FEMS Microbiology Ecology, 95, 2019, fiz086

doi: 10.1093/femsec/fiz086
Advance Access Publication Date: 18 June 2019
Research Article

RESEARCH ARTICLE

Gestation alters the gut microbiota of an oviparous lizard

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One sentence summary: Like mammals, gestation alters the gut microbiota of an oviparous lizard, suggesting that the restructuring of microbial communities during reproduction may be an evolutionarily conserved phenomenon across vertebrate reproductive strategies.

Editor: Julian Marchesi
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ABSTRACT

Mammalian pregnancy can alter the diversity, membership and structure of the maternal gut microbiota, but it is unclear whether this phenomenon occurs in vertebrates with different reproductive strategies. We conducted 16S rRNA bacterial inventories to investigate whether oviparous lizards exhibit shifts in gut microbiota similar to those observed in mammals. Using wild-caught eastern fence lizards from Alabama, USA, we collected and extracted fecal DNA from gravid and non-gravid individuals over 54 days in captivity. We predicted that, like mammals, the alpha diversity of lizard gut microbiota would decrease over gestation, and that inter-individual variation in community composition would increase. Indeed, we found that individuals in late-gestation harbored lower gut bacterial richness compared to non-gravid females. Lizard gut microbial communities of late-gestational females exhibited higher pairwise distances for both community membership and community structure compared to earlier gestation stages, indicating a higher degree of inter-individual variation as gestation progressed. Additionally, we found that the relative abundance and prevalence of the candidate phylum Melainabacteria tended to decrease over the course of gestation. While the consequences of these specific alterations are unknown, our results suggest that a general restructuring of gut microbial communities over gestation may be widespread across vertebrate reproductive strategies.

Keywords: gestation; lizards; microbiome; oviparous

INTRODUCTION

The vertebrate gut harbors a rich and dense microbial community that can provide numerous benefits to the host (Kohl and Carey 2016; McFall-Ngai *et al.* 2013). The composition, and likely function, of these microbial communities often shift in response to major ecological and physiological challenges. Thus, it is thought that the gut microbiota may have influenced host fitness throughout vertebrate evolution (Bäckhed *et al.* 2005). Therefore, characterizing how the diversity and functions of gut microbial communities change in response to various challenges is important for our understanding of host-microbe interactions, and investigating these changes in phylogenetically distant hosts will uncover the potential

Received: 30 November 2018; **Accepted:** 10 June 2019

roles and/or potential consequences of such interactions in an evolutionary context.

One physiological state likely to result in alterations to the gut microbiota is pregnancy. For example, humans harbor gut bacterial communities that are generally unique to individuals, but pregnancy has been shown increase these differences, resulting in greater inter-individual variation in the late stages of gestation (Koren *et al*. 2012). Moreover, women in their third trimester typically exhibit lower gut microbial richness and increased relative abundance of Actinobacteria and Proteobacteria compared to samples collected during early pregnancy (Koren *et al*. 2012). A restructuring of gut microbial communities and loss of alpha diversity during pregnancy has also been observed in laboratory mice (Elderman *et al*. 2018). It is thought that these changes in gut microbial communities may aid in reproductive function in the late stages of gestation. For example, shifts in community structure can result in functional changes in the host, such that germ-free mice inoculated with the gut microbiota of women in late-pregnancy exhibit increased adiposity and insulin insensitivity when compared to mice receiving gut microbiota from women in early-pregnancy (Koren *et al*. 2012). Further studies have shown that maternal colonization of germ-free mice during pregnancy can alter the innate immune system and intestinal gene expression of the developing fetus (de Agüero *et al*. 2016), suggesting that changes in the microbiome during gestation could affect the incipient microbial communities of offspring (Funkhouser and Bordenstein 2013; Perez-Muñoz *et al*. 2017). While the underlying mechanisms driving reduced bacterial richness and greater inter-individual variation in gut microbial communities have yet to be clarified, it has been speculated that the immune system or hormonal changes over pregnancy may be involved (Koren *et al*. 2012; Nuriel-Ohayon, Neuman and Koren 2016; Elderman *et al*. 2018).

To date, changes in gut microbiota over gestation have largely been investigated in placental mammals. These studies are limited to humans (Collado *et al*. 2008; Koren *et al*. 2012; Nuriel-Ohayon, Neuman and Koren 2016), specific strains of laboratory mice (Elderman *et al*. 2018) and general changes between actively reproducing and non-reproducing individuals (Phillips *et al*. 2012; Mallott and Amato 2018). Because vertebrates exhibit a variety of reproductive strategies, such as oviparity (egg laying) or ovoviviparity (internal retention of eggs followed by 'live' birth of offspring), this mammal-focused view limits our understanding of whether gut microbiota might exhibit similar changes in community structure over gestation in animals with different reproductive strategies, such as oviparous lizards.

Numerous physiological mechanisms and consequences of reproduction are shared across vertebrate lineages, and between humans and lizards. For example, the human immune system undergoes substantial restructuring during pregnancy, with a suppression of adaptive immune system and activation of the innate immune system (Sacks, Sargent and Redman 1999; Nuriel-Ohayon, Neuman and Koren 2016). Similarly, studies in lizards have shown decreases in immune function during reproductive periods (French and Moore 2008; Ruiz *et al*. 2011). Further, both humans (Lof *et al*. 2005) and lizards (Angilletta and Sears 2000) have higher metabolic needs during reproduction, which may result in greater food intake. Given these similarities across evolutionarily distant hosts, we predicted that gestating lizards may also exhibit reduced richness and greater inter-individual variation of gut microbiota.

Here, we investigated whether gestation alters the gut microbiota of the eastern fence lizard (*Sceloporus undulatus*). Using wild-caught individuals maintained in captivity, we collected multiple fecal samples from both gravid and non-gravid females, and back-calculated the days before laying for fecal samples from gravid females. Using DNA extracted from lizard fecal material, we conducted bacterial inventories by sequencing the 16S rRNA gene to investigate shifts in the gut microbiota over the course of gestation. As noted above, oviparous lizards exhibit numerous physiological changes during reproduction that are shared across vertebrate lineages, thus we predicted that the gut microbiota of gravid lizards should exhibit qualitatively similar changes over gestation to those that have been previously documented during human pregnancy. Specifically, we predicted that the alpha diversity of the lizard gut microbiota would decrease over gestation, and that inter-individual variation in microbial community composition would increase. Such findings would suggest there may be conserved physiological interactions between the host and gut microbiota during reproduction across vertebrate lineages.

MATERIALS AND METHODS

Lizard capture and housing

We captured female *S. undulatus* from six sites in southern Alabama, USA from April to May 2017 (Supplemental Data S1) as part of larger experiment testing the effects of gestational stress on survival and reproduction (MacLeod *et al*. 2018). Fieldwork was conducted early in the breeding season (April-May) to maximize the likelihood that captured females were gravid for the first time that year, rather than producing their second brood. Gravidity was determined upon capture (monitored weekly thereafter) via abdominal palpation and scored on a scale from 0 to 4 (Graham *et al*. 2012). Lizards were housed separately in plastic tubs (46 × 40 × 30 cm) in a temperature-controlled room (21 ± 1åC). Tubs contained moist sand as a substrate, plastic perches, shelters and water bowls. Heat was provided by a 60-W incandescent light bulb suspended over one end of each tub for 8 hours a day to maintain a daytime temperature of approximately 32åC, with the cooler end of the tub maintaining a temperature of approximately 21åC, allowing lizards to behaviorally thermoregulate. Overhead lights were maintained on a 12-hour light-dark schedule. Food in the form of live crickets (*Acheta domestica*) sourced from a commercial reptile food vendor (ReptileFood.com, Dayton, OH), dusted twice weekly with calcium, vitamins and minerals (Herptivite and Ultrafine Calcium with Vitamin D; Repcal, Los Gatos, CA, USA) was provided every other day. Permits for lizard collections were approved by the Alabama Department of Conservation and Natural Resources. All animal protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of the Pennsylvania State University (protocol no. 44595).

Fecal sample collection

Once per week all traces of feces were removed from lizard housing tubs. Tubs were subsequently checked approximately every 4 hours during the day for signs of fresh fecal matter, with a maximum period of 12 hours overnight between checks. All collected fecal samples were therefore < 12 hours old. Feces were removed from tubs using long tweezers (wiped down with 80% ethanol before each use), stored in 1.5 mL centrifuge tubes, and immediately frozen at −20åC.

DNA extraction and amplification

We isolated DNA from fecal samples using the Qiagen PowerFecal DNA Kit (Qiagen, Hilden, Germany; product number: 12830) with an overnight incubation in lysis buffer at 65°C to increase extraction yields (Trevelline *et al.* 2018). We also extracted DNA from moist sand (prior to contact with housing or animals) as a control for possible bacterial DNA present in the lizards' captive environment. Further, we also conducted four 'blank' extractions to correct for contaminants found in DNA extraction kits (Salter *et al.* 2014). We used polymerase chain reaction (PCR) to amplify a portion of the bacterial 16S rRNA gene for Illumina sequencing using the primers 515F and 806R (modified from the primer set employed by the Earth Microbiome Project (GTGCCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT) targeting the V4 region of microbial small subunit ribosomal RNA gene (Caporaso *et al.* 2011). All primers contained 5′ common sequence tags (known as common sequence 1 and 2, CS1 (ACACTGACGACATGGTTCTACA) and CS2 (TACGGTAGCAGAGACTTGGTCT) as described previously (Moonsamy *et al.* 2013), with forward primers containing the CS1 linker and reverse primers containing the CS2 linker. Amplicons were generated using a two-stage targeted amplicon sequencing protocol (Bybee *et al.* 2011; Green, Venkatramanan and Naqib 2015). First-stage PCR amplifications of 16S rRNA gene fragments were performed in 10 μL reactions in 96-well plates, using the MyTaq HS 2X mastermix (Bioline, London, UK; product number: 25045) and the following thermalcycling conditions: 95°C for 5 minutes, followed by 28 cycles of 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds.

Subsequently, a second PCR amplification was performed in 20 μL reactions in 96-well plates using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; product number: 100–4876). These AccessArray primers contain the CS1 and CS2 linkers at the 3′ ends of the oligonucleotides and can be used without any specific Fluidigm equipment. Thermalcycling conditions were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A final, 7-minute elongation step was performed at 72°C.

Amplicon library preparation and Illumina sequencing

Amplified products were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with 20% phiX, were loaded onto an Illumina MiniSeq mid-output flow cell (2 × 153 base paired-end reads). Based on the distribution of reads per barcode, the amplicons were re-pooled to generate a more balanced distribution of reads. The re-pooled library was purified using AMPure XP cleanup, as described above. The re-pooled bacterial libraries were loaded onto a second MiniSeq flow cell and sequenced (2 × 153 base paired-end reads). In all cases, Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Data from the two runs were concatenated before analysis. All library preparation, pooling, and sequencing was performed at the DNA Services facility at the University of Illinois—Chicago. Sequence reads have been deposited in the NCBI SRA database under PRJNA491710.

Illumina sequencing and bioinformatics

We sequenced bacterial 16S rRNA amplicons from a total of 82 fecal samples from gravid (n = 51 samples from 16 individuals) and non-gravid (n = 31 samples from 8 individuals) female eastern fence lizards, plus an additional 4 environmental contamination controls (sand substrate) and 4 DNA extraction kit controls. Sequence reads were filtered and processed using the DADA2 pipeline (Callahan *et al.* 2016) in QIIME2 version 2018.8 (Bolyen *et al.* 2018). We identified bacterial 16S rRNA sequence variants (hereafter Amplicon Sequence Variants or ASVs) using the Greengenes reference database (version 13.8; DeSantis *et al.* 2006). Illumina sequencing generated a total of 4.53 million reads (mean of 50 327 per sample) and 2395 ASVs after DADA2 processing. These sequences were further processed by removing non-bacterial (archaea, chloroplasts, and mitochondria) and contaminant ASVs (those detected in sand and DNA extraction kit controls), reducing our total number of reads to 3.31 million (mean of 40 311 per sample) and 1865 ASVs. We rarefied ASV tables to 2100 sequences per sample before comparisons of alpha (ASV richness, evenness, Faith's phylogenetic diversity, and Shannon Index) and beta diversity (unweighted and weighted UniFrac; Lozupone and Knight 2005) in QIIME2 (Bolyen *et al.* 2018).

Statistical Analyses

In order to control for the well-documented effects of captivity on the gut microbiome of lizards (e.g. Kohl, Skopec and Dearing 2014; Kohl *et al.* 2017), we investigated for changes in alpha diversity among non-gravid females in response to number of days in captivity using linear mixed effect regression models (LMM) with individual as a random effect. Further, we investigated whether lizards exhibited broad- and/or fine-scale changes in microbial communities in response to time in captivity using phylum- and genus-level relative abundance values, respectively. Here, relative abundances were normalized using the variance stabilizing transformation of arcsin(abundance$^{0.5}$) (Shchipkova, Nagaraja and Kumar 2010; Kumar *et al.* 2012). Then, we compared the relative abundances of bacterial phyla and genera over time in captivity using the Response Screening function with the Robust Fit option to conduct multiple regressions using time in captivity as the main variable, and individual ID as a random variable. All statistical tests, including Benjamini-Hochberg False Discovery Rate (FDR) *P*-value corrections (Benjamini and Hochberg 1995), were conducted in JMP®, version 12.0 (SAS Institute Inc., Cary, NC). For all statistical analyses, *P*-values $\leq$ 0.05 were defined as 'significant', while *P*-values between 0.05-1.0 were defined as 'trends'.

We binned fecal samples occurring during *S. undulatus* gestation (~60 days) into either mid-stage (19-39 days before laying; n = 14 fecal samples) or late-stage (<19 days before laying; n = 37 fecal samples; Supplemental Data S1). We investigated differences in measures of alpha diversity—richness (total number of ASVs), Shannon Index (Shannon 1948), evenness (a component of the Shannon Index) and Faith's phylogenetic diversity (Faith 1992)—between gestation bins using an analysis of variance (ANOVA) with random effects (to account for multiple samples from the same individual). Additionally, we investigated for changes in alpha diversity metrics over the course of gestation with days before laying as a continuous variable using linear mixed models with individual as a random effect. All investigations of changes in alpha diversity, including FDR-corrections for multiple comparisons, were conducted in in JMP® 12.0. For

metrics of beta diversity, we tested for differences in unweighted (community membership) and weighted (community structure) UniFrac distances (Lozupone and Knight 2005) across gestation stages. Differences in microbial beta diversity were visualized by conducting Principal Coordinate Analysis (PCoA) (Lozupone and Knight 2005). Using distance matrices, we conducted a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) with random effects (to account for fecal samples collected from the same individual) in PRIMER with PERMANOVA+ version 7.0.13.

To compare inter-individual variability in beta diversity (unweighted and weighted UniFrac) between gestational stages, we calculated the pairwise distances between each sample in a gestation stage to all other samples in the same gestation stage. These distances were then averaged to become the average pairwise distance for each sample. Thus, each sample only had one average pairwise distance, in order to avoid pseudoreplication. Average pairwise distances were compared across gestational stages (non-gravid, mid, and late) using an ANOVA and differences between stages were tested using a post-hoc Tukey's HSD test in JMP® 12.0. Additionally, changes in the relative abundances of microbial phyla and genera were investigated using days before laying (continuous) and gestational stage (categorical) as the main variables, and individual as a random effect. Again, these *P*-values were FDR-corrected in JMP® 12.0 using the Response Screening function.

RESULTS

Effects of captivity

Using only non-gravid female control lizards, we first investigated for effects of captivity on the gut microbiota, as this has been demonstrated in numerous animals, including lizards (Kohl, Skopec and Dearing 2014; Kohl *et al.* 2017). We collected repeated samples from non-gravid females over 54 days in captivity. Alpha diversity of gut microbial communities (ASV richness, evenness, Faith's phylogenetic diversity and Shannon Index) did not change as an effect of time in captivity (LMM, $P > 0.2$ for all metrics; Fig. S1, Supporting Information). At the phylum level, we found that the relative abundance of Tenericutes in the guts of non-gravid individuals decreased significantly as an effect of time in captivity (LMM, FDR-corrected $P = 0.008$). At the genus level, we observed a significant increase in the abundance of *Phascolarctobacterium* (phylum Firmicutes) in response to time in captivity (LMM, FDR-corrected $P = 0.0004$). Notably, *Phascolarctobacterium* was only detected in 12% of samples during the first 45 days in captivity (3 of 24 samples). However, after 45 days in captivity, this genus was present in 100% of samples (n = 7), with a mean relative abundance of 0.12 ± 0.01% SE.

Effects of gestation

We binned fecal samples into either mid- or late-gestation (*S. undulatus* gestation is roughly 60 days) to compare gut microbial communities of non-gravid females to those in mid- (19–39 days before laying; n = 14) and late-gestational stages (<19 days before laying; n = 37). We did not collect any samples from females in early gestation (>39 days before laying). When binned by these 'gestation stages', we observed that lizards in late gestation exhibited significantly lower ASV richness (Fig. 1A; ANOVA, $F_{2,34.9} = 6.75$, $P = 0.003$), Shannon diversity (Fig. 1C; ANOVA, $F_{2,32.9} = 3.83$, $P = 0.032$) and a trend for lower measurements of Faith's phylogenetic diversity (Fig. 1D; ANOVA, $F_{2,38.1} = 2.84$, $P = 0.071$) compared to non-gravid females. Further, lizards in late gestation exhibited significantly lower ASV richness compared to females in the mid-gestation stage (Fig. 1A; ANOVA, $P = 0.029$). There was no effect of gestation on community evenness (Fig. 1B; ANOVA, $F_{2,30.2} = 0.25$, $P = 0.78$). When investigating the relationship between alpha diversity and gestation as a continuous variable, there was a trend between ASV richness and the number of days before laying (LMM, $R^2 = 0.41$, $P = 0.051$), but

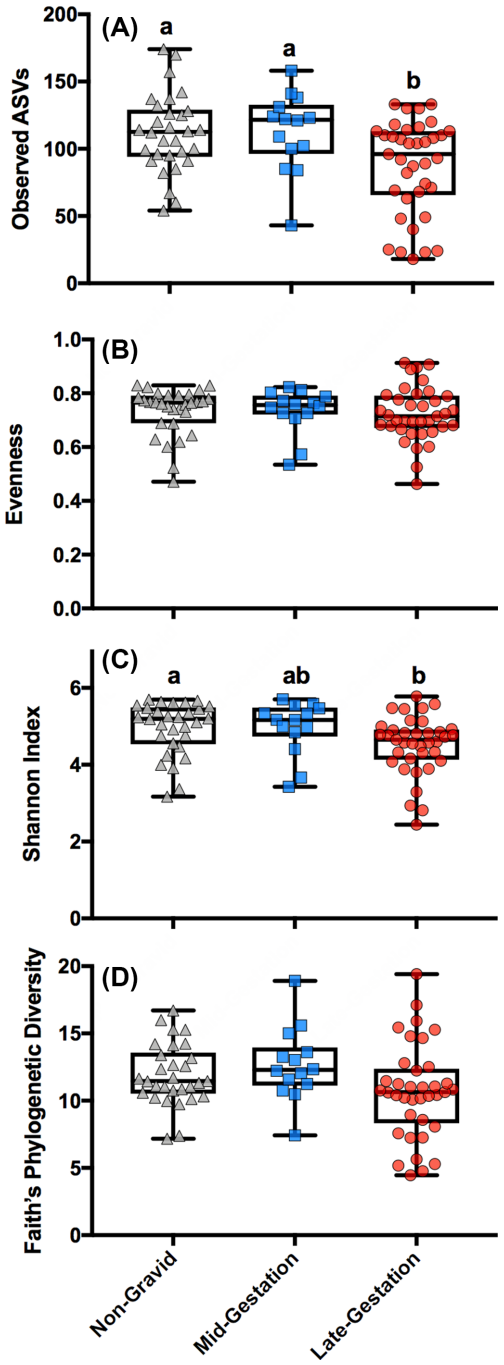

Figure 1. Differences in alpha diversity metrics over gestation stages. A) Observed ASVs; B) Evenness; C) Shannon Index and D) Faith's phylogenetic diversity. Box plots depict the median and interquartile range, with stems representing the maximum and minimum values. Box plots that do not share lower case letters above them are statistically significant from one another according to the Tukey's HSD test.

not for other measurements of alpha diversity. As an additional check, we tested whether the removal of ASVs detected in sand samples could be responsible for these results, but found no significant difference in the number of removed ASVs or sequences across gestation stages (Fig. S2, Supporting Information).

We found that microbial community membership (unweighted UniFrac distances) varied significantly as an effect of gestation stage (Fig. 2A; PERMANOVA, pseudo-F = 1.65, $P = 0.004$). Specifically, non-pregnant females differed significantly from late-gestation individuals (PERMANOVA, pseudo-F = 1.27, FDR-corrected $P = 0.046$). There was a trend for microbial community structure (weighted UniFrac distances) to differ across gestation stages, but no significant differences between gestation stages (Fig. 2B; PERMANOVA, pseudo-F = 1.45, $P = 0.096$). When comparing the average pairwise distances between samples within a given gestation stage, we found significant differences in both community membership (Fig. 2C; ANOVA, $F_{2,36.95} = 7.93$, $P = 0.001$) and community structure (Fig. 2D; ANOVA, $F_{2,36.02} = 5.72$, $P = 0.007$). Specifically, individuals in late gestation exhibited significantly higher pairwise distances for both community membership (Fig. 2C; ANOVA, $F_{1,17.15} = 9.40$, FDR-corrected $P = 0.023$) and community structure (Fig. 2D; ANOVA, $F_{1,14.35} = 5.72$, FDR-corrected $P = 0.035$) compared to non-gravid females, indicating a higher degree of inter-individual variation. In contrast, there were no significant differences in pairwise distances between non-gravid and mid-gestation individuals for either community membership (Fig. 2C; ANOVA, $F_{1,15.29} = 1.23$, FDR-corrected $P = 0.341$) or community structure (Fig. 2D; ANOVA, $F_{1,11.71} = 0.11$, FDR-corrected $P = 0.742$).

No phyla (n = 12) or genera (n = 47) significantly changed in relative abundance in response to time since laying (continuous) or to gestation stages (categorical). However, we observed a trend in the relative abundance of the candidate phylum Melainabacteria to differ among gestation stages (Fig. 3; ANOVA, $F_{2,42.15} = 9.98$, FDR-corrected $P = 0.08$). In general, individuals with a lower relative abundance of Melainabacteria in mid-gestation also had lower relative abundance values in late-gestation (Figure S3, Supporting Informatin). The prevalence of this phylum tended to decrease over the course of gestation, as it was detected in 65% of samples from non-gravid individuals, 71% of samples from mid-gestation individuals, but only 38% of samples from individuals in late gestation.

DISCUSSION

In this study, we investigated whether the gut microbiota of gravid lizards might exhibit qualitatively similar changes over gestation to those previously documented in humans and mice. As predicted, we observed changes in gut bacterial ASV richness and diversity over the course of lizard gestation. Further, we observed increased inter-individual variation in gut bacterial community membership and structure among females in late-gestational stages compared to non-gravid individuals. These changes accompanied reduced relative abundance of Melainabacteria in late-gestational individuals. Below we discuss the potential conserved mechanisms and consequences of these changes in placental mammals and lizards.

We found that gut microbial ASV richness decreased over the course of lizard gestation. This change in alpha diversity was not due to the effects of captivity, as evidenced by the stability of gut bacterial communities among non-gravid individuals over the course of this study. While gut bacterial richness may be associated with host survival (e.g. Bestion *et al.* 2017), an outstanding question is how changes in alpha diversity specifically affect host physiology (Reese and Dunn 2018). For example, previous studies have shown that bacterial community richness is positively correlated with metabolic activity (Patsch *et al.* 2018) and that reduced alpha diversity is associated with phenotypes such as hyperglycemia and increased adiposity among pregnant women (Vijay-Kumar *et al.* 2010; Koren *et al.* 2012). At first glance, these changes in host physiology may appear harmful to a gestating host, but maintaining these metabolic phenotypes in late-gestational stages may increase the availability of nutrients beneficial to both the mother and offspring (Koren *et al.* 2012). Notably, hyperglycemia has also been observed in actively reproducing lizards (Chandavar and Naik 2012), suggesting that changes in the gut microbiota over gestation may provide benefits similar to those predicted in humans. Alternatively, shifts in gut microbial communities may be byproducts of hormonal and immunological changes over the course of pregnancy (Koren *et al.* 2012; Nuriel-Ohayon, Neuman and Koren 2016; Elderman *et al.* 2018), and thus may not provide any specific adaptive benefit.

Inter-individual variation in gut bacterial community membership and structure increased for lizards in late-gestational stages compared to non-gravid females. In pregnant women, inter-individual variation has also been shown to increase over the course of gestation (Koren *et al.* 2012). It is thought that greater inter-individual variation could be induced by hormonal or immunological changes in the late stages of pregnancy (Koren *et al.* 2012; Nuriel-Ohayon, Neuman and Koren 2016; Elderman *et al.* 2018). Regardless of the mechanisms involved in this phenomenon, greater inter-individual variation in could represent a loss of host control over gut microbial community assembly and a stronger overall influence of neutral processes. Given that offspring are thought to inherit microbes from their mothers (Funkhouser and Bordenstein 2013), such late-gestational changes in lizard gut microbiota may influence the incipient microbial communities of offspring. Such effects to transmission dynamics may affect long-term host health, as several studies have found that the early microbiome is critical for proper development and immunity (Arrieta *et al.* 2014; Tamburini *et al.* 2016). While the mechanisms of mother-offspring bacterial transmission are not well understood in wildlife, evidence from human medicine has demonstrated that fetuses may acquire their pioneer microbiota *in utero* (Perez-Muñoz *et al.* 2017) and through post-natal transmission (Mueller *et al.* 2017). Further, recent research has shown that *S. undulatus* may transmit maternal microbiota *in ovo* (Trevelline *et al.* 2018). Together, these studies suggest that maternal microbiota may provide lizard offspring with their incipient gut microbial communities, though it is unknown whether bacteria can be transmitted to a fully-formed egg in late-gestational stages. Regardless, depending on whether inter-individual variation in beta diversity is stochastic, changes in gut bacterial communities could affect which microbial taxa are transmitted to offspring.

We found that lizards in late-stage gestation tended to have a lower relative abundance and reduced prevalence of the bacterial phylum Melainabacteria compared to non-gravid individuals. A similar phenomenon has been observed in humans, where pregnant women exhibit increased relative abundance of Actinobacteria and Proteobacteria over pregnancy (Koren *et al.* 2012), suggesting that lineage-specific interactions may be necessary to meet the specific needs of the host during gestation. Importantly, we did not observe reduced relative abundance of Melainabacteria as an effect of captivity in non-gravid individuals. Melainabacteria (a candidate phylum closely related to Cyanobacteria) is thought to increase the availability of several

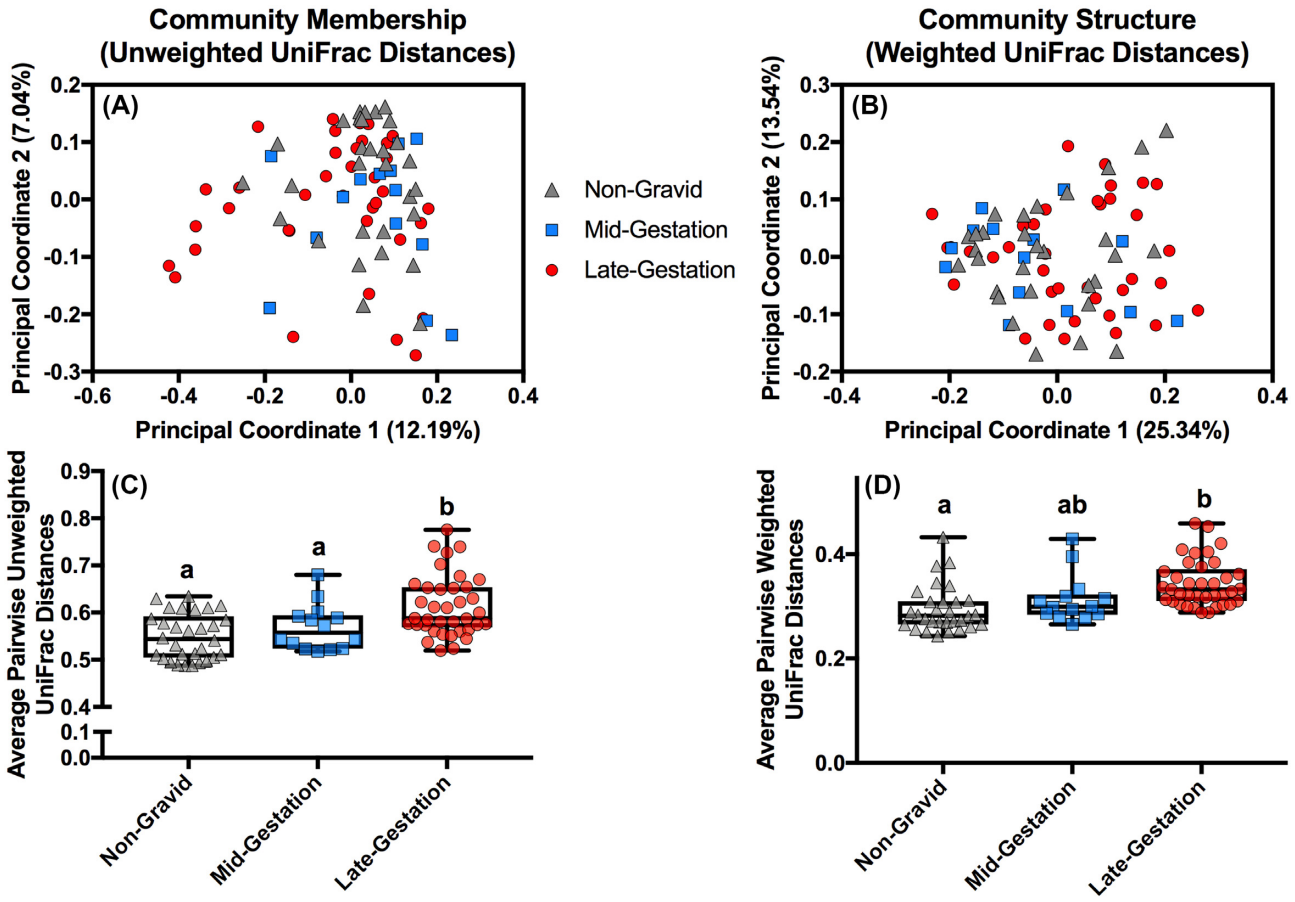

Figure 2. Changes in microbial community membership and structure over gestation. A) Principal coordinate analysis of unweighted UniFrac distances (community membership). B) Principal coordinate analysis of weighted UniFrac distances (community structure). Points are colored according to their gestation stage. C) Pairwise unweighted UniFrac distances between samples within a gestation stage. D) Pairwise weighted UniFrac distances between samples within a gestation stage. Box plots depict the median and interquartile range, with stems representing the maximum and minimum values. Box plots that do not share lower case letters above them are statistically significant from one another according to the Tukey's HSD test.

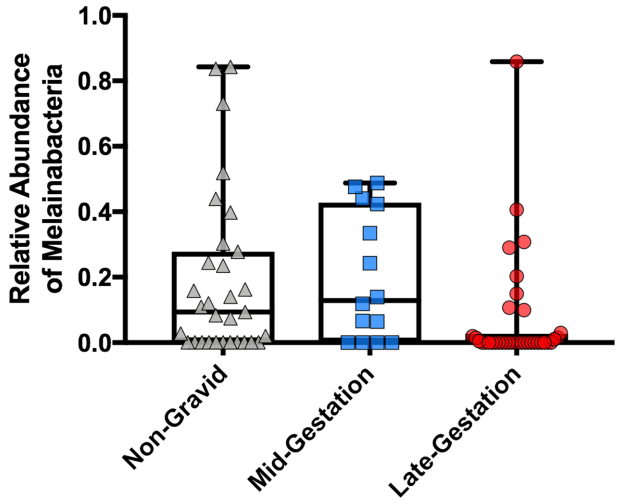

Figure 3. Relative abundance of Melainabacteria in samples from different gestational stages. Box plots depict the median and interquartile range, with stems representing the maximum and minimum values.

vitamins in the gut (Di Rienzi *et al.* 2013), some of which could benefit the host reproduction, especially during early phases of gestation when maternal contributions of nutrients to the unshelled egg are still possible. However, it is worth noting that the reduced relative abundance of Melainabacteria could be caused by hormonal or immunological changes over the course of gestation (Koren *et al.* 2012; Nuriel-Ohayon, Neuman and Koren 2016; Elderman *et al.* 2018), and thus these potential adaptive benefits are highly speculative in nature.

Here, we demonstrated that the gut microbiota of an oviparous lizard changes over the course of gestation, mirroring previous studies on human pregnancy. It is possible that this phenomenon is conserved among vertebrates, with the physiological challenge of reproduction driving changes in the gut microbiota across vertebrate lineages. However, vertebrates as a group exhibit a diversity of reproductive strategies, which may differ in the energetic costs they impose. Therefore, future work should aim to determine whether decreased gut microbial community richness and diversity is universal across reproductive strategies, and what the underlying mechanisms contribute to these changes (e.g. increased energy costs, hormonal signals, restructuring of the immune system). Another major question is whether the observed changes in gut bacterial communities recover after egg laying. In humans, changes in maternal gut bacterial communities persist for at least one month postpartum (Koren *et al.* 2012). While we did not investigate this phenomenon in our study, it is possible gestation-induced changes in lizard gut microbiota may persist long after egg laying,

possibly influencing subsequent broods. Overall, examining the generality of these changes across vertebrate lineages will expand our understanding of host-microbe interactions in an evolutionary context, and allow us to better understand the functional role of the gut microbiome during reproduction.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGEMENTS

We thank the Solon Dixon Forestry Education Center and Land Between the Lakes National Recreation Area for the use of their facilities. David Ensminger and Cameron Venable assisted with wild lizard collection. Dustin Owen, Heather Engler, Thomas Adams and Jennifer Heppner assisted with lizard egg and environmental sample collection. We thank Dr. Stefan Green and the DNA Services Facility of University of Illinois—Chicago for conducting Illumina sequencing. The authors have no conflicts of interest to declare.

FUNDING

This project was funded by NSF grant 1456655.

Conflicts of interest. None declared.

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