Received: 12 May 2021 | Revised: 19 August 2021 | Accepted: 11 October 2021

DOI: 10.1111/mec.16229

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Context-dependent effects of glucocorticoids on the lizard gut microbiome

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Funding information
Directorate for Biological Sciences, Grant/Award Number: IOS 1456655; Marie Sklodowska-Curie Actions, Grant/Award Number: 799025

Abstract

The vertebrate gut microbiota (bacterial, archaeal and fungal communities of the gastrointestinal tract) can have profound effects on the physiological processes of their hosts. Although relatively stable, changes in microbiome structure and composition occur due to changes in the environment, including exposure to stressors and associated increases in glucocorticoid hormones. Although a growing number of studies have linked stressor exposure to microbiome changes, few studies have experimentally explored the specific influence of glucocorticoids on the microbiome in wild animals, or across ecologically important processes (e.g., reproductive stages). Here we tested the response of the gut microbiota of adult female *Sceloporus undulatus* across gestation to ecologically relevant elevations of a stress-relevant glucocorticoid hormone (CORT) in order to determine (i) how experimentally elevated CORT influenced microbiome characteristics, and (ii) whether this relationship was dependent on reproductive context (i.e., whether females were gravid or not, and, in those that were gravid, gestational stage). We show that the effects of CORT on gut microbiota are complex and depend on both gestational state and stage. CORT treatment altered microbial community membership and resulted in an increase in microbiome diversity in late-gestation females, and microbial community membership varied according to treatment. In nongravid females, CORT treatment decreased interindividual variation in microbial communities, but this effect was not observed in late-gestation females. Our results highlight the need for a more holistic understanding of the downstream physiological effects of glucocorticoids, as well as the importance of context (here, gestational state and stage) in interpreting stress effects in ecology.

KEYWORDS
glucocorticoid, lizard, microbiome, stress response

1 | INTRODUCTION

The vertebrate gut microbiota (bacterial, archaeal and fungal communities of the gastrointestinal tract) has profound effects on key physiological processes of their hosts (Kohl, 2017; McFall-Ngai et al., 2013), including nutrient absorption, immune development and regulation of disease onset (Morgan et al., 2013). Changes to microbiome community structure can impact activity and function of microbial communities (Morgan et al., 2013), leading to neurological changes (Cryan & O'Mahony, 2011; Mackos et al., 2016), changes

in behaviour (Bercik et al., 2011; Desbonnet et al., 2014) and disrupted immune function (Hill et al., 2012). Therefore, the structure and functionality of the gut microbiome is increasingly recognized as a key aspect of wildlife health (Trevelline, Fontaine, et al., 2019; West et al., 2019).

Host microbiota are thought to be relatively stable (e.g., in rodents and humans, Bailey, 2014), but transient changes occur due to changes in the environment, including environmental stressors that manifest physiological responses (Mackos et al., 2016; Trevelline, Fontaine, et al., 2019). Relationships between stressors (such as social stressors and food deprivation) and the microbiome have been demonstrated in laboratory studies of mice and primates (Bailey & Coe, 1999; Bailey et al., 2010, 2011; Tannock & Savage, 1974). For example, chronic social defeat in mice was associated with reduced gut microbial community richness and diversity, which led to a shift in the functional profile of the microbiome and changes in immunoregulatory responses (Bharwani et al., 2016). Several studies have also demonstrated increases in interindividual variability in microbiome structure as a result of stressor exposure, probably indicating a loss of ability to successfully regulate community composition (Lesser et al., 2016; Zaneveld et al., 2016, 2017). These trends have led to the so-called Anna Karenina principle (AKP) for animal microbiomes, which suggests that "each unhappy family is unhappy in its own way" (Ma, 2020; Zaneveld et al., 2017). Alterations of microbiome structure and composition as a result of environmental changes can persist over generations given the vertical transmission of these communities (Sonnenburg et al., 2016).

Although changes in the microbiome appear likely to be an important and influential outcome of stressor exposure, little is known of the physiological mechanisms linking the two. A growing body of correlational work linking natural variation in glucocorticoids and gut microbiota suggests that hormones upregulated in response to stressor-exposure, specifically the glucocorticoids, are a likely mechanistic route by which microbiome structure and composition is altered (Levin et al., 2016; Stothart et al., 2016, 2019; Vlčková et al., 2018). However, glucocorticoids are only one part of the complex vertebrate stress response (MacDougall-Shackleton et al., 2019), so correlative data should be interpreted cautiously. One experimental study showed that elevation of glucocorticoids using slow-release hormone implants in the yellow-legged gull *Larus micahellis* resulted in reduced microbial diversity in the gut (Noguera et al., 2020). There remains a need for more such empirical work, particularly in "wild"/free-living systems, to explicitly test potential causative effects of glucocorticoid elevation on the gut microbiome.

Here, we manipulated glucocorticoid levels in wild-caught eastern fence lizards (*Sceloporus undulatus*) to test whether and how glucocorticoid elevation specifically influences the gut microbiota. Glucocorticoid elevation is a well-studied aspect of the physiological response to stressors in this species (Angilletta et al., 2013; Graham et al., 2012; Levy et al., 2015). Increased concentrations of glucocorticoids are linked to reduced immunity (McCormick & Langkilde, 2014; McCormick et al., 2014), but whether this could be mediated by or associated with microbial changes as in other species (Bharwani et al., 2016) is unknown. We focused our experiment on adult female lizards in various stages of gestation to test whether any patterns in microbiome changes are context-dependent (specifically, influenced by gestational stage). This may be important because the microbiome undergoes predictable shifts in structure and composition during pregnancy (Koren et al., 2012)—and these changes are disrupted by exposure to stressors such as restraint and predator odour (Jašarević et al., 2017). Furthermore, alteration of the temporal and spatial dynamics of the maternal microbiome can have consequences for offspring development. The formation of the blood–brain barrier (Braniste et al., 2014) and innate immune development (Gomez de Aguero et al., 2016) depend on metabolites of maternal gut microbial origin. This is facilitated by, for example, transmission of antibodies retaining microbial molecules (Gomez de Aguero et al., 2016) or bacteria to offspring during parturition (Jašarević et al., 2017). Recent work, including in *S. undulatus*, has indicated that oviparous species may also acquire pioneer microbiota from their mothers in ovo (Trevelline, MacLeod, et al., 2018). It has also been previously shown that the gut microbiome of female fence lizards changes over the gestational process (Trevelline, Macleod, et al., 2019). Thus, if glucocorticoid elevation results in alteration of the maternal microbiome during gestation, this could be a mechanistic route by which maternal stress influences offspring traits (e.g., Ensminger et al., 2018) in this species.

We predicted that elevated glucocorticoids would result in reduced diversity of the gut microbiome as in other studies (Bailey et al., 2010; Bharwani et al., 2016; Stothart et al., 2016), but that beta diversity (i.e., interindividual variability) would be increased, following the AKP (Ma, 2020; Zaneveld et al., 2016, 2017). We also predicted that these effects would be strongest in gravid females and, in particular, females in the late stage of gestation, as alpha diversity decreases over gestation in this species (Trevelline, MacLeod, et al., 2019).

2 | METHODS

2.1 | Lizard capture/housing

We captured 44 female *Sceloporus undulatus* from six sites in southern Alabama, USA, from April to May 2017. It was not possible to collect from a single locality; however, these sites are similar in habitat characteristics and no major differences according to site of capture (e.g., in physiological stress responses) have been found in previous studies (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 (Graham et al., 2012). Lizards were housed separately in plastic tubs (46 × 40 × 30 cm) in a temperature-controlled room (21 ± 1°C). 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 h a day to maintain a daytime temperature of ~32°C, with the cooler end of the tub maintaining a temperature of ~21°C, allowing lizards to behaviourally thermoregulate. Overhead lights were maintained on a 12-h light–dark schedule. Food in the form of live crickets (*Acheta domestica*) sourced from a commercial reptile food vendor (ReptileFood.com), dusted twice weekly with calcium, vitamins and minerals (Herptivite and Ultrafine Calcium with Vitamin D; Repcal) 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).

2.2 | Glucocorticoid manipulation experiment

Lizards were assigned randomly to a control or experimental glucocorticoid treatment group. Glucocorticoid-treated females received a transdermal application of corticosterone (hereafter CORT, the primary glucocorticoid in reptiles; Meylan and Clobert, 2005) suspended in sesame seed oil vehicle every second day from capture until laying (a mean duration of 35.5 ± 16.7 days). Dose volume was corrected for lizard body weight (0.2 μl g^{-1} lizard of 4 mg CORT [≥92%, Sigma C2505]) to standardize dose concentration (0.8 μg CORT g^{-1} body mass). Control group females received a dose of the sesame seed oil vehicle only, corrected in the same way for body mass. All treatments were applied by pipette without the need for handling during the lizards' resting period (between 7.30 PM and 8.30 PM) to minimize disturbance. There is no evidence of diel CORT secretion patterns in this species (Trompeter & Langkilde 2011). This procedure and dosage have been shown in prior experiments to result in a short-term (<90 min) increase in plasma CORT after which levels return to baseline (MacLeod et al., 2018), mimicking the CORT increase observed after nonlethal exposure to fire ants (McCormick et al., 2017; Owen et al., 2018), and chasing (Trompeter & Langkilde 2011) and restraint stressors (Graham et al., 2012). This relatively noninvasive treatment (allowing us to avoid confounding effects of handling stress or stress associated with surgery) therefore approximates a general physiological response to short-term, ecologically relevant stressors, rather than the sustained release of hormone implants (Breuner et al., 2008; Crossin et al., 2016) or pharmacologically high levels (Boonstra, 2013).

2.3 | Faecal collection

Once per week all traces of faeces were removed from lizard housing tubs first thing in the morning, and discarded. Tubs were subsequently checked approximately every 4 h during the day for signs of fresh faecal matter, with a maximum period of 12 h overnight between checks. Therefore, all faecal samples collected for molecular analysis were <12 h old. Faeces 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 −20°C. We collected as many samples as possible from each individual during the period of their treatment to maximize the coverage of samples throughout the gestation period (on average, 3.5 samples were collected per individual). Sand samples were also collected from tubs as controls ($N = 4$, collected on two separate dates).

TABLE 1 Sample sizes of faecal microbiome inventories across groups

	Total number of samples	Number of individuals
Control		
Non-gravid	31	7
Mid-gestation	14	9
Late gestation	37	16
CORT		
Non-gravid	26	6
Mid-gestation	5	3
Late gestation	29	15

Note: Some individuals contributed samples during both mid- and late-gestation time points and so are included in both bins. In all analyses we include individual ID as a fixed or random effect.

2.4 | Molecular analyses and bioinformatics

We isolated DNA from faecal samples using the Qiagen PowerFecal DNA Kit (Qiagen; product number: 12830) with an overnight incubation in lysis buffer at 65°C to increase extraction yields (Trevelline, MacLeod, et al., 2018). 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 the V4 region of the bacterial 16S rRNA gene for Illumina sequencing (full molecular protocols are available in Material S1). We sequenced bacterial 16S rRNA amplicons from a total of 142 faecal samples, four sand samples and four DNA extraction kit controls (sample sizes per treatment are given in Table 1). Sequence reads were filtered and processed using the DADA2 pipeline (Callahan et al., 2016) in QIIME2 version 2018.8 (Bolyen et al., 2019) and have been deposited in the NCBI SRA database under PRJNA491710. We removed sequences that were identified as archaea, chloroplasts, mitochondria and contaminant amplicon sequence variants (ASVs; those detected in DNA extraction kit controls). We rarefied ASV tables to 1980 sequences before comparisons of alpha (ASV richness, evenness, Faith's phylogenetic diversity and Shannon index) and beta diversity (unweighted and weighted UniFrac metrics [Lozupone & Knight, 2005] in QIIME2 [Bolyen et al., 2019]). After quality control and removing nonbacterial sequences, our sequencing efforts resulted in a total of 7.6 million sequences (mean ± SE: 53,560 ± 4012 sequences per sample). These sequences were grouped into a total of 2776 ASVs.

2.5 | Statistical analyses

We binned faecal samples occurring during *S. undulatus* gestation (~60 days) into either midstage (19–39 days before laying) or late-stage (<19 days before laying) by back-counting from the date of laying. 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)—across groups. We removed a single CORT-treated sample in the late-gestation bin from analysis as it exhibited much higher diversity than all other samples (>6 SD higher than the mean of all other samples). Furthermore, we first investigated whether the number of treatments impacted aspects of alpha diversity, both using the full data set and in each group. However, none of these results was significant and so in the end this variable was not included in final analysis. The final comparisons were conducted using an analysis of variance (ANOVA) with gestation bin, treatment and the interaction between these variables as main effects, and random effects of both lizard ID (to account for multiple samples from the same individual) and collection site.

For metrics of beta diversity, we tested for differences in unweighted (community membership) and weighted (community structure, membership-weighted by abundance) UniFrac distances (Lozupone & Knight, 2005) across gestation bins. Differences in microbial beta diversity were visualized by conducting principal coordinate analysis (PCoA) (Lozupone & Knight, 2005). Using distance matrices, we conducted tests for homogeneity of variation/dispersion across groups using the PERMDISP function within QIIME2 (Anderson & Walsh, 2013), by each treatment/gestational stage being coded as a "group." Furthermore, we conducted permutational multivariate analysis of variance (PERMANOVA: Anderson, 2001) using the adonis2 function in R version 4.1.2 (R Core Team) with the following effects: gestation bin, treatment, the interaction between these variables, individual ID (to account for faecal samples collected from the same individual) and the number of treatments. Given the potential importance of site-specific differences in the microbiome (Gillingham et al., 2019; Montoya-Ciriaco et al., 2020) we also control for sampling site.

To compare interindividual variability in beta diversity (unweighted and weighted UniFrac), we calculated the pairwise distances between each sample in a group (gestation bin and treatment group) to all other samples in the same group. 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 groups using an ANOVA with gestation bin and treatment as main effects, and individual ID as a random effect. Here we excluded the midgestation time point (both control and CORT), as we had relatively few samples in these groups. We conducted targeted pairwise comparisons between select groups and corrected for multiple comparisons using the Bonferroni correction.

We investigated whether lizards exhibited broad- and/or fine-scale changes in microbial communities in response to CORT and gestation using phylum- and genus-level relative abundance values. Here, relative abundances were normalized using the variance stabilizing transformation of arcsin(abundance$^{0.5}$) (Kumar et al., 2012; Shchipkova et al., 2010). Then, we compared the relative abundances of bacterial phyla and genera using the Response Screening function with the Robust Fit option to conduct multiple regressions using gestation bin and treatment as main effects, and individual ID as a random effect. All statistical tests, including Benjamini–Hochberg false discovery rate (FDR) *p*-value corrections (Benjamini & Hochberg, 1995), were conducted in JMP version 13.0 (SAS Institute). For all statistical analyses, $p \leq .05$ were defined as significant.

3 | RESULTS

Samples were collected opportunistically over the course of our experiment, without knowing when lizards would lay eggs, thus causing the number of treatments to range from one to 29 treatments for any particular sample, with the median and mean being seven and 9.13 treatments respectively (Figure S2a). There was a significant difference in the number of treatments across gestational stages, with the Late-Gestation treatment receiving four (based on median) to six (based on means) fewer doses than those that were not gravid (ANOVA: Gestation bin: $F_{2,136} = 4.36$, $p = .01$; CORT: $F_{1,136} = 1.35$, $p = .25$, Gestation bin × CORT: $F_{2,136} = 2.36$, $p = .10$).

Treatment with CORT changed the dynamics of microbial diversity over gestation. In nongravid individuals there were no significant effects of CORT treatment on alpha diversity metrics (observed ASVs, evenness, Shannon index or Faith's phylogenetic diversity). There was a significant interaction between gestation bin and CORT treatment on the number of observed ASVs (Figure 1a; Gestation bin: $F_{2,60.2} = 2.15$, $p = .13$; CORT: $F_{1,53.9} = 0.22$, $p = .64$, Gestation bin × CORT: $F_{2,64\cdot6} = 3.15$, $p = .049$) and Faith's phylogenetic diversity (Figure 1b; Gestation bin: $F_{2,51.0} = 0.48$, $p = .48$; CORT: $F_{1,55.1} = 0.58$, $p = .62$, Gestation bin × CORT: $F_{2,56.0} = 4.30$, $p = .018$). The number of ASVs hosted by CORT-treated individuals at late gestation was 1.34 times higher than the untreated individuals (Figure 1a; post-hoc test, FDR-corrected $p = .013$). There were no direct or interacting effects of CORT on evenness or the Shannon index.

Next we investigated aspects of variation in the faecal microbiome across treatments and gestational stages. Here, we coded samples to groups depending on combination of treatment/gestational stage. We found significant differences in beta dispersion across "groups" for community membership (unweighted UniFrac distances; PERMDISP: $F = 3.46$; $p = .02$), but not community structure (weighted UniFrac: $F = 1.42$; $p = .24$). Using means of interindividual distances, we found that individual variation in community membership was higher in late-gestation gravid females than in nongravid individuals as previously reported (Figure 2a). Samples collected during late gestation also exhibited higher interindividual variation in community structure (the relative abundance of observed organisms), though these results were not statistically significant (Figure 2b). Within nongravid individuals, CORT treatment seemed to stabilize

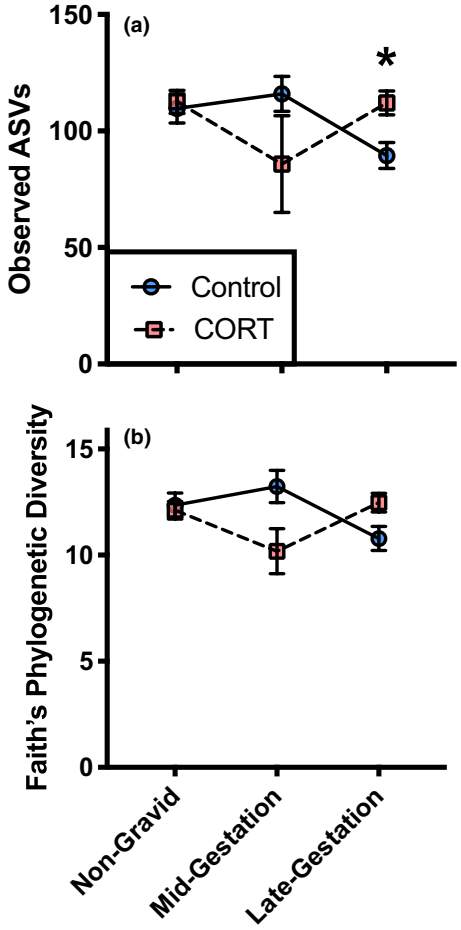

FIGURE 1 There was a significant interaction between gestation bin and CORT treatment on the number of observed ASVs (a) and Faith's phylogenetic diversity (b). Post-hoc analyses show that the number of ASVs hosted by CORT-treated individuals was significantly higher than in untreated individuals in late gestation (denoted with *). Points represent mean ± SE. Individual values are depicted in Figure 2b

the microbiome, as they exhibited significantly lower interindividual variation in terms of both community membership and community structure (Figure 2a,b). However, this effect was not apparent in late-gestation gravid females, where no differences were observed between control- and CORT-treated individuals (Figure 2a,b). Note that this comparison was not tested for midgestation females due to low sample sizes.

Microbial community membership and structure were both largely dictated by individual identity (ID) and the original site from which lizards were collected (Table 2). However, while controlling for these factors, microbial community membership (based on unweighted UniFrac distances) exhibited significant structuring by CORT treatment, gestation bin and an interaction between CORT treatment × gestation bin, though these factors exhibited smaller effect sizes and explained less variance (Table 2). Community membership also varied according to the number of treatment doses an individual received (Table 2). Again, this analysis did not meet the assumption of homogeneity of dispersion, though PERMANOVAs are much more robust to violation of this assumption as compared to other analyses (Anderson & Walsh, 2013. Microbial community structure (weighted UniFrac distances, where the assumption of homogeneity of dispersion was met) also differed according to CORT treatment, and was also influenced by gestation bin, though with no significant interaction term (Table 2). Inspection of individual values for these analyses show substantial variation (Figure S2c).

No microbial phyla exhibited significant changes in relative abundance as a result of gestational stage or CORT treatment. At the genus level, we identified several genera that exhibited statistically significant differences across groups. However, substantial individual variation underlies these differences, which are shown in Figure S2d. We detected one taxon (*Erysipelatoclostridium*) that exhibited significant changes in relative abundance as a result of gestational bin, decreasing through gestation (Figure 3a; FDR-corrected $p = .003$), but there was no effect of CORT treatment, nor any significant interaction between these factors. CORT treatment significantly increased the abundance of the genus *Catabacter* in the lizard gut (Figure 3b; FDR-corrected $p = .034$), with no significant effect of gestation bin or any significant interaction term between these factors. Last, we identified three genera that exhibited significant CORT × gestation bin effects: *Butyricimonas* (Figure 3c; Gestation bin: $p = .83$; CORT: $p = .45$, Gestation × CORT: $p = .018$), *Bilophila* (Figure 3d; Gestation bin: $p = .70$; CORT: $p = .20$, Gestation bin × CORT: $p = .033$) and *Anaerofustis* (Figure 3e; Gestation bin: $p = .022$; CORT: $p = .012$, Gestation bin × CORT: $p = .022$). The first two of these were less abundant in the presence of CORT in nongravid or early-gravid females but more abundant in the presence of CORT late in gravidity. The third, *Anaerofustis*, increased in abundance in the presence of CORT only during early gestation.

4 | DISCUSSION

Despite an increasing interest in how perturbations of the microbiome (e.g., as a result of environmental stressors) can influence host physiology (Mackos et al., 2016; Veru et al., 2014), there remains a relative lack of integration into ecological research (Kohl, 2017). Here we tested the response of the gut microbiota of wild-caught adult female fence lizards, *Sceloporus undulatus*, at various stages of gestation to ecologically relevant elevations of a glucocorticoid hormone (CORT) and whether this relationship was context-dependent (i.e., altered by gestational status). We show that the effects of CORT on gut microbiota are complex, and depend on both gestational state and stage (i.e., gravid vs. nongravid, and stage of gravidity). Given the importance of glucocorticoid elevation as one part of the complex vertebrate stress response, these results highlight the need for a more holistic understanding of the downstream physiological effects of stressor exposure, as well as the importance of context (here, gestational state and stage) in understanding and interpreting stress effects in ecology (Sheriff et al., 2017).

Although we predicted that CORT treatment would result in a decline in microbial diversity (Bailey et al., 2010; Bharwani et al., 2016; Stothart et al., 2016), gut microbiome diversity in late-gestation females treated with CORT was increased relative to control females, while CORT treatment had no effect on microbiome diversity in nongravid females. Our results in gravid females are contrary to the general pattern of reduced diversity following stressor exposure reported in the majority of studies, as confirmed by meta-analyses (Rocca et al., 2019): for example, restraint and social stressors are associated with reduced microbial richness and diversity in laboratory rodents (Bailey et al., 2010; Galley et al., 2014). This may be a good example of how the effects of "stress" and the effects of glucocorticoids are not synonymous and should not be conflated (MacDougall-Shackleton et al., 2019); the reductions in microbial diversity shown in response to stressor exposure may be caused by other elements of the physiological stress response, including behaviour. Alternatively, it is possible that here the interactive effects of gestation on the microbiome might counter any potential reduction in diversity. For example, reduced microbial diversity as a result of stress is thought to be linked to the association between stress and immunity—elevated glucocorticoids boost innate immunity, resulting in an uptick in circulating concentrations of macrophages and cytokines, which have antimicrobial effects (Bailey et al., 2009; Fleshner et al., 1995). However, pregnancy also affects and commonly suppresses the immune system, which might counter or even reverse these effects (Cox et al., 2010; French et al., 2007; French & Moore, 2008; Saad & El Deeb, 1990; Stahlschmidt et al., 2013; Veiga et al., 1998), leading to the increased microbial diversity we observed in late-gestation CORT-treated females. For example, gravid female garter snakes (*Thamnophis elegans*) show reduced immune function relative to nongravid females in terms of T-leukocyte proliferation (Palacios & Bronikowski, 2017). Further work explicitly testing the outcome of these trade-offs for microbial diversity is needed to further disentangle these complicated dynamics.

We predicted that in response to CORT treatment, we would see an increase in interindividual variation in treated individuals, compared with relative stability/similarity in the microbiomes of healthy lizards. This is based on the AKP for animal microbiomes (Zaneveld et al., 2017). For example, corals exposed to above-average temperatures show increased beta diversity (Zaneveld et al., 2016), while ocean acidification leads to increased variability in sponge microbiomes (Lesser et al., 2016). Our results, however, indicate reduced interindividual variability (beta diversity) in (nongravid) females under CORT treatment, and no effect of CORT treatment on beta diversity in gravid females. Thus, we do not provide support for the AKP in this experiment, again contrary to our predictions based on previous work on the outcomes of stressor exposure (Ma, 2020; Zaneveld et al., 2017), and again suggesting that glucocorticoid and stressor exposure effects may be divergent. However, our results from nongravid females do match those of Lavrinienko et al. (2020), which showed that wild bank voles (*Myodes glareolus*) exposed to an environmental stressor (radionuclide contamination in Chernobyl) had more similar gut microbiota composition than unexposed voles. Clearly more work is needed to further test the role of glucocorticoids in generating this pattern of reduced variability between individuals. Additionally, the differences we observed in the response of gravid and nongravid females to CORT treatment in beta diversity again indicate that the effects of glucocorticoid elevation are more context-dependent than previously thought.

We saw changes in a number of microbial phyla as a result of CORT treatment that have the potential to have downstream physiological and neurological effects on individuals. For example, the relative abundances of *Catabacter* were higher in CORT-treated individuals generally, and the abundance of both *Butyricimonas* and *Bilophila* increased across gestation in CORT-treated lizards. These groups are associated with increased inflammation in humans and rodents (Jangi et al., 2016; Natividad et al., 2018; Petrov et al., 2017). The relative abundance of *Anaerofustis*, associated with an increased susceptibility to stressors and depressive behaviour in laboratory rats (Zhang et al., 2019), was also increased in CORT-treated individuals relative to controls. It is increasingly understood that the gut microbiome can have profound effects on cognitive and neurological functioning (including depressive behaviours, and the stress response) via the "microbiome–gut–brain axis" (Cryan & O'Mahony, 2011; Grenham et al., 2011). For example, stressful situations dysregulate gut microbiota, leading to further disruption of the HPA (hypothalamic–pituitary–adrenal) axis (Carlessi et al., 2019), including increases in anxiety-like behaviour (Bailey, 2014; Crumeyrolle-Arias et al., 2014) and changes in stress reactivity (Sudo et al., 2004). Thus, the microbiome/stress physiology relationship is bidirectional (Farzi et al., 2018; Foster et al., 2017; Sudo et al., 2004). Indeed, this bidirectionality could potentially explain the effects we saw of the number of treatment doses on beta diversity (unweighted Unifrac); glucocorticoid effects on the microbiome early in treatment could have feedback effects on host physiology (including HPA axis function, Carlessi et al., 2019) that could lead to changes in how subsequent glucocorticoid exposure influences host microbiota. The potential for the gut microbiota to regulate the HPA axis and physiological adaptation to stress, to our knowledge, remains untested beyond the rodent literature (Sudo, 2012), though our results tentatively suggest this could be a rich area of future investigation in a broader range of taxa. In particular, measuring changes to blood circulating glucocorticoids alongside microbiota changes could help disentangle the complex bidirectional relationships between glucocorticoid physiology and the microbiome.

There is increasing evidence that microbial colonization begins *in utero* (Aagaard et al., 2014; Neu & Rushing, 2011) and perhaps in ovo (Dietz et al., 2019; Trevelline, MacLeod, et al., 2018) or through the eggshell microbiome (van Veelen et al., 2018), so the changes we demonstrate here in gravid lizards could also be a mechanism by which downstream products of the maternal stress response (in this case, CORT) influence offspring traits via maternal effects. Altered community structure in mothers can alter what microbes are transmitted between generations (Aatsinki et al., 2020; Jašarević et al., 2017; Mueller et al., 2015; Sonnenburg et al., 2016), as well as the metabolites available to developing embryos during development

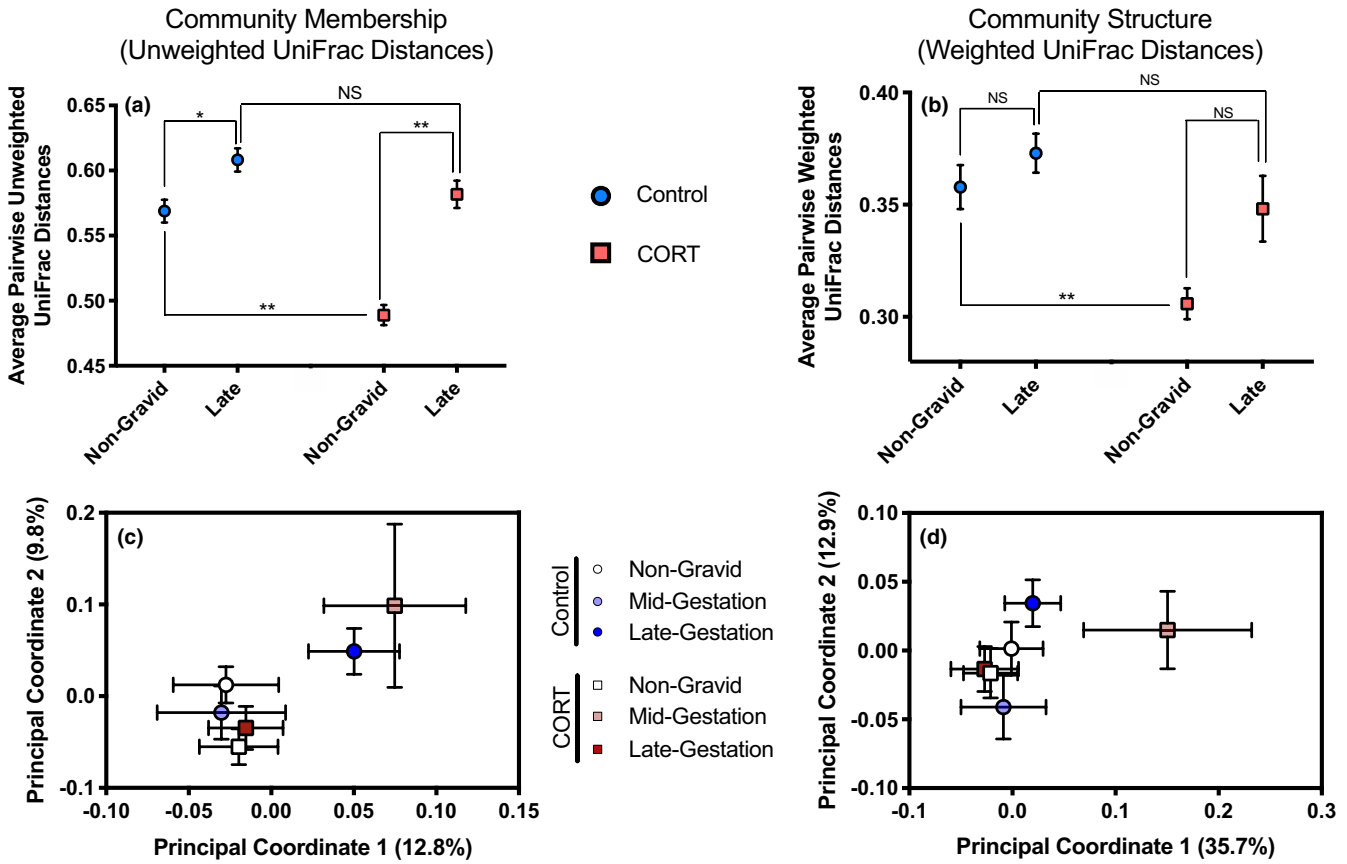

FIGURE 2 Differences in interindividual variation in community membership (a) and structure (b) of nongravid and late-gravid females treated with CORT (shades of red) or a control (shades of blue). Midgestation females were excluded from these analyses due to low sample sizes. (c, d) Principal coordinate analyses (PCoA) of these microbial communities. All points represent mean ± SE. Individual values are depicted in Figure 2c

TABLE 2 Results from PERMANOVA testing beta diversity according to treatment (CORT vs. control) and gestation bin, accounting for individual ID.

	Community membership[a] (unweighted UniFrac)			Community structure (weighted UniFrac)		
	R^2	Pseudo-*F*	*p*	R^2	Pseudo-*F*	*p*
Individual	.38	2.47	**.0002**	.41	2.65	**.0002**
Collection site	.12	3.34	**.0002**	.12	3.31	**.0002**
Treatment	.02	4.27	**.0002**	.01	2.37	**.044**
No. of treatments	.01	2.81	**.0002**	.01	1.54	.15
Gestation stage	.03	2.87	**.0002**	.02	1.83	.051
Treatment × gestation stage	.01	1.72	**.024**	.004	0.99	.39

Bold values indicate statistically significant results ($p \leq .05$).

[a]Assumption of homogeneity of dispersion not met, though PERMANOVAs are quite robust to violations of this assumption (Anderson & Walsh, 2013).

(Koren et al., 2012; Lv et al., 2018), both of which can have substantial influence on offspring phenotypes (Braniste et al., 2014; Gomez de Aguero et al., 2016), including immune (Winter & Bäumler, 2014) and hypothalamic development (Jašarević et al., 2018). Prenatal stress-induced changes in offspring gut microbiota, probably mediated by changes initially to maternal microbiota, have been linked to behavioural disorders in laboratory studies: for example, acquisition of an altered microbiome from gestationally stressed mouse mothers alters bacterial metabolite profiles of exposed offspring, resulting in an increase in colonic hippuric acid, associated with neuropsychiatric disorders (Jašarević et al., 2015). There remains a dearth of studies linking maternal microbial disruption with offspring traits in wild and nonmodel organisms, which will be important in testing the generality of these patterns.

Our results, as well as demonstrating significant and multifarious effects of gestational glucocorticoid increase on microbiome

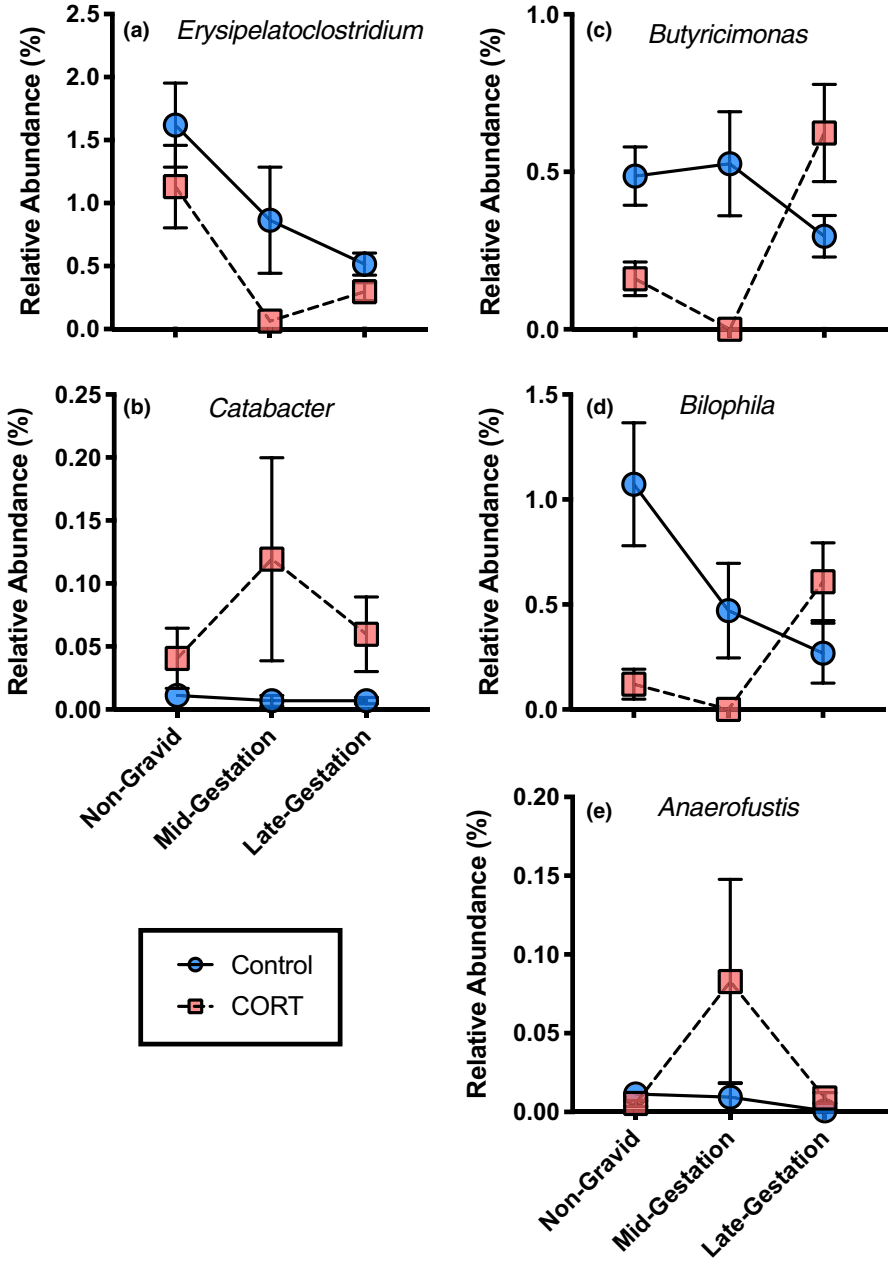

FIGURE 3 Relative abundance (%) of (a) *Erysipelatoclostridium*, (b) *Catabacter*, (c) *Butyricimonas*, (d) *Bilophila* and (e) *Anaerofustis* in the gut microbiota of control (control) females (blue filled circles) and CORT-treated (red filled squares) females at three stages of gestation (nongravid, midgestation, late gestation). Points represent mean ± SE. Individual values are depicted in Figure S2d

composition and variability, add to a growing literature emphasizing the importance of contextual differences (e.g., in reproductive state) to better link physiology with ecology in the study of prenatal stress. For example, a study linking gut microbial diversity and the stress response in barn swallows reported nuanced relationships between the two, dependent on sex, as well as social interactivity (Levin et al., 2016), and differences in the effects of prenatal stress exposure on offspring via the vaginal microbiome also show sex-specific effects (Jašarević et al., 2015). Our results showing CORT differences were robust even accounting for variation based on predictable sampling site differences (Gillingham et al., 2019; Montoya-Ciriaco et al., 2020). Nevertheless, the lasting significance of site-specific differences, even under temporary captive conditions, highlights the need for more microbiome studies including wild animals in order to account for the effects of such ecological variation in experimentation (Cusick et al., 2021). Such contextual variation will be key to understanding the role of the microbiome in mediating the influence of environmental stressors on individual fitness, and offspring traits, in wild animals.

ACKNOWLEDGEMENTS

This project was funded by the National Science Foundation (IOS 1456655 to T.L.). K.J.M. received additional funding from the European Union's Horizon 2020 research and innovation programme (Marie Skłodowska Curie grant no. 799025).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the study. Kirsty J. MacLeod and Tracy Langkilde conducted the prenatal glucocorticoid manipulation and all associated fieldwork and sample collection. Brian K. Trevelline and Kevin D. Kohl conducted all sequencing and data analysis. Kirsty J. MacLeod wrote the manuscript and all authors edited and approved it.

DATA AVAILABILITY STATEMENT

Sequence data are available in the NCBI SRA database under PRJNA491710. Data used for analysis are available at https://doi.org/10.5281/zenodo.5207491.

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How to cite this article: MacLeod, K. J., Kohl, K. D., Trevelline, B. K., & Langkilde, T. (2022). Context-dependent effects of glucocorticoids on the lizard gut microbiome. *Molecular Ecology*, 31, 185–196. https://doi.org/10.1111/mec.16229