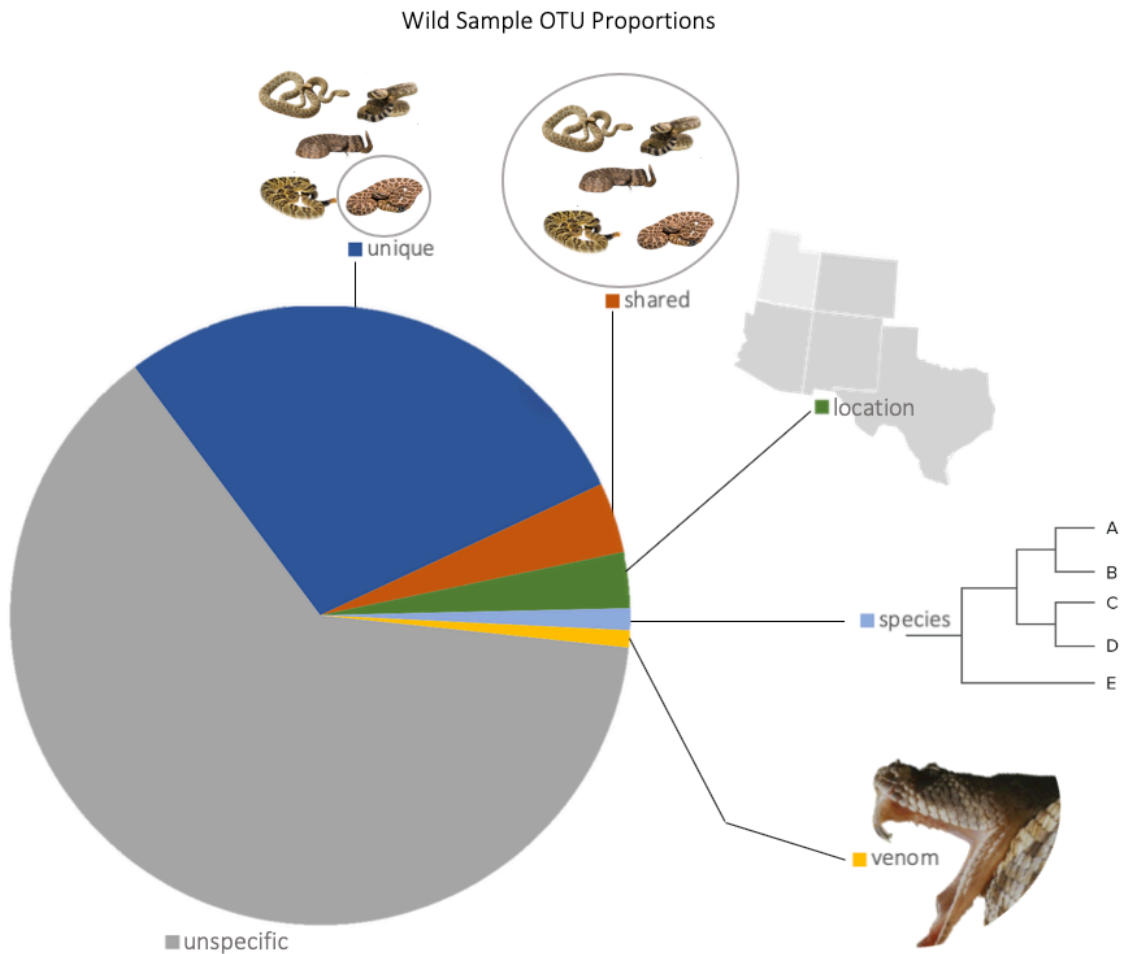
Figure A1: The pie chart represents the total number of OTUs across all wild samples (789 in total). Presence/absence was calculated for each OTU in all wild samples. Unique (223 OTUs) indicates an OTU that was found in one individual snake and not found in any other. Shared (29 OTUs) indicates OTUs that were in >70% of all individual. Location (23 OTUs), Species (9 OTUs), and Venom (7 OTUs) are OTUs that met a strict cutoff of >= 50% in one group and are never seen in another group. e.g. For venom type, an OTU would have to be in at least half of venom Type A individuals and never seen in a venom type B individual to be able to be considered a venom type OTU, etc. Unspecified OTUs (498) are those that did not fall into a specific category. Any OTU that was only in two individuals was automatically placed in this category because it was not unique to an individual nor would it meet the 50% cutoff for any of the group categories.
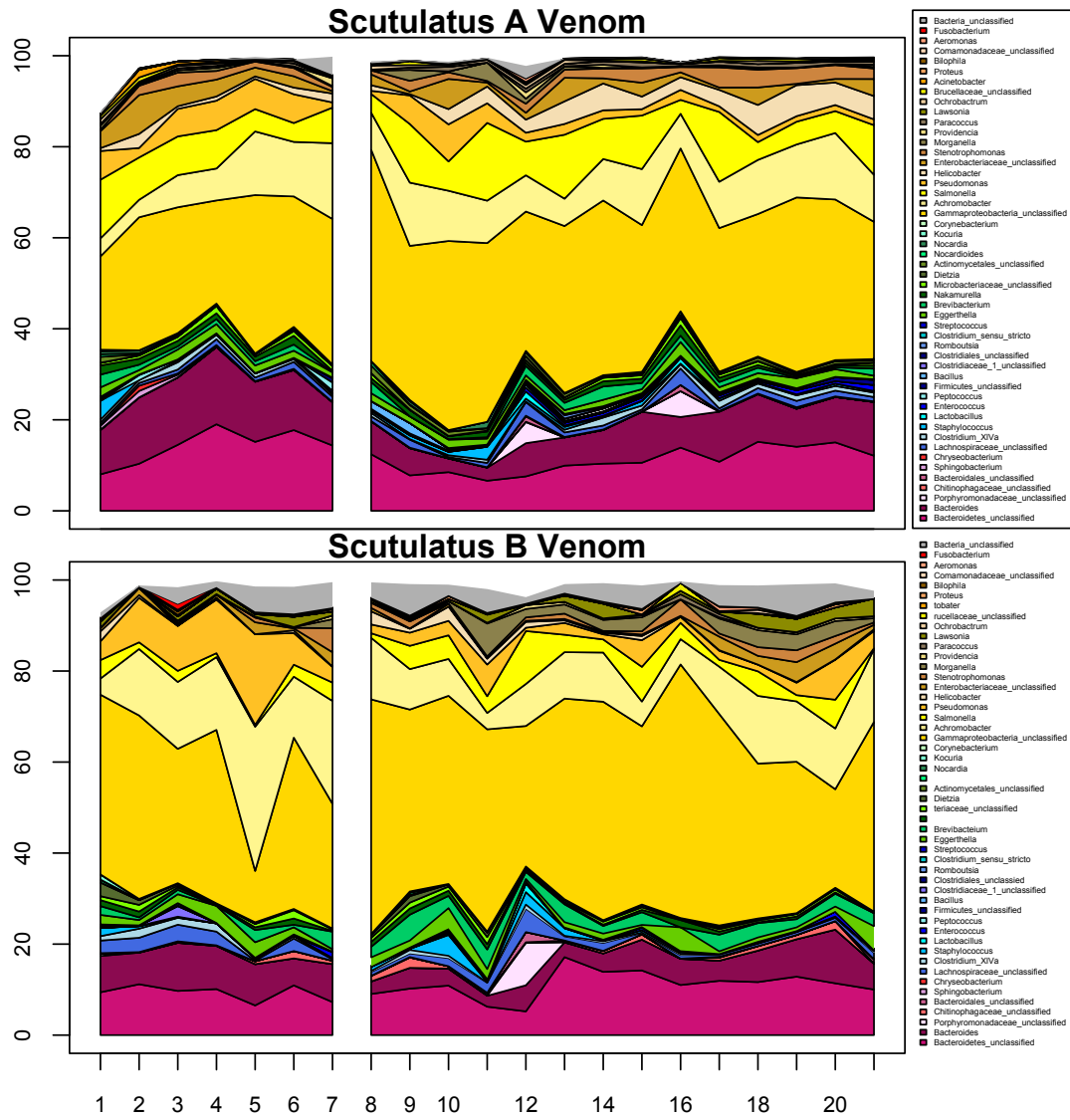
Figure A2. Genus-level stream plot of top 50 genera across all samples, comparing only the venom Type A and venom Type B *C. scutulatus* samples.
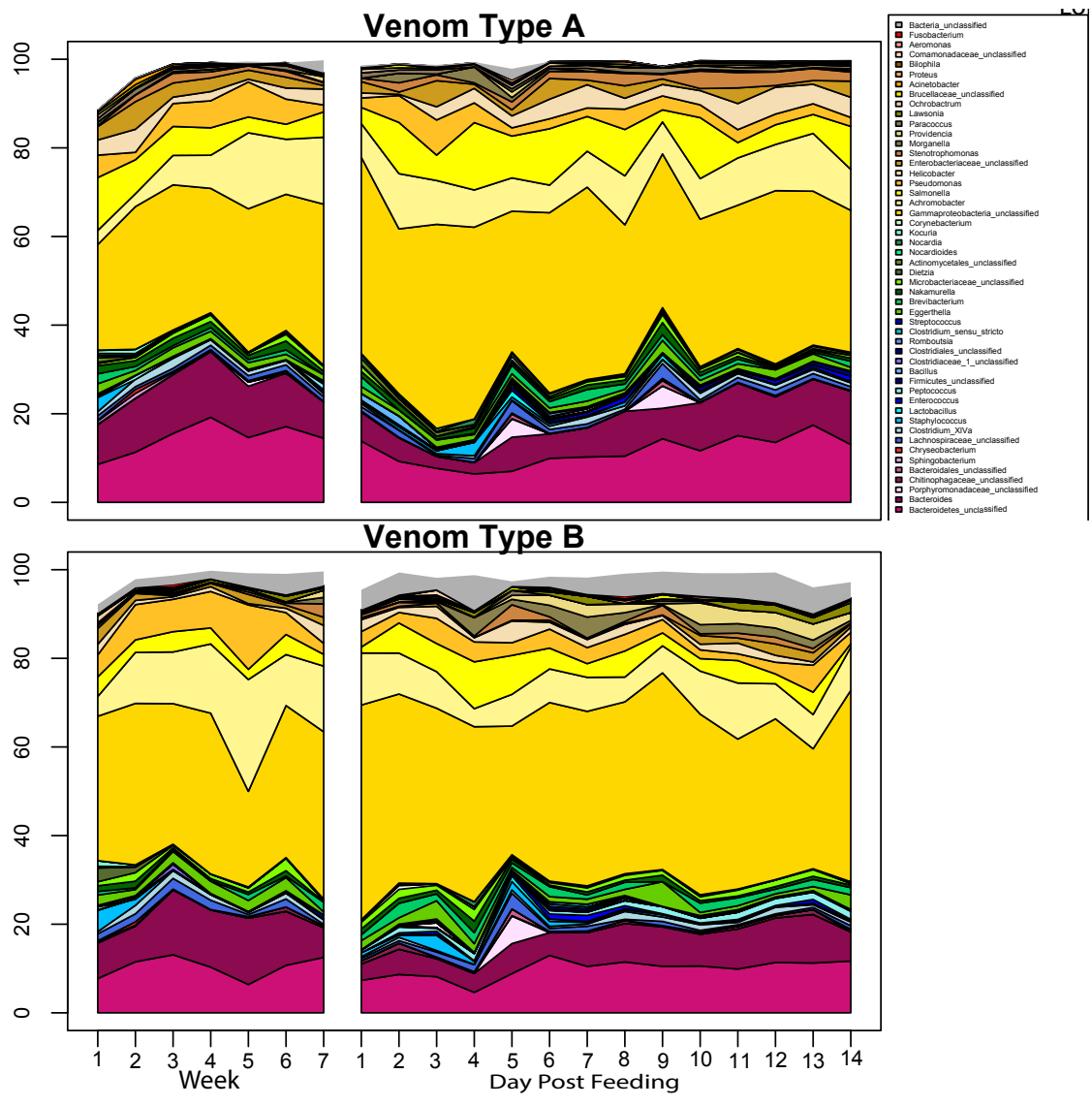
Venom Type A

Venom Type B

Bacteria_unclassified
Fusobacterium
Aeromonas
Comamonadaceae_unclassified
Bilophila
Proteus
Acinetobacter
Brucellaceae_unclassified
Ochrobactrum
Lawsonia
Paracoccus
Providencia
Morganella
Stenotrophomonas
Enterobacteriaceae_unclassified
Helicobacter
Pseudomonas
Salmonella
Achromobacter
Gammaproteobacteria_unclassified
Corynebacterium
Kocuria
Nocardia
Nocardioides
Actinomycetales_unclassified
Dietzia
Microbacteriaceae_unclassified
Nakamurella
Brevibacterium
Eggerthella
Streptococcus
Clostridium_sensu_stricto
Romboutsia
Clostridiales_unclassified
Clostridiaceae_1_unclassified
Bacillus
Firmicutes_unclassified
Peptococcus
Enterococcus
Lactobacillus
Staphylococcus
Clostridium_XIVa
Lachnospiraceae_unclassified
Chryseobacterium
Sphingobacterium
Bacteroidales_unclassified
Chitinophagaceae_unclassified
Porphyromonadaceae_unclassified
Bacteroides
Bacteroidetes_unclassified

Week

Day Post Feeding

Figure A3. Genus-level stream plot of top 50 genera across all samples, comparing all samples with both venom Types.
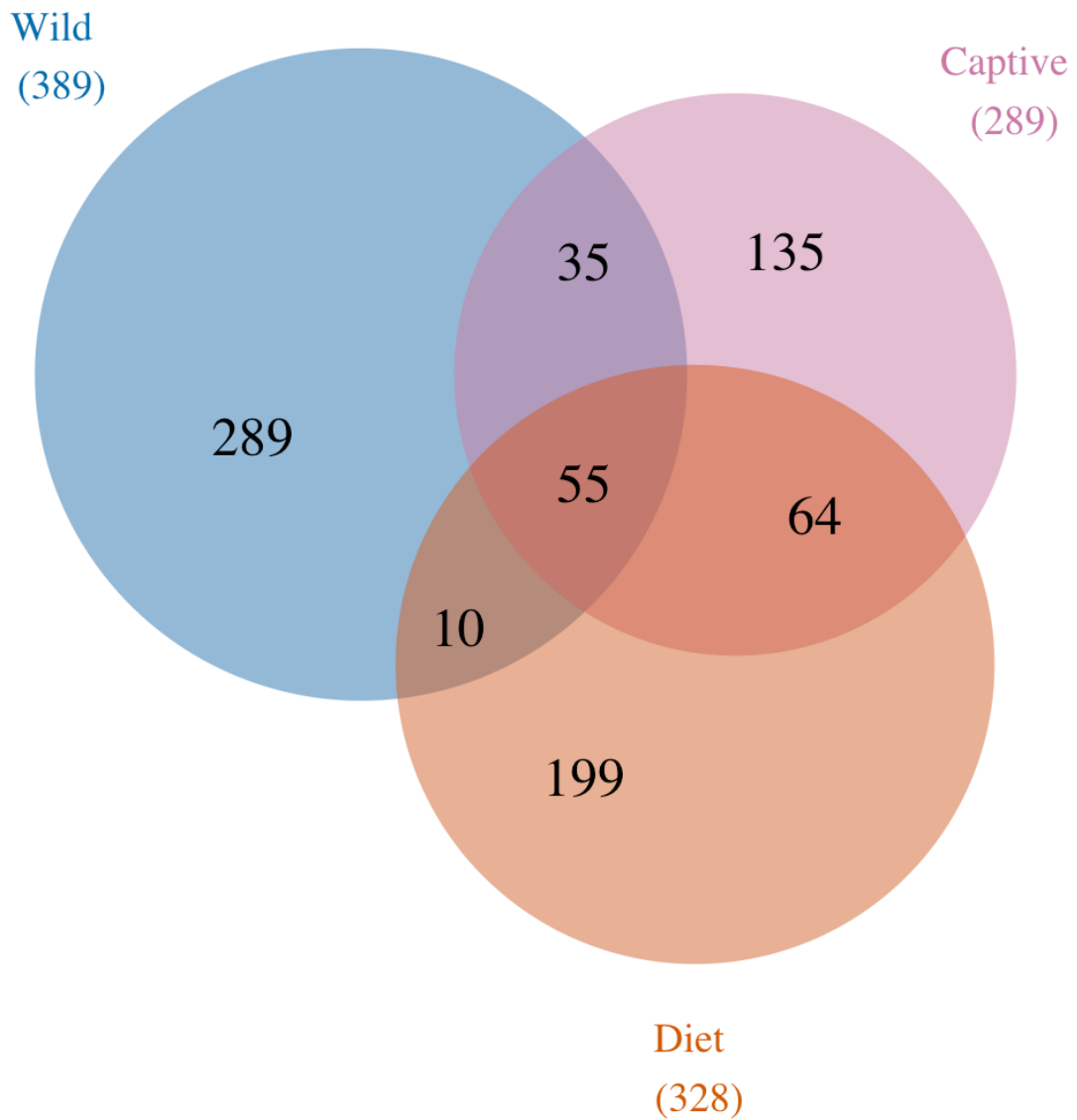
Figure A4. OTU presence/absence comparison of the average number of OTUs seen within an individual, and how many of the OTUs are exclusive to 'wild', 'captivity', 'diet' and how many are shared between these categories.
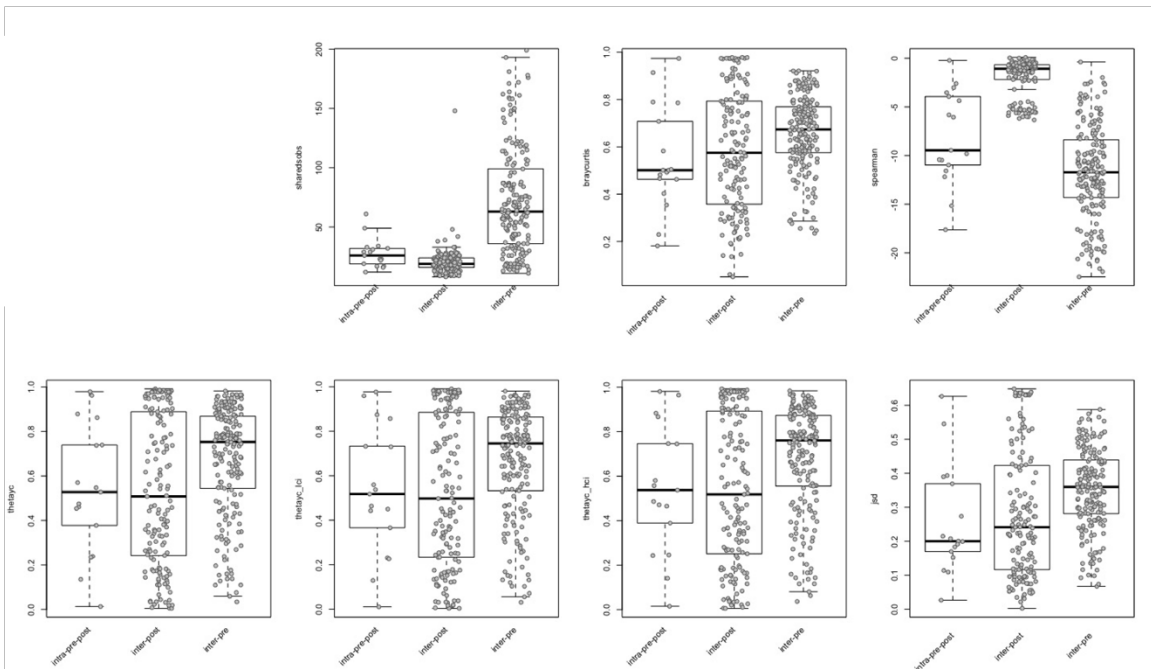
Figure A5. All statistics performed on box plots showing one individual compared to themselves pre- and post-captivity (intra-pre-post), all combinations of post-captivity samples compared to each other (inter-post), and all combinations of pre-captivity samples compared to each other (inter-pre).
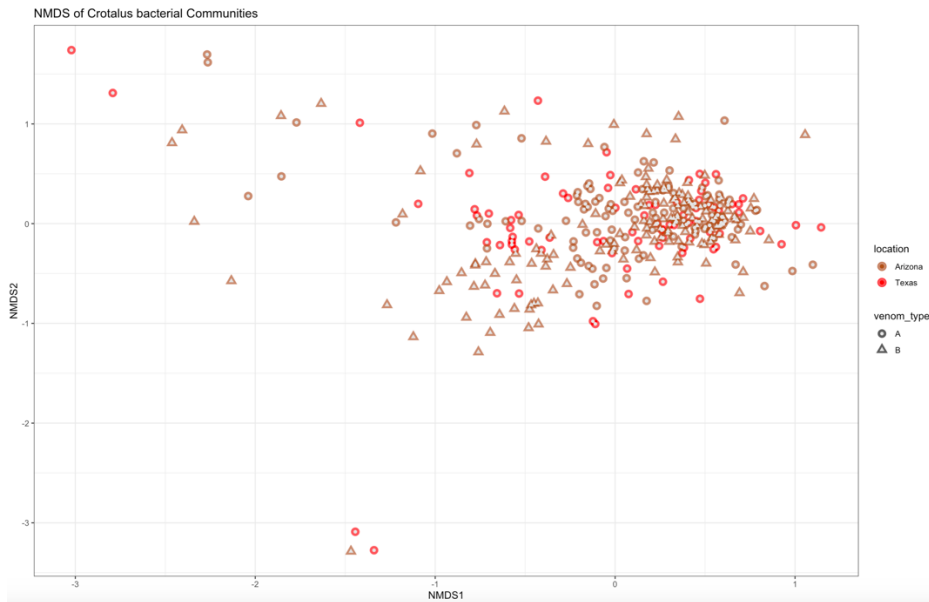
Figure A6. NMDS plots comparing location and venom type. PERMANOVA tests indicated significantly different centroids for venom type and location ($P < 0.001$), however dispersion was no significant for either (Levene's test).
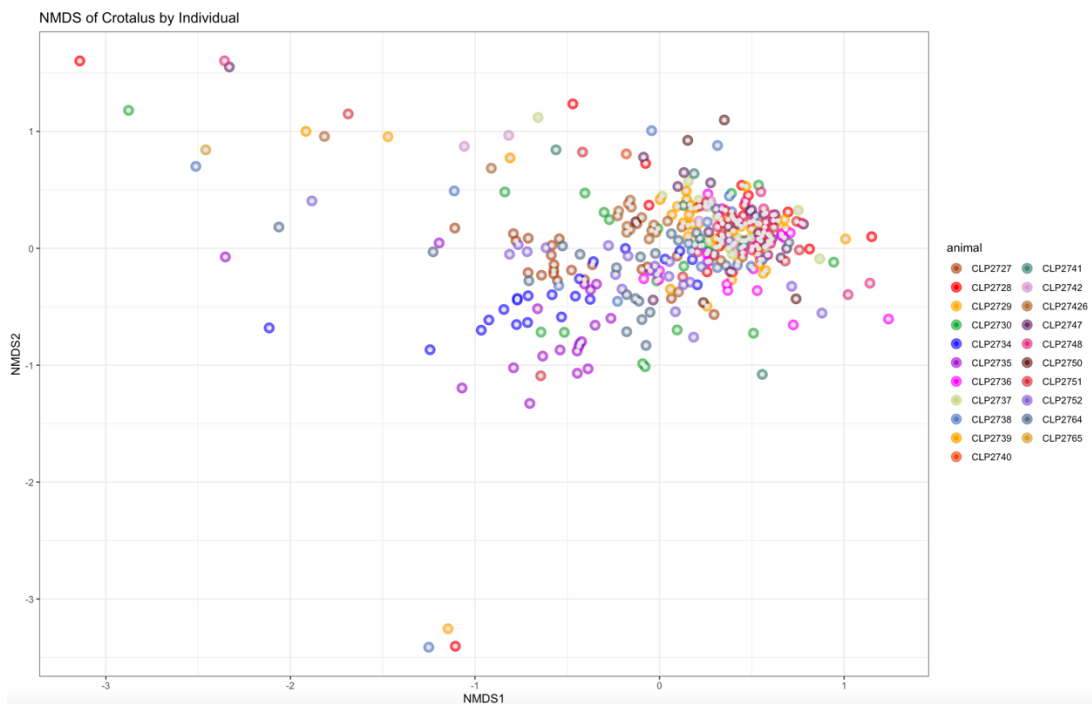

Figure A7. NMDS plots comparing all individuals. Both PERMANOVA and Levene's tests indicated significantly different centroids ($P < 0.001$) and dispersion ($P < 0.001$) between groups
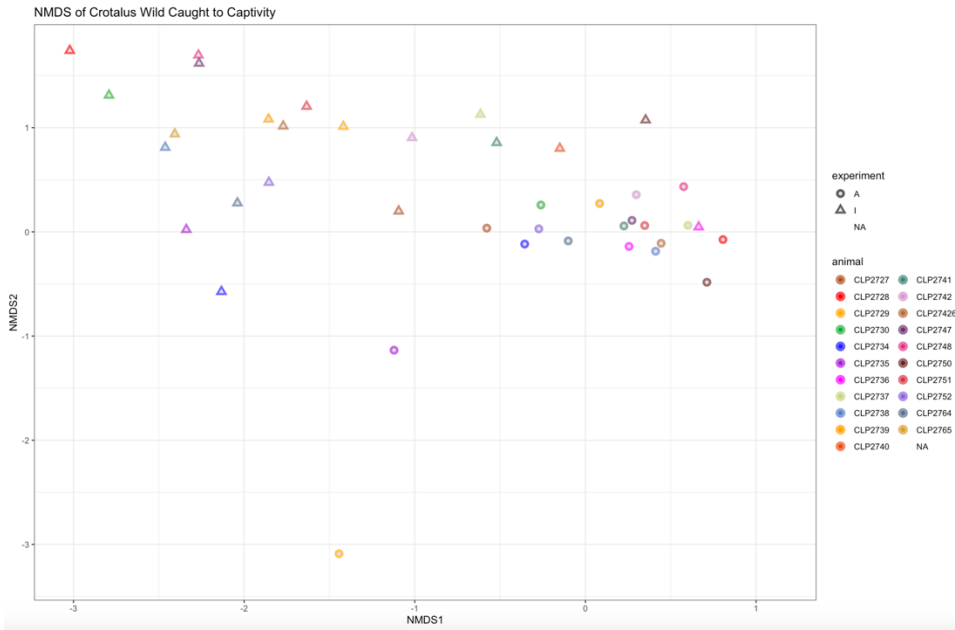
Figure A8. NMDS plots comparing the wild sample to the last captive samples for each snake. Triangles represent the first time point and circles represent that final time point.
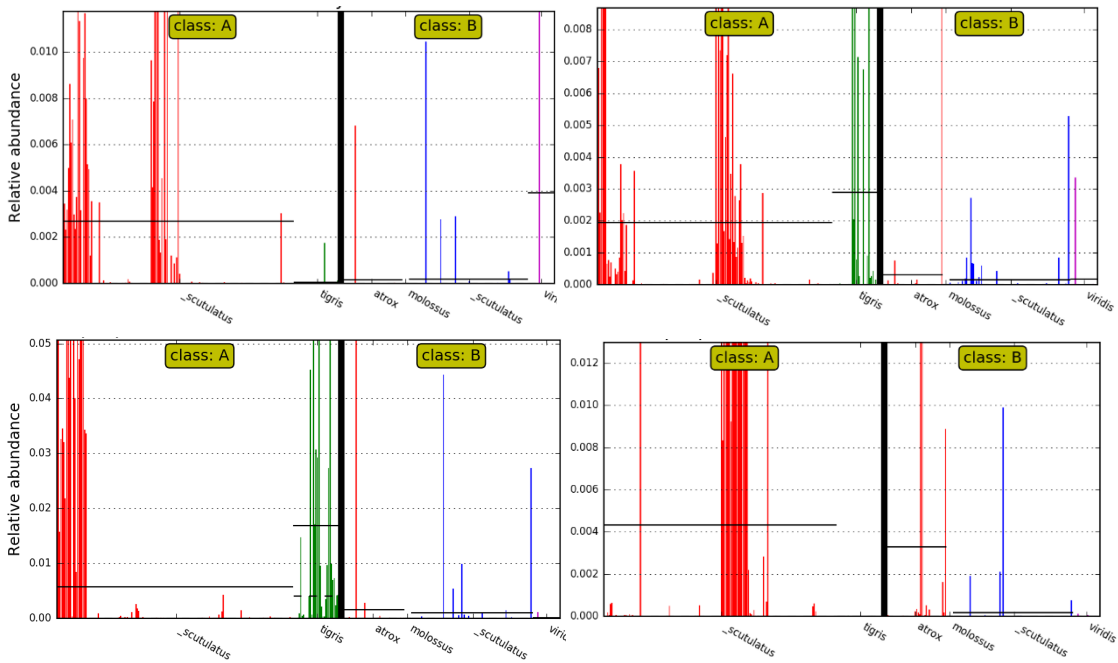

Figure A9. LEfSe generated plots showing notable differences in OTU relative abundance in samples with Type A venoms; OTU0041 *Nocardia* (top left), OTU0039 *Sphingobacterium* (top right), OTU0020 *Paracoccus* (bottom left), and OTU0029 *Ochrobactrum* (bottom left).