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ORIGINAL ARTICLE

Taxonomy, not locality, influences the cloacal microbiota of two nearctic colubrids: a preliminary analysis

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Abstract

Background The gut microbiota is an emerging frontier in wildlife research and its importance to vertebrate health and physiology is becoming ever more apparent. Reptiles, in particular snakes, have not received the same attention given to other vertebrates and the composition of their wild gut microbiome remains understudied. The primary goal of this work was to describe the cloacal microbiota of two Colubrids, the Eastern Gartersnake (*Thamnophis sirtalis sirtalis*) and the Northern Watersnake (*Nerodia sipedon sipedon*), and if their cloacal microbiota differed as well as if it did between a wetland and upland population of the former species.

Methods and results We utilized next-generation sequencing of cloacal swabs—a non-destructive proxy for the gut microbiota. The cloacal microbiome of Eastern Gartersnakes (N = 9) was like those of other snakes being comprised of Proteobacteria, Bacteroidetes, and Firmicutes, while that of Northern Watersnakes (N = 6) was dominated by Tenericutes. Seven microbial operational taxonomic units (OTUs), all members of Proteobacteria, were shared among all individuals and were indicative of a core microbiome in Eastern Gartersnakes, but these OTUs were not particularly relevant to Northern Watersnakes. The latter had greater OTU richness than did Eastern Gartersnakes, and habitat did not have any apparent effect on the microbial community composition in Eastern Gartersnakes.

Conclusions Our findings suggest host taxonomy to be a determining factor in the cloacal microbiota of snakes and that Tenericutes are associated with aquatic habitats. This is the first report to examine the cloacal microbiome of these species and provides a useful foundation for future work to build upon.

Keywords Cloacal microbiota · Eastern Gartersnake · Northern Watersnake · Habitat · Tenericutes

Introduction

Animals exhibit a complex relationship with their microbiome—the microorganisms living both externally and internally with a host. The role of the gut microbiome is multi-faceted as it has been identified to assist the host with a variety of physiological functions including nutrient acquisition, behavior, immune function, development, and maintenance of homeostasis [1, 2]. Due to its importance in animal health, the gut microbiome has become an increasingly important facet in animal conservation efforts [3] and animal ecology in general. With the advent of next-generation-sequencing (NGS), inventories of gut microbial communities have risen exponentially in recent years; however, the great majority of these research efforts have focused on a narrow range of animal taxa with < 10% of studies on non-mammals [4]. This lack of information regarding non-mammal microbiomes calls for the need of a better understanding on the microbial community of these taxa.

Reptiles are nearly twice as diverse as mammals and occupy a diverse array of habitats where they serve as important prey and predators. Some limited research is available on the gut microbiota of lizards [5–7], turtles [8], and crocodilians [9], while snakes have received even less attention with Timber Rattlesnakes (*Cortalus horridus*; [10, 11]) and Cottonmouths (*Agkistrodon piscivorus*; [12]) being

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the only wild North American taxa examined. Colubridae, despite being the largest family of snakes, have been vastly overlooked regarding their microbiome (but see [13, 14]), indicating a clear void in current knowledge.

The composition of the gut microbiota is known to be influenced by a variety of factors including diet [7, 15], host taxonomy [16–18], and habitat [19, 20]. Few studies have examined how an environmental gradient can affect the microbiome composition, with some authors finding great overlap between habitats [21], while others noted shifts in microbiota concurrent with habitat variation [19, 20]. The ability of a host's microbiome to be highly plastic provides a mechanism that allows large shifts in diet without compromising its functionality [22]. Monitoring the difference in the gut microbiome community between disparate habitats could highlight microbial taxa associated with specific functions necessitated by the animal behavior and diet.

In this study, we aimed to increase the overall understanding on the cloacal microbiota community of two common North American Colubrid snakes: the Eastern Gartersnake (*Thamnophis sirtalis sirtalis*) and the Northern Watersnake (*Nerodia sipedon sipedon*) (Fig. S1). The Eastern Gartersnake is the most common species in the northeastern United States and is both a habitat and dietary generalist [23]. The Northern Watersnake is more of a habitat and dietary specialist only occupying aquatic/semi-aquatic habitats in the northeastern United States and preying nearly exclusively on fish and anurans [23].

The objectives of this study were three-fold: (1) describe the cloacal microbiome composition of both Eastern Gartersnakes and Northern Watersnakes, (2) compare the microbiome of Eastern Gartersnakes from a wetland and upland habitat to determine what role diet and habitat have on microbiome composition, and (3) compare the microbiome of sympatric Eastern Gartersnakes and Northern Watersnakes to elucidate how host taxonomy influences differences in the microbiome. As both species can be ubiquitous, this information could be used to determine what aspects influence their respective gut microbiomes.

Materials and methods

Location

Snakes were sampled from two sites within Powdermill Nature Reserve located in Rector, Westmoreland County, Pennsylvania, USA in July 2018 (Fig. S2). Since 2003, the snake population has been sampled using 1 × 3 m corrugated metal coverboards. The two sites surveyed were Crisp Pond (N: 40.1635, W: 79.2669), a wetland habitat comprised of multiple artificial ponds with a large abundance of Northern Green Frogs (*Lithobates clamitans melanotus*) and emergent aquatic vegetation, and Friedline Right Entrance (N: 40.1346, W: 79.2859), an old field habitat consisting of mixed rangeland surrounded by mixed forest. The two sites are > 2 km apart indicating there is likely little chance of individuals moving between the sites based on home ranges of both species [23].

Microbiome sampling

All snakes were captured under coverboards and snout-vent length (SVL), sex, and presence of embryos/food were recorded. Only individuals exceeding 50 cm SVL were selected for microbiome analysis to ensure that adult individuals were sampled because the gut microbiota stabilizes with age in vertebrates [24, 25]. Furthermore, only females were investigated to limit the potential of sex-associated differences in the microbiome. The protocol for collection of cloacal samples followed, with minor modifications, that of Colston et al. [12]. Briefly, a sterile alcohol wipe was used on the outside of the cloaca to minimize the chance of transient environmental microbes contaminating the cloacal swab. A sterile cotton-tipped applicator was inserted approximately 2 cm into the cloaca and rotated 5 times to ensure coverage on the swab. The applicator swab was placed in an autoclaved 2 mL tube, stored on ice in the field, and then placed in a − 4 °C freezer within 2 h of collection. A field control was taken by waving an applicator in the air before placing in a 2 mL tube. All snakes were released at site of capture upon completion of microbiome sampling. Swabs were transported to Southern Illinois University Carbondale on ice and then stored at − 80 °C until DNA extraction.

Microbial DNA extraction

Microbial DNA was extracted from thawed swabs using the GenCatch™ Plasmid DNA Mini-Prep Kit (Epoch Life Science) following manufacturer instructions with minor modifications. Reagent volumes were tripled to ensure coverage of the swab and the initial incubation at 60 °C was increased to 3 h. Lab controls were used to identify any microbial DNA present in the extraction kit reagents. Extracted DNA was quantified using Take3™ Microvolume Plate on a Microplate Spectrophotometer (BioTek Instruments INC). Cloacal samples averaged 47.65 µg/µL (range 13.05–221.36), while the field control value was 12.32 µg/µL and lab controls averaged just 5.34 µg/µL. All extracted DNA samples were stored at -80 °C prior to sequencing.

Microbiota sequencing

PCR products of a portion of the bacterial 16S rDNA gene were prepared from each extracted DNA sample in triplicate and pooled into one amplicon library using bacterial

universal primers (515F/806R) and Earth Microbiome Project protocols [26], with minor modifications: PCR was run for 30 cycles instead of 35, and 0.04% Bovine Serum Albumin was included in each reaction. The library was spiked with 10% PhiX and sequenced through 2150 paired-end cycles using the Illumina MiSeq at the Kansas State Integrated Genomic Facility. Raw sequence data were processed using the QIIME software package [27]: Sequences were quality filtered, joined and demultiplexed, and assigned to operational taxonomic units (OTUs) based on 97% DNA sequence similarity using the open-reference workflow. The Ribosomal Database Project classifier [28] was used to assign taxonomy, representative OTU sequences were aligned to the GreenGenes v. 13.8 16S rDNA gene reference database, and nonaligned OTUs, singletons and doubletons were removed prior to further analysis. Furthermore, any chimeric sequences were identified via ChimeraSlayer and removed. The dataset coming out of pre-processing included a mean ± one standard deviation and median number of reads per library of 9454 ± 3690 and 11,103, respectively, but all samples were reduced to 1650 reads to ensure all samples were included in the analyses. The mean ± one standard deviation and median number of OTUs per sample at this rarefaction depth was 36 ± 15 and 36, respectively, reflecting OTU collection curves that reached a saturation point. Also, the relative abundance of OTUs among all represented taxonomic groups was exported from QIIME for further analysis.

Data analyses

Snakes were placed into three categories for all analyses: Northern Watersnakes (N = 6), Upland Eastern Gartersnakes (N = 4), and Wetland Eastern Gartersnakes (N = 5). The core microbiota represents microbes that are shared in a majority of the sample population and are believed to be evolutionarily tied to the host, perform essential functions in the microbiome, and large-scale changes in their abundance are thought to be associated with health consequences [29]. Due to the small sample sizes, we defined the core cloacal microbiota of the snakes to be the OTUs found in 100% of swab samples. We used the Adonis function in the Vegan package [30] for an analysis of similarity (ANOSIM) in R [31] to compare these categorical data. A principal coordinates analysis (PCoA) ordination analysis was undertaken using QIIME to explore patterns of heterogeneity among all samples included in the study. The output model explained 48.3% and 21.9% of the variation among all samples on Axis 1 and Axis 2. ANOSIM tests were run using Bray–Curtis distance based on the relative abundance of OTUs among the species and habitats. One-way ANOVAs were used to identify differences in alpha diversity metrics among the groups with Tukey HSD post-hoc tests to examine pairwise comparisons. The alpha diversity metrics used were bacterial OTU richness, Faith's Phylogenetic Diversity (PD), and Shannon Diversity index. All values, unless specified otherwise, written as mean ± one standard error.

Results

There were 141,804 total reads from all samples representing 173 unique bacterial OTUs from 16 phyla (Online Resource 1). The number of total reads for each group ranged 1651–13,354 for Northern Watersnakes, 11,103–13,054 for upland Eastern Gartersnakes, and 2981–12,232 for wetland Eastern Gartersnakes.

In Northern Watersnakes, Tenericutes were the most prevalent phyla followed by Proteobacteria, Firmicutes, and Bacteroidetes with no other phylum representing > 1% of the microbiota composition (Fig. 1). In contrast, the cloacae of Eastern Gartersnakes were dominated by Proteobacteria followed by Bacteroidetes, Firmicutes, Tenericutes, and Fusobacteria although the relative abundance of these phyla was influenced by the habitat (Fig. 1). Individuals from the upland habitat had a greater relative abundance of Proteobacteria (81.8 to 67.7%) and Bacteroidetes (11.2 to 9.8%) but lower relative frequencies of Firmicutes (3 to 9.8%) and Tenericutes (1.4 to 9%) when compared to wetland counterparts.

Only seven OTUs were shared among all 15 individuals, indicative of a possible core microbiome among both species. These shared OTUs were *Bordetella petrii*, an unidentified Enterobacteriaceae species, *Morganella morganii*, *Pseudomonas veronii*, an unidentified Pseudomonadaceae

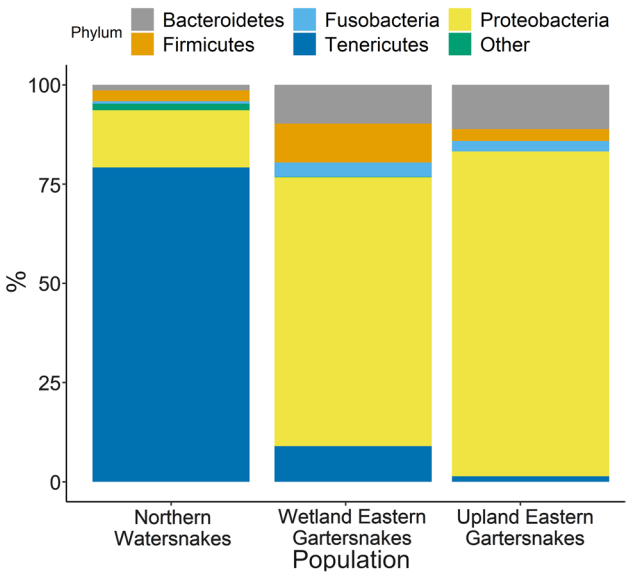

Fig. 1 The relative abundance of bacterial phyla in the cloacal microbiome samples that represented > 1% of reads

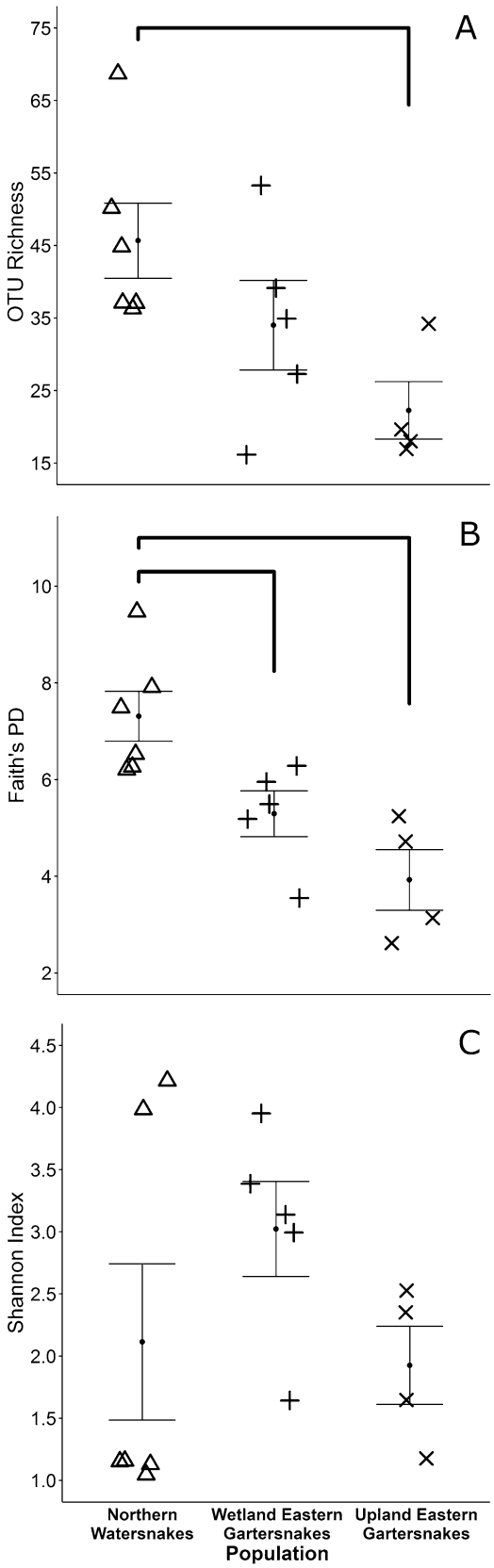

Fig. 2 Comparisons of selected alpha diversity metrics of the cloacal microbiome among Northern Watersnakes (*Nerodia s. sipedon*) and both populations of Eastern Gartersnakes (*Thamnophis s. sirtalis*). Diversity Metrics include: **A** OTU richness, **B** Faith's Phylogenetic Diversity, and **C** Shannon's Index. The center point represents the mean with the error bars ± 1 standard error. Lines between groups represent significant pairwise comparisons (Tukey HSD; $P < 0.05$)

species, *Acinetobacter guillouiae*, and *Sphingomonas echinoides*. The percentage of total reads these OTUs comprised 53.4 ± 3.6% in wetland Eastern Gartersnakes, 74.6 ± 6.0% in upland Eastern Gartersnakes, and only 8.4 ± 0.3% in Northern Watersnakes. Four OTUs were shared among all Northern Watersnakes and upland Eastern Gartersnakes representing 1.3% and 0.2%, respectively, a single OTU (an unidentified *Mycoplasma* species) was shared among all Northern Watersnakes and wetland Eastern Gartersnakes, and only the previously described seven OTUs were shared among all Eastern Gartersnakes.

Examining bacterial OTUs found in all samples within each group revealed that Northern Watersnakes had the highest core microbiota OTU richness (N = 28). Wetland Eastern Gartersnakes (10) and upland Eastern Gartersnakes (12) had a substantially lower proportion of core microbial species. The core microbes within each group accounted for 95.5 ± 2.9% in Northern Watersnakes, 79 ± 2.4% in wetland Eastern Gartersnakes, and 74.9 ± 3.7% in upland Eastern Gartersnakes.

The unidentified *Mycoplasma* species dominated the cloacal microbiome of Northern Watersnakes with a relative abundance of 78.9% with no other bacterial OTU representing > 2.2%. Among wetland Eastern Gartersnakes, an unidentified Enterobacteriaceae species had the greatest relative abundance (21.5%) with *Bordetella petrii* (19.4%), *Morganella morganii* (10%), an unidentified *Mycoplasma* species (9%), an unidentified *Bacteroides* species (8.6%), an unidentified *Providencia* species (8.1%) being the other species with relative abundance > 4%. These species also comprised a large portion of the upland Eastern Gartersnake cloacal microbiome, but their relative abundances differed with *Bordetella petrii* (42.8%), *Morganella morganii* (17.8%), the unidentified Enterobacteriaceae species (13.1%), the unidentified *Bacteroides* species (11.1%), and the unidentified *Providencia* species (6%) with no other OTU representing > 3%.

Alpha diversity

Bacterial OTU richness of all cloacal samples ranged from 16 – 69 with a median of 36. The OTU richness (Fig. 2A; $F_{2,12} = 4.557$, $P = 0.034$) and Faith's phylogenetic diversity (Fig. 2B; $F_{2,12} = 10.11$, $P = 0.003$) were, on average, highest in Northern Watersnakes (45.667 ± 5.188, 36–69) with wetland Eastern Gartersnakes (34 ± 6.164, 16 – 53) and upland

Eastern Gartersnakes (22.25 ± 3.966, 17–34) exhibiting lower values. Tukey post-hoc tests revealed that Northern Watersnakes had greater OTU richness than upland Eastern Gartersnakes ($P < 0.05$) and greater PD than either of the Eastern Gartersnake groups ($P < 0.05$). The Shannon's diversity was similar across among the three groups (Fig. 2C; $F_{2,12} = 1.245$, $P = 0.323$) but Northern Watersnakes had a greater range (1.045–4.215) compared to both upland (1.177–2.528) and wetland (1.642–3.953) Eastern Gartersnakes.

Beta diversity

The community composition of the cloacal microbiota was distinct between the two snake species based on Bray–Curtis distances (Fig. 3; $r^2 = 0.604$, $P = 0.001$). However, this outcome did not hold true between Eastern Gartersnake samples as both upland and wetland Eastern Gartersnakes were similar in community structure (Fig. 3; $r^2 = -0.088$, $P = 0.577$). These findings indicate that the presence of rare OTUs drove the observed differences between both species, but Eastern Gartersnakes retained similar OTUs regardless of habitat.

Discussion

This was the first study to examine the effects of host taxonomy and diet/habitat on the cloacal microbiota of North American Colubrids—a highly underrepresented group in microbiome studies [4]. While the cloacal microbiome of Eastern Gartersnakes was comprised primarily of Proteobacteria, Firmicutes, and Bacteroidetes, that of Northern Watersnakes was dominated by Tenericutes. Northern Watersnakes had higher alpha diversity metrics than Eastern Gartersnakes, among which those from upland habitat generally had the lowest diversity. Microbiome community structure was distinct between the species, but the cloacal microbiome community of Eastern Gartersnakes was independent of habitat. Our results indicate that host taxonomy rather than habitat as the more important factor in the cloacal microbiome of snakes, such that in a highly generalist species this is similar across disparate habitats.

As the gut microbiota of snakes generally harbors high levels of Proteobacteria, Bacteroidetes, and Firmicutes [10–12, 14, 32, 33], it appears that Eastern Gartersnakes reflect a common community composition, although the relative proportion of Proteobacteria in Eastern Gartersnakes was greater than those in Cottonmouths [12], Burmese Pythons (*Python bivittatus*; [34]), and Red-necked Keelbacks (*Rhabdophis subminiatus*; [14]). However, Northern Watersnakes were highly divergent with other snakes as the

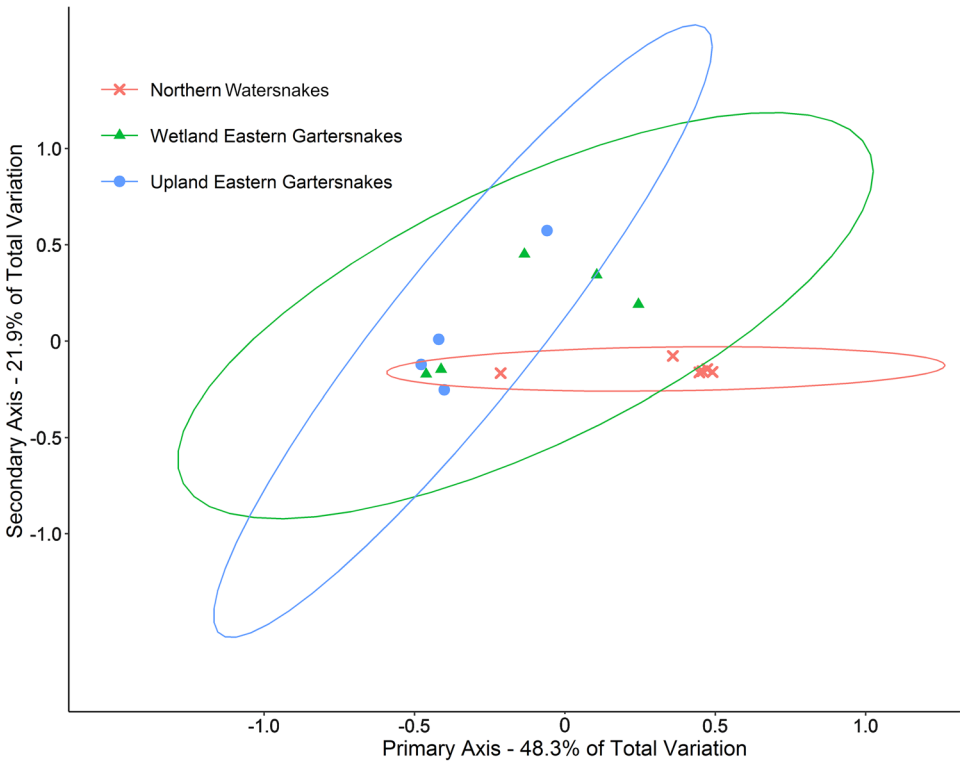

Fig. 3 Principal coordinate analysis (PCoA) using Bray–Curtis dissimilarity of cloacal samples. Each point represents a single individual, and the ellipses represent a 95% confidence interval

Tenericutes were exceedingly prominent. As this phylum is not common in terrestrial vertebrates [16], this result came as a surprise. [4] reviewed that Tenericutes are relatively abundant in fish which may indicate that aquatic habitats can assist members of this phylum in colonizing the gut of resident vertebrates. This could also explain why the relative abundance of Tenericutes in wetland Eastern Gartersnakes was 6.4 × greater than upland individuals. Nonetheless, Cottonmouths are aquatic snakes that lacked any Tenericutes [12] suggesting colonization mechanisms beyond habitat influence. Identifying the microbiome of the water these species lived in could indicate if Tenericutes are transient and acquired from the environment or originate from another source.

Determining the core microbiome is a key aspect in understanding the microbes that are unlikely to be associated with transient acquisition. In both species, it consisted of seven OTUs—all Proteobacteria—that comprised > 50% of total microbial abundance in both Eastern Gartersnake groups but represented just a small fraction for Northern Watersnakes. However, when examining the core microbiome of Northern Watersnakes on their own, the core microbiota was more diverse, including members of Proteobacteria, Firmicutes, Bacteroidetes, and Tenericutes, and represented nearly ~ 95% of the microbial community. Because we did not examine the function of these microbes, we can only speculate on their importance to the host, but their widespread abundance may indicate that they assist in structuring the microbiota through competitive inhibition of transient, potentially pathogenic, microbes (e.g., [35]). The relative abundance of non-core microbes was < 25% in each sampled group, which suggests these core communities could actively exclude colonization by more transient microbes and thereby contribute to maintaining community stability and host health.

Representing the Tenericutes phylum, an unidentified *Mycoplasma* species was the most abundant OTU detected in Northern Watersnakes and was also common in Eastern Gartersnakes, particularly wetland individuals. Members of this genus are among the smallest eubacteria in both cell and genome size and lack a cell wall [36], and are pathogenic in reptiles [37, 38]. Despite this, *Mycoplasma* can be common in the gut microbiota of lizards [7], turtles [39], snakes [14], and fish [40, 41] without the host exhibiting any signs of disease. These species, along with Northern Watersnakes and wetland Eastern Gartersnakes from this study, are associated with aquatic habitats furthering the potential link between these habitats and Tenericutes colonization of the host gut microbiota. As there was no evidence of illness in any snake sampled, it is likely that the observed *Mycoplasma* was non-pathogenic. While *Mycoplasma* are hypothesized to assist in nutrient processing of larval amphibians detritivores [4], its functional role remains unknown in these snakes. The remarkably high abundance of *Mycoplasma* in Northern Watersnakes is unique among reptiles and suggests it imparts a significant, albeit unidentified, benefit to the host.

Members of the family Enterobacteriaceae were prevalent in both species but especially in Eastern Gartersnakes. Enterobacteriaceae was prevalent along the gastrointestinal tract of Cottonmouths [12] and Red-necked Keelbacks [14]. The high prevalence of Enterobacteriaceae in the gut microbiota of Red-necked Keelbacks was indicative of a carnivorous diet which could be the case for our sampled snakes. The bacterium *Morganella morganii* has been found in the oral cavity [32] and colon [11, 34] of healthy snakes. It appears that snakes naturally host Enterobacteriaceae but their function within the gut microbiome are not well described and require further examination to understand their importance.

The presence of *Bordetella petrii* in both species and its prominence in Eastern Gartersnakes was surprising as there is no current evidence indicating its presence in the squamate microbiota. While its function in the microbiome is unknown, *in* silica analysis revealed that *B. petrii* has a highly diverse set of metabolic functions [42] capable of degrading aromatic compounds and detoxifying heavy metals. Despite *B. petrii* being present in all samples, its relative abundance varied wildly suggesting that it may be a transient member of the cloaca rather than a true resident of the gut.

Cloacal microbiome composition appeared to be driven by host taxonomy as it was partitioned between the species. As host phylogeny is a well-established mechanism behind microbiota composition [6, 15, 17, 18], and so this outcome was expected. The high degree of community overlap between the Eastern Gartersnake populations, despite being isolated for > 50 years, suggests the cloacal microbiome is conserved within the species and not affected by diet and/or habitat. Diet generally accounts for a large percentage of microbial sourcing in most vertebrates but [33] found that the gut microbiome of snakes retained few microbes from their diet. This would minimize the importance of dietary microbes in sourcing the snake gut microbiome, but this requires further exploration. While habitat has been shown to have a role in structuring the gut microbiome [20, 43], no such effect was found for Eastern Gartersnakes. We cannot rule out that our small sample size and the inherent variability of the cloacal microbiota [44] reduced our ability to detect a statistically significant difference. Therefore, we suggest that diet and habitat may have a smaller role in the composition of the cloacal microbiome of snakes when compared to host taxonomy, especially for a generalist species, but larger sample sizes are needed to better assess this conclusion.

As microbiome richness and diversity of fecal samples have been found to be influenced by the storage method with the 'gold standard' being freezing the sample at − 80 °C immediately following collection [45, 46], the inability to

rapidly freeze cloacal swabs could have negatively impacted the observed results. However, the OTU richness of Cottonmouth cloacal swabs from [12], which were immediately stored at − 80 °C in the field, were comparable to those of Northern Watersnakes in this study. This suggests that our methodology was unlikely to have resulted in a significant loss of OTUs although further analysis should examine how storage techniques can influence microbiota swab samples in a similar manner to those of fecal samples.

It is important to note that although the cloaca represents a region in which effective non-destructive microbiota sampling can be conducted, its viability as a proxy for the intestinal microbial community may be overstated. One important reason is that the cloaca tends to harbor lower OTU richness than other gastrointestinal regions in snakes [12, 14] although the same was not found in birds [47–49]. Our OTU richness was comparable to Cottonmouths [12] and tropical snakes [50] but was far below Red-necked Keelbacks [14]. The cloaca also has lower microbial mass when compared to other regions of the gastrointestinal tract and feces [48], and [44] suggested that this could cause high variability among samples and reduce their repeatability. As the total reads among the cloacal swabs varied on an order of magnitude (1651–13,354), it may be that a larger sample size is needed to reduce any effect of low OTUs and microbial mass. Furthermore, the cloaca tends to have a unique microbial composition compared to the different areas of the gastrointestinal tract [14, 47–49] indicating that variability in microbial habitat likely influences what taxa can persist. Based on the available evidence, it does appear that the cloacal swabs are a useful proxy in place of destructive sampling, but any conclusions made solely through this method should be approached with caution. Future research should include some degree of destructive sampling to better identify how the cloacal microbiome fits within the entirety of the gut and how effective a proxy it is for snakes.

Conclusions

Our study is the first to examine the cloacal microbiome of two North American Colubrids. We identified that Proteobacteria was abundant in Eastern Gartersnakes, but Tenericutes was the dominant phylum in Northern Watersnakes, and that host taxonomy accounted for differences in microbial composition and diversity. In both species, many of the prominent bacterial taxa are known to be pathogenic—especially *Mycoplasma*—but there were no signs of disease in any sampled individual, so these taxa did not negatively affect host health. Expansion upon our findings through the inclusion of functional analyses would answer several questions raised here, and further assessing how the snake gut microbiome varies across dietary and environmental gradients will aid in addressing its resiliency to external microbes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06645-x.

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Author contributions Conceptualization: JD, RW; Methodology: JD, LZ, RW; Formal Analysis and Investigation: JD and LZ; Writing—original draft preparation: JD; Writing—reviewing and editing: JD, WM, RW; Funding acquisitions: RW; Resources: WM; LZ, RW; Supervision: WM; LZ, RW.

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Data availability Cloacal microbiota data is available in online resource.

Declarations

Conflicts of interest The authors report no conflicts of interest.

Ethical approval Animals were handled under Pennsylvania Fish and Boat Permit 02–0119 and under Southern Illinois Institutional Animal Use and Care Permit 21–016.

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