Hormones and Behavior 141 (2022) 105146

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Maternal stress and the maternal microbiome have sex-specific effects on offspring development and aggressive behavior in Siberian hamsters (*Phodopus sungorus*)

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ARTICLE INFO

Keywords:
Maternal effects
Gut-brain axis
Gut microbiota
Aggression
Host-microbiome interactions
Stress

ABSTRACT

The gut microbiome, a community of commensal, symbiotic and pathogenic bacteria, fungi, and viruses, interacts with many physiological systems to affect behavior. Prenatal experiences, including exposure to maternal stress and different maternal microbiomes, are important sources of organismal variation that can affect offspring development. These physiological systems do not act in isolation and can have long-term effects on offspring development and behavior. Here we investigated the interactive effects of maternal stress and manipulations of the maternal microbiome on offspring development and social behavior using Siberian hamsters, *Phodopus sungorus*. We exposed pregnant females to either a social stressor, antibiotics, both the social stressor and antibiotics, or no treatment (i.e., control) over the duration of their pregnancy and quantified male and female offspring growth, gut microbiome composition and diversity, stress-induced cortisol concentrations, and social behavior. Maternal antibiotic exposure altered the gut microbial communities of male and female offspring. Maternal treatment also had sex-specific effects on aspects of offspring development and aggressive behavior. Female offspring produced by stressed mothers were more aggressive than other female offspring. Female, but not male, offspring produced by mothers exposed to the combined treatment displayed low levels of aggression, suggesting that alteration of the maternal microbiome attenuated the effects of prenatal stress in a sex-specific manner. Maternal treatment did not affect non-aggressive behavior in offspring. Collectively, our study offers insight into how maternal systems can interact to affect offspring in sex-specific ways and highlights the important role of the maternal microbiome in mediating offspring development and behavior.

1. Introduction

The gut microbiota consists of a complex, ecological microbial community composed of living microorganisms, including commensal, symbiotic and pathogenic bacteria, fungi, and archaea (Sylvia and Demas, 2018; Berg et al., 2020). Their genes and the molecules produced by the microorganisms (e.g., structural elements, metabolites, phages, viruses) are collectively called the microbiome (Berg et al., 2020). The microbiome connects many physiological systems (e.g., endocrine, immune, central nervous systems Garcia-Reyero, 2017; Sylvia and Demas, 2018; Cusick et al., 2021b) resulting in bidirectional, functional relationships with these systems (Collins and Bercik, 2014; Cryan and O'Mahony, 2011). These bidirectional relationships can influence a large variety of outcomes, from early development (Diaz et al., 2011; Erny et al., 2015), to immune system function (Sylvia and Demas, 2018), to behavior and survival (Williams et al., 2020). Throughout an individual's lifetime, exposure to antibiotics (Sylvia et al., 2017), changes in diet (Myles et al., 2014; Bruce-Keller et al., 2017), stress (Partrick et al., 2018; Bastiaanssen et al., 2021), ambient temperature (Kohl and Yahn, 2016), seasonal and spatial patterns (e.g., dispersal or photoperiod, Ren et al., 2017; Xiao et al., 2019; Ren et al., 2020), and social interactions (Archie and Tung, 2015; Munger et al., 2018; Cusick et al., 2021b) can impact both the composition and diversity of the gut microbiome to affect behavior (Sylvia and Demas, 2018), cognitive

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https://doi.org/10.1016/j.yhbeh.2022.105146
Received 8 June 2021; Received in revised form 18 February 2022; Accepted 21 February 2022

performance, and social learning (Davidson et al., 2018). Both the gut microbiome and the physiological systems that interact with the gut microbiome can be shaped by early experiences (Sachser et al., 2020; Warne et al., 2019), suggesting differences in early development may be an important source of organismal variation affecting these physiological systems and behavior.

Experiences during the prenatal period, like maternal stress (Seckl and Meaney, 2004; Duckworth et al., 2015) and exposure to different maternal microbiomes (Jasarevic et al., 2017), are important sources of organismal variation that can have long-term, and often sex-specific, effects on offspring. Manipulation of the maternal microbiome or maternal stress can independently impact offspring's immune and neurodevelopment (e.g., Joëls et al., 2008; Dickens et al., 2009; Jasarevic and Bale, 2019), the foundation and development of offspring's microbiome (Jasarevic et al., 2017; Golubeva et al., 2015; Dominguez-Bello et al., 2010), and offspring behavior (Shapiro et al., 2013; Hartman et al., 2019; de Kloet et al., 2005; Ahmed et al., 2014; Tochitani et al., 2016). These maternal physiological systems do not function in isolation and alterations of one system can affect the other. Activation or alterations of the HPA axis (Wei et al., 2020; Partrick et al., 2018), which is responsible for regulating many homeostatic functions (e.g., energy, immune) and neuroendocrine-microbiome bidirectional communication, can result in changes in the gut microbiome (e.g., Noguera et al., 2018; Gur et al., 2015; Jasarevic et al., 2015) that affect behavior (e.g., Partrick et al., 2018). Similarly, alterations of the gut microbiome can influence HPA activity (Tetel et al., 2018), the sensitivity of the HPA response (Sudo et al., 2004), and can attenuate the impacts of stress (Provensi et al., 2019; Kuti et al., 2020; Langgartner et al., 2018). Maternal stress and the maternal microbiome (e.g., gut microbiome) could therefore affect offspring development independently or have an interactive effect on offspring development.

Knowledge about the interactive role of the maternal microbiome and maternal stress on offspring development and its long-term effects on offspring social behavior is needed (Treichel et al., 2019) and may offer insight into the complex role of the maternal environment in shaping offspring phenotypes. Offspring social behavior is of particular interest because these behaviors influence how individuals interact with conspecifics and have consequences for their reproduction and survival. For example, aggressive behavior is observed when competing for resources (e.g., mates, territory, food Boesch, 2002; Holtmann et al., 2019; Gould and Zeigler, 2007; Soma et al., 2015) or defending offspring (e.g., Cusick et al., 2021a), and can be important for signaling condition (Brown et al., 2006; Bertram and Rook, 2012). Avoidance of and escape from potential conspecific competitors may be essential for appropriate competitive interactions and failure to do so could be fatal (e.g., Capbel et al., 2001; Cooper and Frederick, 2010; Cooper and Peréz-Mellado, 2004; Blumstein et al., 2016). Investigation is particularly important for recognizing and identifying characteristics about conspecifics, including their sex or reproductive status (Smale et al., 1990; Rendon et al., 2016; Pellis and Pellis, 1988), and investigation of conspecifics can influence subsequent social interaction (i.e., decision to attack, attempt mating e.g., Pryke et al., 2001; Bertram and Rook, 2012). Stress and manipulations of the microbiome can affect these behaviors in adults (Cusick et al., 2021b; Earley et al., 2006; Earley et al., 2013; Takahashi et al., 2018; Rogers-Carter et al., 2018; Zalaquett and Thiessen, 1991). Considering how maternal stress and the maternal microbiome interact to shape these behaviors in offspring is important for understanding how early development affects adult behavioral phenotypes and for determining whether early development has long-lasting effects on behaviors critical for reproduction and survival.

In this study we investigated the interactive effects of maternal stress and manipulations of the maternal microbiome on male and female offspring development and social behavior using Siberian hamsters, *Phodopus sungorus*. Siberian hamsters are an excellent, non-model system in which to test the effects of the maternal environment on offspring development and social behavior. In this species, investigation and aggression are essential for interactions with conspecifics and for reproduction (Rendon et al., 2017; Munley et al., 2018), have been well documented in the lab (e.g., Munley et al., 2020; Sylvia et al., 2017; Scotti et al., 2008), and are comparable to behaviors observed in the wild (Ross, 1998). Antibiotics, which are an excellent tool for manipulating the microbiome (Archie and Theis, 2011), have been successfully used in Siberian hamsters to modify the adult gut microbiome, resulting in sex-specific changes in adult behavior (Sylvia et al., 2017).

To understand how maternal stress and the maternal microbiome interact to influence variation in offspring development and social behavior, we exposed pregnant females to one of the following treatments: stressor only, antibiotics only, combination of both the stressor and antibiotics, or no treatment. We quantified male and female offspring growth, gut microbiome composition and diversity, stress-induced cortisol concentrations, and social behavior. We hypothesized that the combined treatment would have an additive effect, influencing offspring development more than either treatment alone. We also predicted that the effects of these manipulations would differ for male and female offspring, predicting female offspring to be more susceptible to treatments based on previous data from adult females.

2. Methods

2.1. Animal housing

All hamsters were housed in polypropylene cages (28 × 17 × 12cm) in a 16:8 light and dark photoperiod. Ambient temperature was maintained at 22 ± 2 °C and relative humidity was maintained at 55 ± 5%. Hamsters were given ad libitum access to tap water and standard laboratory rodent chow (Envigo Teklad Global Diets 18% Rodent Diet). To control for any differences in food across batches, all animals in this study were fed food from the same lot number (2018Exp1-18-2020). All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Bloomington Institution Animal Care and Use Committee BIACUC 19-023 at Indiana University.

2.2. Maternal treatment

Adult male and female hamsters were paired across five days ($n = 34$ pairs total). Twenty-four hours after pairing, the male was removed from the female's cage and housed separately. Females were then randomly assigned to one of four treatments: (1) Antibiotic Only ($n = 9$ females), (2) Stress Only ($n = 8$ females), (3) Antibiotic and Stress (i.e., combined treatment "Antibiotic + Stress", $n = 9$ females), or (4) no treatment (i.e., "Control," $n = 8$ females). Maternal treatment lasted a total of 10 days, beginning five days after pairing and ending four days before pups' expected birth date. During the maternal treatment period, females were weighed daily because antibiotic doses were based on the individual weight of each female. Mean body weight change across treatment period for each treatment group is presented in Table S1.

Females assigned to the Antibiotic Only treatment received a broad-spectrum antibiotic daily (0.3 μl of enrofloxacin [Baytril, Elanco Animal Health Inc., Greenfield, Indiana] 10% oral solution per gram of body mass). Treatment was administered between 14:30 and 16:30 ET each day and administered orally via sterile pipette following established protocol (Sylvia et al., 2018; Sylvia et al., 2017). Enrofloxacin (Baytril) is a broad-spectrum antibiotic and a fluoroquinolone antimicrobial agent that inhibits DNA synthesis and does not easily cross the blood brain barrier (Alvarez et al., 2010; Ooie et al., 1997a; Ooie et al., 1997b; Slate et al., 2014). The use of this antibiotic to alter the gut microbiome of adult Siberian hamsters has been validated previously (Sylvia et al., 2017).

Females assigned to the Stress Only treatment were exposed to a social stressor a total of five times during the 10-day maternal treatment period, specifically occurring on Day 1, Day 3, Day 4, Day 8, and Day 10.

The social stressor consisted of exposure to an adult, weight-matched conspecific female "intruder" in the home cage of the experimental animal (i.e., "resident") for 15 min. This social stressor treatment was chosen based on pilot data indicating that this manipulation significantly elevated serum cortisol levels measured 30 min after the trial. Intruder exposure occurred within the first 3 h of the dark phase under low red light illumination. At least 24 h before the trial, the intruder had a small patch of fur shaved on their dorsal surface for identification purposes. The home cage of the resident female had not been changed for at least three days prior to behavioral testing. Females in this group also received sterile water each day during the maternal treatment period between 14:30 and 16:30 ET (0.3 μl of sterilized water per gram of body mass administered orally via sterilized pipette) to control for any stress associated with receiving liquid orally via sterilized pipette.

Females assigned to the Antibiotic + Stress treatment received both the broad spectrum antibiotic administered daily via sterile pipette (0.3 μl of enrofloxacin [Baytril] 10% oral solution per gram of body mass) starting at 14:30 ET and were exposed to the social stressor treatment.

Females assigned to the Control group only received sterilized water daily during the 14:30 to 16:30 ET administration period (0.3 μl of sterilized water per gram of body mass administered orally via sterilized pipette) to control for any stress associated with receiving liquid orally via sterilized pipette.

Of the 34 females that received the maternal treatment, 27 females produced pups (N = 9 Antibiotic Only mothers, N = 6 Stress Only mothers, N = 7 Antibiotic + Stress mothers, N = 5 Control mothers). Pups remained in the litters until they were weaned at postnatal day (PND) 21. At weaning, offspring were sexed and were individually housed for the remainder of the study. Our desired sample size was 7–10 female offspring and 7–10 male offspring per maternal treatment group. We randomly selected 1–3 male and 1–3 female offspring per adult female per treatment. A total of 67 offspring were used for the remainder of the study: 17 pups from Control mothers (N = 8 females, N = 9 males), 18 pups from Antibiotic Only mothers (N = 10 females, N = 8 males), 16 pups from Stressed Only mothers (N = 7 females, N = 9 males), and 16 pups from Antibiotic + Stress mothers (N = 7 females, N = 9 males).

2.3. Assessing offspring phenotype

Offspring gut microbiomes were assessed at PND40 and social behaviors were assessed when offspring were PND51-PND56, during adolescence and late adolescence, respectively. Siberian hamsters in long-day conditions begin the pubertal transition at approximately PND30 (males) and PND50 (females) and complete this transition around PND60 (Paul et al., 2010).

2.3.1. Fecal sample collection

Fecal samples were collected from offspring at PND40. To collect fecal samples, each individual was removed from their home cage and held over a sterile container. Fecal samples were placed into 1.5 ml sterile vials with screw caps using sterile forceps. The fecal sample was immediately frozen using liquid nitrogen, placed on dry ice, and then stored at −80 °C until the samples were processed. Animals were then weighed and returned to their home cage.

2.3.2. Behavioral trials

We quantified offspring social behavior once when individuals were in late adolescence (at PND51-PND56; n = 67 individuals) using a 15-min same-sex resident-intruder trial. This age marks the ending of the pubertal transition, which occurs at approximately PND60 (Paul et al., 2010). This period is marked by a decline in play behavior (occurring between PND 30-PND 50) and an increase in aggressive behavior (between PND30-PND55, Paul et al., 2010), making this an appropriate time period to assess the development of aggressive and other social behaviors.

Staged interactions were comprised of the experimental focal animal (i.e., resident) and the intruder. The intruder was the same-sex, of similar age and weight (± 3.0 g), and came from a different parental line. Focal experimental animals and intruders were weighed the day before their trial. Each intruder had a small shaved patch on their dorsum for the purpose of identification and was used a maximum of twice per day. Intruders were housed with one same-sex individual and were handled minimally (e.g., only during weekly cage changes or when used in behavior trials). Researchers performing the trial and later quantifying the social behavior of each experimental animal were blind to their maternal treatment and identifying characteristics (e.g., sex).

Trials occurred within the first 3 h of the dark phase. The intruder was introduced into the home cage of the experimental animal, which had not been changed for at least three days prior (this allows the resident animal to establish its territory). Behavioral trials were conducted under low red light illumination and each behavioral trial was video recorded. After the 15 min trial, the intruder was returned to its home cage. The resident was then brought into a separate dark room. A blood sample was collected 15 min later (i.e., 30 min after the start of the resident-intruder trial).

2.4. Blood sampling

Cortisol is the primary glucocorticoid found in Siberian hamsters. To assess the effect of maternal treatment on differences in offspring stress-induced cortisol (SI-CORT) concentrations we collected blood samples from late adolescents 30 min after the start of behavioral trials. Individuals were lightly anesthetized using isoflurane (Isothesia; Henry Schein Animal Health, Covetrus Portland, ME USA) and blood was drawn from the retro-orbital sinus into microcapillary tubes within 1 min. Handling was minimized and consistent across animals; less than 3 min elapsed between removal and return to the animal's home cage. Blood samples were left for 1 h to clot, clots were removed, and samples were centrifuged at 4 °C for 25 min at 2500 rpm. Serum was collected and stored in sealable polypropylene microcentrifuge tubes at −20 °C. Samples were collected from 64 of the 67 offspring; we were unable to collect a sufficient blood sample from three offspring.

2.5. Sample and behavior processing

2.5.1. Microbiome DNA extraction, 16S rRNA sequencing, and bioinformatics

DNA extractions and sequencing procedures were performed in the Center for Genomics and Bioinformatics (CGB) at Indiana University. DNA was extracted from the fecal material using a QIAsymphony PowerFecal Pro DNA Kit (Qiagen, Germantown MD) following the manufacturer's instructions. We used 515F (Parada)/806R(Apprill) universal primers (Caporaso et al., 2018; Project E.M., 2020) to amplify the V4 region of the 16S rRNA gene. A unique barcode was added to each primer to tag the samples. PCR reactions were conducted in triplicate following the Earth Microbiome Project protocols (Caporaso et al., 2018; Project E.M., 2020). PCR reaction mixtures had a final volume of 25 μl and included PCR grade water (13 μl), PCR master mix (10 μl), forward primer 10 μM (0.5 μl), reverse primer 10 μM (0.5 μl), and sample (1 μl). Thermocycling conditions were initiated at 94 °C for 3 min, followed by Stage 2 (32 cycles): 94 °C for 45 s, 50 °C for 60s, and 72 °C for 90s, and ending with Stage 3: 72 °C for 10 min. Three of the 64 samples were run for 30 cycles during Stage 2 and two of the 64 samples were run for 35 cycles during Stage 2. PCR reactions were pooled to prepare for sequencing (Caporaso et al., 2018; Project E.M., 2020). Samples were sequenced (spiked with 30%phiX control in sequencing running) using Illumina MiSeqV3(600).

To generate amplicon sequence variants (ASVs), sequences were demultiplexed using 'demux' command with quality filtering using DADA2 (Callahan et al., 2016) in QIIME 2 (release 2020.8 Bolyen et al., 2019) with the parameters "–p-trunc-len-f 210 –p-trunc-len-r 125." Reads identified as anything other than bacteria or archaea were

identified and removed by aligning reads to the RDP (training set v.9 Cole et al., 2014; following Mothur MiSeq SOP Kozich et al., 2013). Remaining reads were imported back into QIIME 2 and chimeras were removed using the vsearch ("uchime-denovo" subcommand, Rognes et al., 2016). ASVs were classified against the Silva SSU138.1 database 138.1 in QIIME 2 ("classify-sklearn" command, Quast et al., 2013).

2.5.2. Behavioral analysis

Video recordings of social behaviors were scored using the program BORIS v7.9.6 (Friard and Gamba, 2016) by an unbiased observer (JAC). We scored the frequency and/or duration of behaviors performed by the experimental focal individual (i.e., resident) during the first 5 min of the resident-intruder trial, following established protocol in our lab. Detailed descriptions and definitions of the behaviors scored are provided in Table S2. We scored the focal individual's (1) aggressive (e.g., attack, chase) and non-aggressive (e.g., intruder investigation) interactions with the intruder, (2) behaviors associated with escaping from the intruder (e.g., jump and run), (3) paw, and (4) grooming behaviors.

2.5.3. Serum cortisol

Serum SI-CORT concentrations ($n = 64$ individuals) were measured using Enzo Cortisol ELISA kits (ADI-901-071; Enzo Life Sciences, Farmingdale, NY, USA; assay sensitivity 56.72 pg/ml), according to the manufacturer's instructions. This kit was previously validated in Siberian hamsters by Carlton and Demas (2015) and is highly specific for cortisol (100%), with corticosterone cross-reactivity 27.7% and low cross-reactivity (<4%) for other steroid hormones. Samples were diluted 1:80 with assay buffer and run in duplicate. Each plate included samples from both sexes and each treatment condition. Absorbance was determined using BioRad xMark Microplate Spectrophotometer at 405 nm wavelength. The intra-assay coefficient of variation was 3.64% and the inter-assay coefficient of variation was 7.22%.

2.6. Statistical analysis

All statistical analyses were conducted in R v 4.0.2. (R Core Team, 2020) and we report mean ± standard error of the mean unless stated otherwise. Significance was assessed at $p \leq 0.05$. We estimated effect sizes for generalized linear mixed model (GLMM) as the coefficient of determination (i.e., pseudo-R^2) and report both conditional R^2_{GLMM} (variance explained by the entire model) and marginal R^2_{GLMM} (variance explained by the fixed effects) for each model (Nakagawa et al., 2013), which we calculated using the *r.squaredGLMM* function in the MuMIn package. For generalized linear models (GLMs) we also estimated effect size of the model using the coefficient of determination (i.e., pseudo-R^2) and report both likelihood-ratio based R^2 and KL-Divergence-Based R^2 for each model, which we calculated using the rsq package. We do not report traditional parametric effect size estimates for the non-parametric comparisons as parametric effect sizes are negatively affected by data that do not meet parametric assumptions (Leech and Onwuegbuzie, 2019).

2.6.1. Offspring growth rate

We assessed offspring growth rate as the difference in weight between the day they were first weighed (PND40) and the day they were exposed to the resident intruder paradigm (PND51-PND56) divided by the number of days passed. Individual growth rate was normally distributed (Shapiro-Wilk Normality Test $p > 0.05$) and homogeneity of variance was confirmed (Levene's Test $p > 0.05$). To determine the effects of maternal treatment on offspring growth rate, we ran a GLMM with identity link function. We included the interaction of maternal treatment and offspring sex as fixed effects. Litter ID was included as a random effect. Each individual ($n = 67$ individuals) was included in the dataset once.

Table 1
Principal component loadings derived from offspring behavior during the resident-intruder trial ($n = 63$ individuals).

	PC1 Escape score	PC2 Aggression score	PC3 Non-contact aggression score
Eigenvalue	*2.50*	*1.31*	*1.13*
Variation explained	*31.19*	*16.38*	*14.07*
Attack	−0.2343	0.4026	0.3769
Chase	−0.2424	0.4005	0.5126
Received Aggression	0.3210	0.4977	−0.1898
Investigation	−0.5044	−0.2840	−0.1628
Jump	0.4140	−0.3043	0.2171
Run	0.4578	0.3398	−0.1327
Paw Display	−0.1695	0.2332	−0.6693
Grooming	−0.3452	0.2945	−0.1413

2.6.2. Offspring social behavior

To reduce the number of statistical tested conducted, and to avoid making arbitrary judgments about how these behaviors relate to one another in adolescent as opposed to adult individuals, we derived individual social behavior composite scores for each offspring based on the behaviors they performed during the resident-intruder trials. To accomplish this, we centered and scaled the social behavior data as *Z*-scores using the scale function in R and conducted a principal components analysis (PCA) using a correlation matrix, a method commonly employed in animal behavior analyses (e.g., Kanda et al., 2012; Budaev, 2010). Three of the eight PC's were used for the behavioral analyses because they had an eigenvalue greater than one and cumulatively explained 61.65% of the variance (Table 1). PC1, which we refer to as an individual's "escape score," was positively associated with jumping and running, while negatively associated with conspecific investigation (Table 1). PC2, which we refer to as an individual's "aggression score," was positively associated with behaviors associated with aggressive interactions (e.g., attack, chase and received aggression, Table 1). PC3, which we refer to as an individual's "non-contact aggression score" and was positively associated with chase behavior (Table 1).

To determine whether maternal treatment explained variation in offspring escape scores, aggression scores, and non-contact aggression scores, we conducted three separate GLMMs with identity link function. For each analysis, we included the interaction between offspring sex and maternal treatment, offspring weight, and offspring SI-CORT concentration as fixed effects. Intruder ID was included as a random effect because intruders were used more than once. Escape scores and non-contact aggression scores were normally distributed (Shapiro-Wilk Normality Test $p > 0.05$) and displayed homogeneity of variance (Levene's Test $p > 0.05$). Aggression scores also displayed homogeneity of the variance (Levene's Test $p > 0.05$) and to normalize aggression scores we transformed the data by adding the absolute value of the smallest score (2.2080435) to each individual's score and then performing a square root transformation (Shapiro-Wilk Normality Test $p > 0.05$). Overall, seven individuals were excluded from these analyses because four individuals were identified as outliers using Tukey's IQR rule (Kannan et al., 2015) and for three individuals we did not have SI-CORT concentrations. Each individual ($n = 60$ individuals) was included in the dataset once. We also provide mean (±SEM) duration and frequency of attacks, duration of chase, and duration of jump behaviors in the Supplementary files (Table S3).

As a control of our behavioral measures, we evaluated whether maternal treatment affected offspring's total activity and found no differences in total activity in male and female offspring from different maternal treatment groups (Table S4).

2.6.3. Offspring microbiome

The ASV table, taxonomic table, and metadata files were analyzed using phyloseq. Four individuals were not included in these analyses

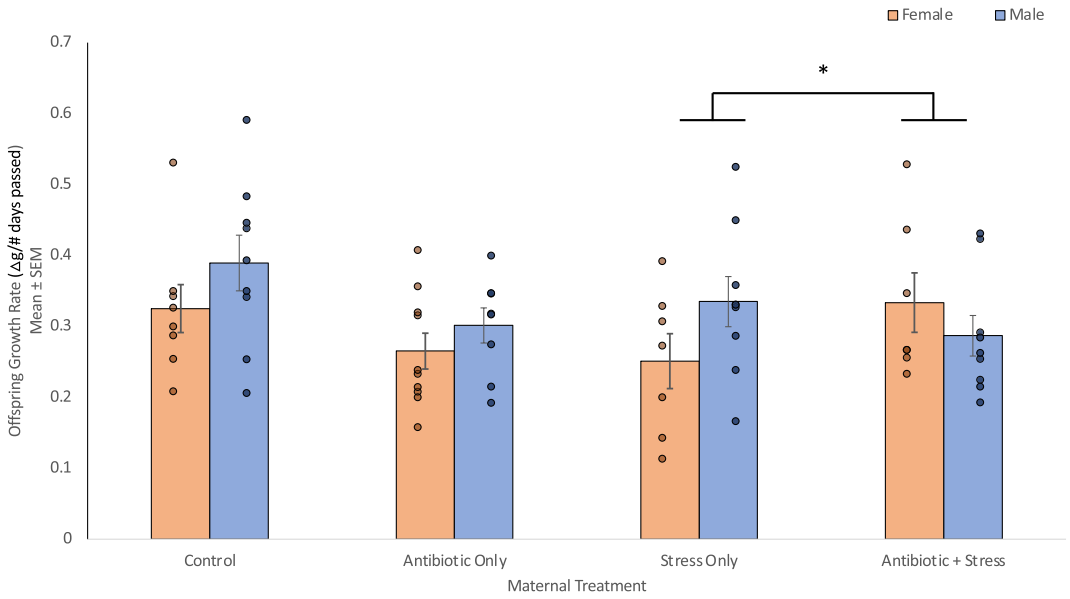

Fig. 1. Interactive effect of maternal treatment and offspring sex on female (orange) and male (blue) offspring growth rate. We detected a significant interaction between maternal treatment and offspring sex (indicated by "*"). Male and female offspring from Stress Only mothers differed significantly in their growth rate compared to male and female offspring from Antibiotic + Stress mothers (GLMM: Male vs. Female: Stress Only vs. Antibiotic + Stress: 0.13 ± 0.07, df = 55.28, t = 1.99, p = 0.05). Individual growth rate was calculated as the difference in weight between weight at PND40 and the day they were exposed to the resident intruder paradigm (PND51-PND56) divided by the number of days passed. Points represent individual datapoints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

because DNA could not be extracted or amplified from their sample or samples contained <40,000 ASVs. The output from the remaining individuals was subject to rarefaction through random subsampling of sequences resulting in 46,637 reads per sample, which corresponded to the lowest sequencing depth across samples. Therefore, all analyses in this study were conducted on normalized abundance data (i.e., normalized ASV table). From this point on, we refer to normalized abundance as "abundance." Shannon diversity index for each sample was calculated using the *estimate_richness* function. To determine whether maternal treatment and offspring characteristics (e.g., sex, weight) influenced offspring microbiome alpha diversity based on the Shannon Index, we conducted a GLM with an identity link function (Shapiro-Wilk test $p > 0.05$, Levene's Test $p > 0.05$). Maternal treatment, offspring sex, offspring escape, aggression, and non-contact aggression scores, offspring SI-CORT concentrations, and offspring weight were included as fixed effects.

We used the *mvabund* package to test the effects of maternal treatment and other factors on offspring gut microbiome composition at the lowest mapped ID: the ASV level. The *mvabund* method provided a model-based analyses of multivariate abundance data (Wang et al., 2012). Using the *manyglm* function, we conducted a negative binomial GLM (log link function) to test for an effect of the interaction of maternal treatment and offspring sex on ASV abundance. We tested whether maternal treatment, offspring sex, or their interaction had a significant effect on the abundance of each ASV using the *anova* function with adjusted p-values (e.g., resampling-based implementation of Holm's step-down multiple testing procedure, Westfall and Young, 1993 as cited in Wang et al., 2012). For ASVs that were found to be significantly impacted by maternal treatment, we identified to which Order these ASVs could be classified. We conducted pairwise comparisons using the Wilcoxon rank sum test to determine which maternal treatment groups differed from one another.

2.6.4. Offspring stress-induced serum cortisol

To determine whether offspring SI-CORT concentrations were affected by maternal treatment we conducted a GLMM with identity link function. SI-CORT was not normally distributed (Shapiro-Wilk Normality Test $p < 0.05$), which was corrected by log transforming the data (Shapiro-Wilk Normality Test $p > 0.05$) and resulted in homogeneity of variance (Levene's Test $p > 0.05$). The log of offspring SI-CORT concentration was the response variable, and offspring weight, maternal treatment, offspring sex, and the interaction of offspring sex and maternal treatment were included as fixed effects. Litter ID was included as a random effect. Each individual (n = 64 individuals) was included in the dataset once.

3. Results

3.1. Effect of maternal treatment on offspring growth rate

We detected a significant interaction between maternal treatment and offspring sex on offspring growth rate, specifically among male and female offspring produced by Stress Only mothers compared to offspring produced by Antibiotic + Stress mothers (GLMM: Male vs. Female, Stress Only vs. Antibiotic + Stress: 0.13 ± 0.07, df = 55.28, t = 1.99, p = 0.05, Fig. 1). Female offspring produced by Stress Only mothers grew at a slower rate (0.25 ± 0.04) compared to female offspring produced by Antibiotic + Stress mothers (0.33 ± 0.04). In contrast, male offspring from Stress Only mothers grew at a faster rate (0.33 ± 0.04) compared to male offspring produced Antibiotic + Stress mothers (0.29 ± 0.03). Control mothers tended to produced offspring that differed in their growth rate from offspring produced by Antibiotic + Stress mothers, but the interaction was not significant (Table S5). Male offspring from Antibiotic + Stress mothers (0.29 ± 0.03) grew at a slower rate compared to male offspring produced by Control mothers (0.39 ± 0.04). Female offspring produced by Control mothers (0.33 ± 0.03) had a similar growth rate compared to female offspring from Antibiotic + Stress mothers (0.33 ± 0.04). The remaining interaction terms and main effects of sex and maternal treatment were not significant (Table S5).

3.2. Effects of maternal treatment on offspring gut microbiome

The microbial community composition of fecal samples collected at PND 40 from male and female offspring in different maternal treatments

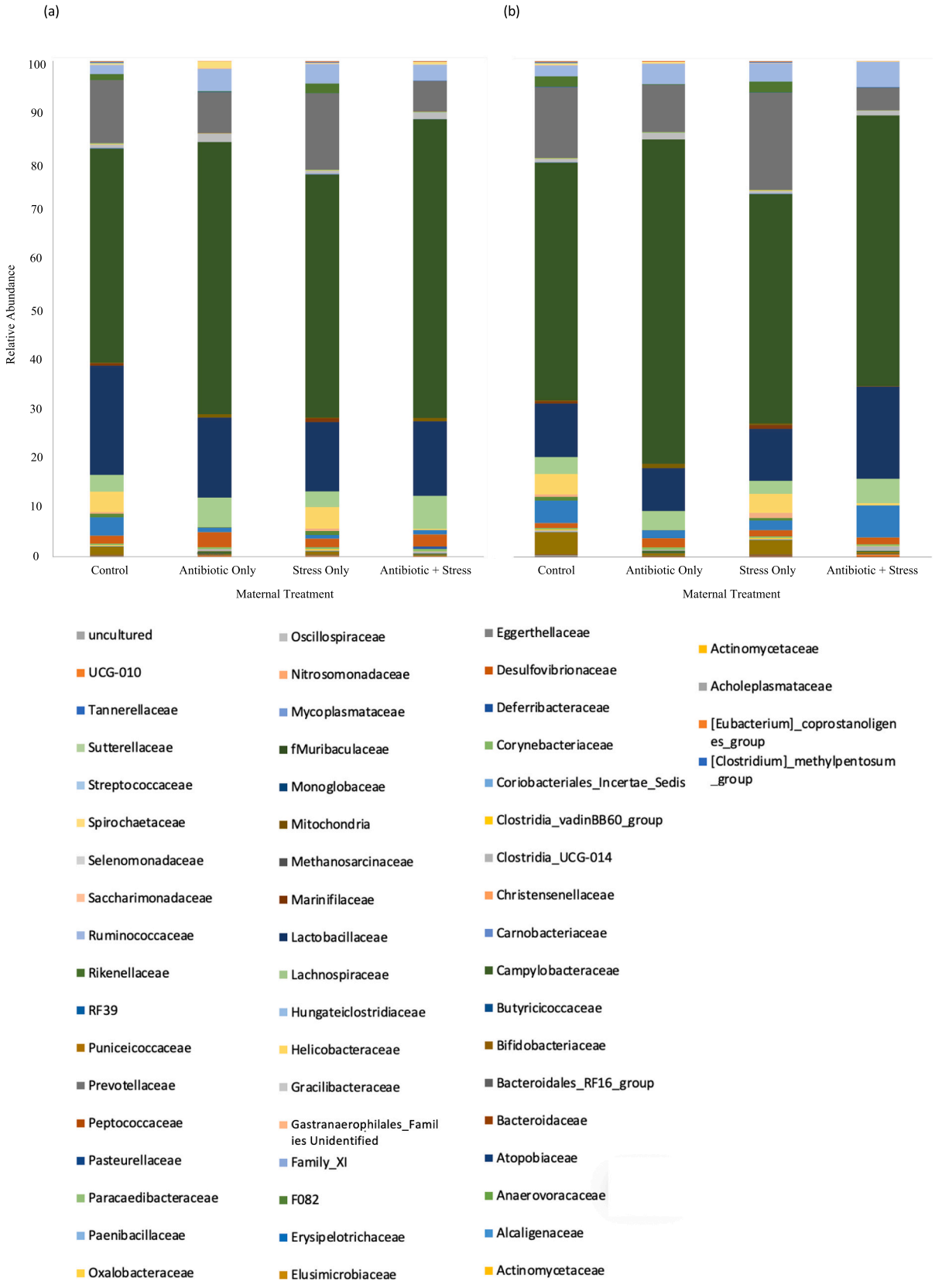

Fig. 2. The microbial community composition in fecal samples of female (a) and male (b) offspring across treatment groups at 40 PND. ASVs are represented at the Family level, with the exception of ASVs belonging to the Order *Gastranaerophilales*. The microbiome was made up of ASVs from 57 Families.

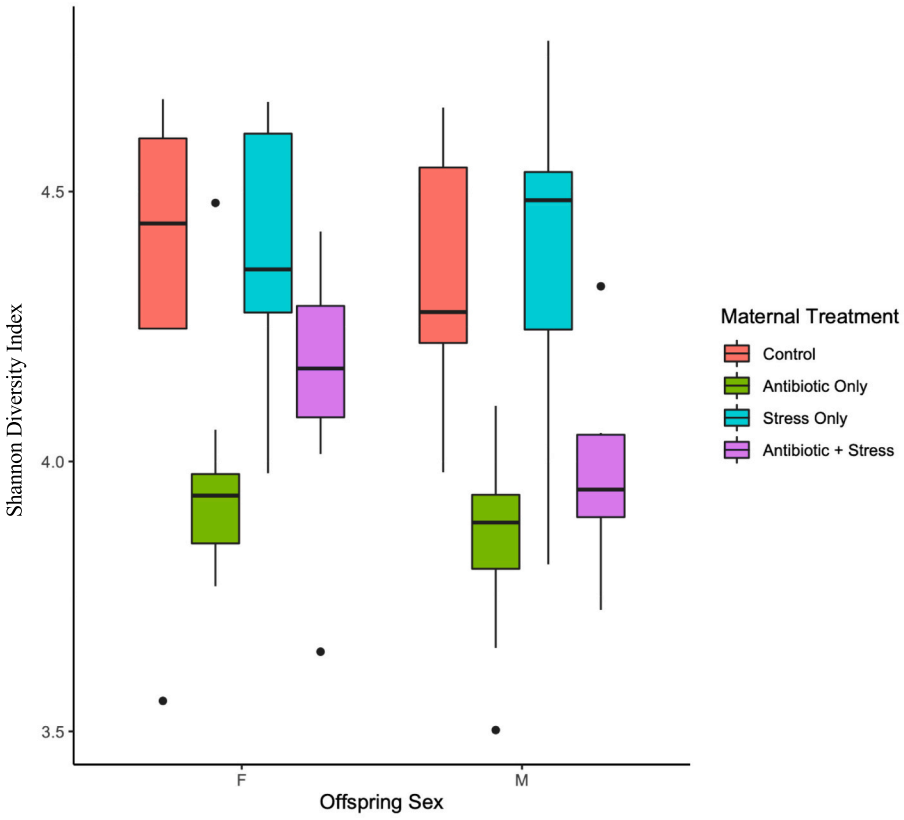

Fig. 3. Maternal treatment had a significant effect on female ("F") and male ("M") offspring microbial communities' Shannon Diversity Index. GLM analyses revealed the Shannon Index of the gut microbiome communities of male and female offspring produced by Control mothers and Stress Only mothers differed significantly from the Shannon Index of the gut microbiome communities of male and female offspring produced by Antibiotic Only mothers and Antibiotic + Stress mothers (Table 2). The Shannon index of samples did not differ between sexes and was unrelated to other offspring characteristics (Table 2).

Table 2
GLM coefficients investigating the effects of maternal treatment on offspring's gut microbiome alpha diversity based on the Shannon Index.

Parameters	Estimate	Std. error	t value	*p* value
Offspring Sex Male vs. Female	−0.05	0.10	−0.52	0.60
Offspring Weight (g)	−0.003	0.01	−0.31	0.76
Offspring SI-CORT Concentration	0.0000013	0.0000015	0.88	0.38
Maternal Treatment				
Antibiotic Only vs. Antibiotic + Stress	−0.10	0.10	−1.35	0.18
Control vs. Antibiotic + Stress	**0.30**	**0.11**	**2.64**	**0.01**
Stress Only vs. Antibiotic + Stress	**0.31**	**0.111**	**2.89**	**0.006**
Control vs. Antibiotic Only	**0.44**	**0.11**	**3.98**	**0.0002**
Stress Only vs. Antibiotic Only	**0.045**	**0.10**	**4.40**	**0.00006**
Stress Only vs. Control	0.0091	0.12	0.078	0.94
Offspring Escape Score	0.016	0.024	0.68	0.50
Offspring Aggression Score	−0.012	0.037	−0.32	0.75
Offspring Non-Contact Aggression Score	0.023	0.038	0.61	0.54

A generalized linear model (GLM) with an identity link function was conducted to assess the effects of maternal treatment, offspring sex, offspring escape score (PC1), offspring aggression score (PC2), offspring non-contact aggression score (PC3), offspring SI-CORT concentrations, and offspring weight on offspring Shannon index (N = 56 individuals). Shannon index for each sample was calculated using the *estimate_richness* function in the *phyloseq* package. R^2_{GLM} (likelihood-ratio) =0.42 and R^2_{GLM} (Kullback-Leibler-divergence-based) = 0.41. Bold indicates significant parameters ($p \leq 0.05$).

is shown in Fig. 2. The microbial community of male and female offspring was primarily dominated by ASVs belonging to the Families *Lactobacillaceae, Muribaculaceae* and *Prevotellaceae,* regardless of maternal treatment.

Maternal treatment had a significant effect on offspring microbial communities' alpha diversity based on the Shannon Index (Fig. 3, Table 2). The Shannon Index of the gut microbiome communities of male and female offspring produced by Control mothers (male: 4.36 ± 0.08, female: 4.30 ± 0.20) and Stress Only mothers (male: 4.38 ± 0.11, female: 4.40 ± 0.10) was greater than the Shannon Index of the gut microbiome communities of male and female offspring produced by Antibiotic Only mothers (male: 3.85 ± 0.07, female: 3.96 ± 0.07) and Antibiotic + Stress mothers (male: 3.97 ± 0.07, female: 4.14 ± 0.10). The Shannon index did not differ between sexes and was unrelated to offspring weight, measures of offspring stress, or offspring social behavior (Table 2).

Maternal treatment significantly affected the mean abundance of ASVs (Dev = 13,351, $p = 0.001$), but there was no effect of offspring sex (Dev = 2159, $p = 0.09$). An interaction of maternal treatment and offspring sex was detected (Dev = 3501, $p = 0.006$). Univariate analyses revealed maternal treatment had a significant effect on 96 unique ASVs belonging to 16 different Orders (Table 3), but did not affect the remaining ASVs (see Supplementary files) Notably, some ASVs belonging to these Orders were completely absent (i.e., present in ≤1 individual) in offspring produced by Antibiotics Only and Antibiotic + Stress mothers, including *Acholeplasmatales*, *Clostridia_vadinBB60_group*, *Gastranaerophilales, Rhodospirillales,* and an unidentified Order from the Class *Alphaproteobacteria* (Table 4). Other ASVs belonging to these Orders were significantly reduced in offspring from these maternal treatment groups (e.g., ASV from *Bacteroidales*, Table 4) while in some cases, certain ASVs belonging to these Orders (e.g., ASVs from *Desulfovibrionales* and *Lachnospirales,* Table 4) were detected in higher abundances in offspring produced by Antibiotic mothers or Antibiotic + Stress mothers. Means (±SEM) and pair-wise comparisons are presented in Table 4 and Table 5, respectively.

In addition, we also observed a significant interaction between maternal treatment and offspring sex on one unique ASV:

Table 3
Analyses of deviance table displaying ASVs that were significantly impacted by maternal treatment, offspring sex, or the interaction of these terms. ASVs are summarized at the Order level and when possible, further classification is provided. All significant and non-significant ASVs analyzed with full taxonomic classification are displayed in supplementary files.

		Maternal treatment		Offspring sex		Maternal treatment * offspring sex	
Order	Family, genus, species (if identifiable)	DEV	*p* Value	DEV	*p* value	DEV	*p* value
Acholeplasmatales	Acholeplasmataceae, Anaeroplasma, uncultured_bacterium	44.89	**0.001**	0.84	1	0.54	1
Bacteroidales	Bacteroidaceae, Bacteroides, uncultured_bacterium	44.86	**0.001**	0.05	1	2.84	1
	F082, F082, uncultured_bacterium	48.93	**0.001**	0.36	1	0.15	1
	Marinifilaceae, Odoribacter, unidentified	38.25	**0.001**	0.39	1	1.81	1
	Marinifilaceae, Odoribacter, unidentified	40.34	**0.001**	0.45	1	0.16	1
	Marinifilaceae, Odoribacter, uncultured_bacterium	56.07	**0.001**	0.80	1	1.09	1
	Muribaculaceae, Muribaculaceae,	54.98	**0.001**	0.15	1	0.00	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	110.33	**0.001**	0.22	1	0.00	1
	Muribaculaceae, Muribaculaceae,	42.13	**0.001**	0.51	1	0.57	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	51.13	**0.001**	0.01	1	0.01	1
	Muribaculaceae, Muribaculaceae,	92.81	**0.001**	0.05	1	0.24	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	56.20	**0.001**	4.64	1	40.23	**0.041**
	Muribaculaceae, Muribaculaceae, unidentified	44.65	**0.001**	0.20	1	0.36	1
	Muribaculaceae, Muribaculaceae,	47.56	**0.001**	0.01	1	3.79	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	54.50	**0.001**	4.68	1	34.67	*0.08*
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	56.95	**0.001**	0.01	1	0.98	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	57.66	**0.001**	1.24	1	3.08	1
	Muribaculaceae, Muribaculaceae,	56.35	**0.001**	7.96	0.99	30.72	0.10
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	41.86	**0.001**	1.70	1	16.57	0.65
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	47.60	**0.001**	1.16	1	12.91	0.97
	Muribaculaceae, Muribaculaceae,	43.64	**0.001**	0.33	1	0.87	1
	Muribaculaceae, Muribaculaceae,	50.41	**0.001**	0.31	1	1.88	1
	Prevotellaceae, Prevotella_9, uncultured_bacterium	57.77	**0.001**	0.83	1	11.03	1.00
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	78.67	**0.001**	0.02	1	0.29	1
	Prevotellaceae, Prevotella, uncultured_bacterium	53.22	**0.001**	0.22	1	0.14	1
	Prevotellaceae, Prevotella, uncultured_bacterium	54.70	**0.001**	0.69	1	0.01	1
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	104.45	**0.001**	0.48	1	0.00	1
	Prevotellaceae, Prevotellaceae_NK3B31_group, uncultured_bacterium	71.40	**0.001**	1.66	1	6.48	1
	Prevotellaceae, Prevotella,	101.95	**0.001**	0.28	1	13.96	0.93
	Rikenellaceae, Alistipes, uncultured_bacterium	94.83	**0.001**	3.07	1	5.75	1
	Rikenellaceae, Rikenellaceae_RC9_gut_group,	105.49	**0.001**	2.58	1	0.10	1
	Rikenellaceae, Alistipes, uncultured_bacterium	56.81	**0.001**	0.35	1	0.18	1
	Rikenellaceae, Rikenella, uncultured_bacterium	66.50	**0.001**	0.72	1	0.45	1
	Rikenellaceae,	56.03	**0.001**	0.39	1	0.58	1
	Rikenellaceae, Alistipes,	43.73	**0.001**	0.20	1	3.28	1
	Rikenellaceae, Rikenellaceae_RC9_gut_group, uncultured_bacterium	80.39	**0.001**	0.02	1	0.09	1
	Tannerellaceae, Parabacteroides,	123.31	**0.001**	0.01	1	0.04	1
	Muribaculaceae, Muribaculaceae,	35.35	**0.002**	0.93	1	6.78	1
	Rikenellaceae, Alistipes, uncultured_bacterium	34.20	**0.002**	0.10	1	0.91	1
	Rikenellaceae, Rikenellaceae_RC9_gut_group, uncultured_organism	35.58	**0.002**	0.20	1	2.62	1
	Muribaculaceae, Muribaculaceae, uncultured_organism	32.21	**0.003**	0.04	1	0.04	1
	Muribaculaceae, Muribaculaceae,	33.04	**0.003**	0.69	1	3.89	1
	Muribaculaceae, Muribaculaceae,	30.92	**0.004**	1.91	1	16.95	0.61
	Muribaculaceae, Muribaculaceae,	31.40	**0.004**	0.61	1	0.12	1
	Muribaculaceae, Muribaculaceae,	31.03	**0.004**	1.28	1	2.32	1
	Marinifilaceae, Odoribacter, uncultured_bacterium	30.13	**0.005**	0.61	1	0.31	1
	Prevotellaceae, Prevotella, uncultured_Prevotellaceae	30.28	**0.005**	0.07	1	0.01	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	29.57	**0.006**	3.99	1	0.94	1
	Rikenellaceae, Alistipes, uncultured_bacterium	29.67	**0.006**	1.53	1	1.12	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	28.24	**0.009**	0.44	1	0.01	1
	Muribaculaceae, Muribaculaceae, uncultured_Bacteroidales	27.39	**0.01**	3.93	1	13.76	0.94
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	26.75	**0.01**	0.44	1	12.08	1.00
	Marinifilaceae, Odoribacter, unidentified	25.69	**0.02**	0.00	1	0.33	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	25.51	**0.02**	0.08	1	1.55	1
	Prevotellaceae, uncultured, uncultured_bacterium	25.35	**0.021**	0.00	1	0.03	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	24.98	**0.022**	0.02	1	2.27	1
	Rikenellaceae, Alistipes,	25.10	**0.022**	0.42	1	0.00	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	24.34	**0.025**	0.50	1	7.26	1
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	24.20	**0.027**	0.05	1	0.31	1
	Prevotellaceae, Prevotellaceae_UCG-003, uncultured_bacterium	24.18	**0.027**	0.05	1	0.00	1
	Prevotellaceae,	23.49	**0.038**	0.10	1	0.13	1
	Rikenellaceae, Alistipes, uncultured_bacterium	22.87	**0.046**	2.37	1	8.56	1
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	22.84	**0.048**	0.82	1	1.13	1
	Rikenellaceae, Rikenellaceae_RC9_gut_group,	22.79	**0.049**	0.53	1	0.43	1
Burkholderiales	Oxalobacteraceae, Oxalobacter, uncultured_bacterium	32.00	**0.003**	0.05	1	0.81	1
	Sutterellaceae, Parasutterella, uncultured_bacterium	23.02	**0.043**	0.27	1	0.32	1
Campylobacterales	Helicobacteraceae, Helicobacter,	56.80	**0.001**	6.91	1	17.94	0.50
	Helicobacteraceae, Helicobacter, Helicobacter_sp.	47.31	**0.001**	1.36	1	2.91	1
	Helicobacteraceae, Helicobacter, Helicobacter_bilis	23.99	**0.028**	0.20	1	3.45	1

(*continued on next page*)

Table 3 (*continued*)

		Maternal treatment		Offspring sex		Maternal treatment * offspring sex	
Order	Family, genus, species (if identifiable)	DEV	*p* Value	DEV	*p* value	DEV	*p* value
Clostridia_vadinBB60_group	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, unidentified	42.75	**0.001**	1.00	1	1.85	1
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_bacterium	33.40	**0.003**	0.00	1	0.32	1
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, unidentified	29.92	**0.006**	0.02	1	1.79	1
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_Clostridia	27.59	**0.009**	1.07	1	0.46	1
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_bacterium	24.67	**0.023**	0.03	1	0.88	1
Coriobacteriales	Coriobacteriales_Incertae_Sedis, uncultured,	32.03	**0.003**	1.22	1	4.52	1
Deferribacterales	Deferribacteraceae, Mucispirillum,	27.14	**0.01**	1.72	1	5.84	1
Desulfovibrionales	Desulfovibrionaceae, Bilophila, uncultured_bacterium	49.73	**0.001**	0.12	1	0.32	1
	Desulfovibrionaceae, uncultured, uncultured_bacterium	41.81	**0.001**	0.06	1	3.39	1
Gastranaerophilales	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	65.09	**0.001**	3.93	1	1.33	1
	Gastranaerophilales, Gastranaerophilales,	50.53	**0.001**	2.14	1	0.37	1
	Gastranaerophilales, Gastranaerophilales, uncultured_rumen	51.23	**0.001**	0.03	1	0.06	1
	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	99.09	**0.001**	0.62	1	0.02	1
	Gastranaerophilales, Gastranaerophilales,	90.64	**0.001**	0.31	1	0.00	1
	Gastranaerophilales, Gastranaerophilales,	28.86	**0.009**	0.11	1	0.84	1
	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	25.34	**0.021**	0.05	1	0.02	1
	Gastranaerophilales, Gastranaerophilales,	23.22	**0.042**	2.49	1	4.11	1
Lachnospirales	Lachnospiraceae, [Eubacterium]_ventriosum_group, uncultured_rumen	35.73	**0.001**	0.38	1	3.20	1
	Lachnospiraceae, Acetatifactor,	26.50	**0.013**	0.41	1	0.88	1
Mycoplasmatales	Mycoplasmataceae, Mycoplasma, uncultured_rumen	39.05	**0.001**	2.81	1	7.75	1
	Mycoplasmataceae, Mycoplasma, Malacoplasma_penetrans	27.98	**0.009**	8.31	0.98	19.33	0.35
Oscillospirales	UCG-010, UCG-010, uncultured_bacterium	40.19	**0.001**	0.01	1	0.25	1
	Oscillospiraceae, uncultured, Clostridium_sp.	32.61	**0.003**	0.20	1	2.31	1
Paracaedibacterales	Paracaedibacteraceae, uncultured, uncultured_Alphaproteobacteria	29.53	**0.006**	0.39	1	1.95	1
Peptococcales	Peptococcaceae, Peptococcus, uncultured_bacterium	25.00	**0.022**	1.45	1	5.72	1
Rhodospirillales	uncultured, uncultured, gut_metagenome	50.86	**0.001**	0.17	1	0.05	1
Alphaproteobacteria_Order Unidentified	Unclassified ASV	23.89	**0.03**	1.04	1	0.68	1

A negative binomial GLM (log link function) was conducted with maternal treatment, offspring sex and their interaction included as the fixed effects. ASV normalized abundance was included as the response variable. Multivariate and univariate hypotheses were calculated using the anova function on the GLM model with adjusted *p*-values (e.g., resampling-based implementation of Holm's step-down multiple testing procedure, Westfall and Young, 1993). Bold indicates significant parameters ($p \leq 0.05$) and italics indicates non-significant parameters ($0.05 < p < 0.1$).

"uncultured_bacterium" belonging to the Order *Bacteroidales,* Family *Muribaculaceae.* This ASV was detected in male (168 ± 24) and female (176 ± 40.24) offspring produced by Control Mothers and offspring produced Stress Only mothers (male: 166.11 ± 29.21; female: 172.57 ± 25.93). This ASV was not detected in both male and female offspring produced by Antibiotic Only mothers, and was not detected in male offspring produced by Antibiotic + Stress mothers, but was detected and lower abundances in female offspring produced by Antibiotic + Stress mothers (78.86 ± 35.06).

3.3. Effects of maternal treatment on social behavior

We did not detect an effect of maternal treatment, offspring sex, offspring weight, or offspring SI-CORT concentrations on offspring escape score (Table S6).

There was a significant interaction between maternal treatment and offspring sex on offspring aggressive score (Table 6; Fig. 4). Female offspring produced by Stress Only mothers were more aggressive (i.e., had a higher aggression score: 0.36 ± 0.36, Fig. 4) than both female offspring produced by Control mothers (aggression score: −0.77 ± 0.37) and female offspring produced by Antibiotic + Stress mothers (aggression score:-0.88 ± 0.25). Female offspring produced by Antibiotic + Stress mothers were more similar in their aggressive behavior to female offspring produced by Control mothers, displaying low levels of aggression (Fig. 4). In contrast, male offspring produced by Stress Only mothers displayed levels of aggression (aggression score: 0.34 ± 0.52) more similar to that of male offspring produced by Control mothers (aggression scores: 0.38 ± 0.32, Fig. 4). Unlike female offspring, male offspring produced by Antibiotic + Stress mothers (aggression score: 0.63 ± 0.21) and male offspring produced by Antibiotic Only mothers (aggression score: 0.74 ± 0.50) were more aggressive compared to male offspring from other maternal treatment groups. We detected a significant main effect of sex and maternal treatment on offspring aggression score, but the remaining fixed effects and interactions were not significant (Table 6).

Offspring non-contact aggression score tended to be negatively related to SI-CORT, suggesting offspring with lower SI-CORT engaged in more chasing of the intruder (GLMM SI-CORT: −0.27 ± 0.15, df = 47.23, t value = −1.80, $p = 0.08$, Table S7). We did not detect a relationship between offspring non-contact aggression score and offspring sex, maternal treatment, offspring weight, or the interaction of offspring sex and maternal treatment (Table S7).

3.4. Effect of maternal treatment on SI-cortisol concentrations

Maternal treatment and offspring sex had a significant interactive effect on the log of offspring SI-CORT concentrations, specifically for offspring produced by Stress Only mothers compared to offspring produced by Antibiotic + Stress mothers (GLMM Male vs. Female, Stress Only vs. Antibiotic + Stress: −0.34 ± 0.18, df = 54.67, $t = -1.93$, $p = 0.05$, Fig. 5, Table 7, Fig. S1). Female offspring from Stress Only mothers had higher SI-CORT concentrations (log SI-CORT 5.13 ± 0.05) compared to female offspring from Antibiotic + Stress mothers (log SI-CORT 5.03 ± 0.04). In contrast, male offspring from Stress Only mothers had lower SI-CORT concentrations (log SI-CORT 4.99 ± 0.04) compared to male offspring produced by Antibiotic + Stress mothers (log SI-CORT 5.03 ± 0.03,). Male and female offspring from Antibiotic Only mothers tended to differ in their SI-CORT concentrations when

Table 4
Average abundance (mean ± SEM) of the significant ASVs belonging to 16 Orders detected in offspring fecal samples found to be significantly affected by maternal treatment.

Order	Family, Genus, Species (if identifiable)	Control	Antibiotic Only	Stress Only	Antibiotic + Stress
Acholeplasmatales	Acholeplasmataceae, Anaeroplasma, uncultured_bacterium	26.86 ± 10.95	0 ± 0	24.5 ± 9.18	0 ± 0
Bacteroidales	Bacteroidaceae, Bacteroides, uncultured_bacterium	49.64 ± 17.6	0.17 ± 0.17	65.5 ± 15.53	0 ± 0
	F082, F082, uncultured_bacterium	345 ± 72.92	30.83 ± 16.74	296.5 ± 76.63	0 ± 0
	Marinifilaceae, Odoribacter, unidentified	9.29 ± 5.13	0 ± 0	113.88 ± 45.14	0 ± 0
	Marinifilaceae, Odoribacter, unidentified	82.14 ± 22.72	0 ± 0	50.69 ± 27.69	0 ± 0
	Marinifilaceae, Odoribacter, uncultured_bacterium	42.07 ± 5.79	0 ± 0	63.94 ± 15.77	9.27 ± 5.87
	Muribaculaceae, Muribaculaceae,	54.29 ± 15.27	0 ± 0	11.38 ± 2.86	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	36.21 ± 4.16	0 ± 0	40.69 ± 6.47	0 ± 0
	Muribaculaceae, Muribaculaceae,	6.79 ± 3.04	0 ± 0	18.31 ± 5.1	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	21.43 ± 5.62	0 ± 0	42.06 ± 7.48	0 ± 0
	Muribaculaceae, Muribaculaceae,	55.43 ± 9.78	0 ± 0	47.06 ± 10.42	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	171 ± 20.17	0 ± 0	168.94 ± 19.35	34.47 ± 18.52
	Muribaculaceae, Muribaculaceae, unidentified	211.86 ± 62.39	0 ± 0	451.19 ± 64.79	338.33 ± 130.92
	Muribaculaceae, Muribaculaceae,	56.5 ± 11.12	420.39 ± 76.43	58.25 ± 10.47	198.2 ± 33.43
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	56.64 ± 8.16	0 ± 0	48.13 ± 6.56	12.53 ± 7.54
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	14.71 ± 3.5	0 ± 0	14.25 ± 3.48	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	93.5 ± 19.37	0 ± 0	76 ± 13.84	25.67 ± 9.44
	Muribaculaceae, Muribaculaceae,	70.29 ± 11.78	0 ± 0	74.44 ± 8.89	12.8 ± 8.8
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	85.71 ± 21.2	0 ± 0	42.75 ± 9.27	5.53 ± 2.97
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	77.93 ± 17.45	0 ± 0	91.25 ± 22.03	2.4 ± 1.81
	Muribaculaceae, Muribaculaceae,	58.57 ± 14.19	575 ± 137.29	57.44 ± 19.99	307.6 ± 54.19
	Muribaculaceae, Muribaculaceae,	131.29 ± 26.34	1087.72 ± 181.43	104.5 ± 31.67	702.87 ± 142.57
	Prevotellaceae, Prevotella_9, uncultured_bacterium	444.93 ± 72.8	1.5 ± 1.12	515 ± 246.04	0 ± 0
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	29.07 ± 7.74	0 ± 0	129.38 ± 31.84	0 ± 0
	Prevotellaceae, Prevotella, uncultured_bacterium	74.5 ± 23.4	0 ± 0	76.25 ± 16.6	0 ± 0
	Prevotellaceae, Prevotella, uncultured_bacterium	97.93 ± 28.61	0 ± 0	217.38 ± 58.99	0 ± 0
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	51.07 ± 11.78	0 ± 0	152.38 ± 30.91	0 ± 0
	Prevotellaceae, Prevotellaceae_NK3B31_group, uncultured_bacterium	1165.43 ± 339.53	1.5 ± 0.85	1656.19 ± 240.2	0.4 ± 0.27
	Prevotellaceae, Prevotella,	940.14 ± 234.6	0.94 ± 0.65	1392.5 ± 349.35	0 ± 0
	Rikenellaceae, Alistipes, uncultured_bacterium	113.43 ± 14.69	0.44 ± 0.35	121.25 ± 28.54	0 ± 0
	Rikenellaceae, Rikenellaceae_RC9_gut_group,	20.79 ± 4.73	0 ± 0	26.63 ± 7.72	0 ± 0
	Rikenellaceae, Alistipes, uncultured_bacterium	51.43 ± 18.31	0 ± 0	52.75 ± 12.47	0 ± 0
	Rikenellaceae, Rikenella, uncultured_bacterium	23.93 ± 8.74	0 ± 0	36.13 ± 8.78	0 ± 0
	Rikenellaceae,	255.36 ± 150.63	0 ± 0	127.5 ± 56.27	0 ± 0
	Rikenellaceae, Alistipes,	19.21 ± 9.29	0 ± 0	7.56 ± 2.16	0 ± 0
	Rikenellaceae, Rikenellaceae_RC9_gut_group, uncultured_bacterium	23.14 ± 5.07	0 ± 0	37.94 ± 8.36	0 ± 0
	Tannerellaceae, Parabacteroides,	80.29 ± 15.7	0 ± 0	68.44 ± 10.61	0 ± 0
	Muribaculaceae, Muribaculaceae,	37 ± 3.06	102.11 ± 17.59	40.25 ± 3.4	74.33 ± 14.21
	Rikenellaceae, Alistipes, uncultured_bacterium	37.07 ± 14.74	0 ± 0	26.31 ± 9.63	0 ± 0
	Rikenellaceae, Rikenellaceae_RC9_gut_group, uncultured_organism	147.71 ± 49.73	0.22 ± 0.22	336.69 ± 82.85	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_organism	255.29 ± 109.75	0 ± 0	13.75 ± 5.4	0 ± 0
	Muribaculaceae, Muribaculaceae,	0.5 ± 0.5	134.17 ± 46.2	7.25 ± 3.59	44.33 ± 9.68
	Muribaculaceae, Muribaculaceae,	12.57 ± 3.3	0 ± 0	18.63 ± 3.52	6.07 ± 4.55
	Muribaculaceae, Muribaculaceae,	13.07 ± 4.11	0 ± 0	28.19 ± 9.81	0 ± 0
	Muribaculaceae, Muribaculaceae,	124 ± 16.76	442.61 ± 68.19	68.63 ± 13.38	117.47 ± 32.25
	Marinifilaceae, Odoribacter, uncultured_bacterium	1.5 ± 0.55	0 ± 0	9.25 ± 4.32	0 ± 0
	Prevotellaceae, Prevotella, uncultured_Prevotellaceae	191.79 ± 82.82	0 ± 0	24.25 ± 13.04	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	620.21 ± 156.44	1878.72 ± 514.63	173.88 ± 34.5	511.33 ± 164.31
	Rikenellaceae, Alistipes, uncultured_bacterium	2.29 ± 0.77	0 ± 0	2 ± 0.67	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	5 ± 1.73	0 ± 0	4.31 ± 1.33	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_Bacteroidales	18.71 ± 5.48	0 ± 0	27.13 ± 7.18	31.07 ± 14.74
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	289.64 ± 107.25	2.94 ± 2.83	158.44 ± 27.21	418.6 ± 143.3
	Marinifilaceae, Odoribacter, unidentified	52.21 ± 18.19	0 ± 0	38.81 ± 15.8	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	0.71 ± 0.22	0 ± 0	0.94 ± 0.38	0 ± 0
	Prevotellaceae, uncultured, uncultured_bacterium	4.79 ± 1.93	0 ± 0	7.06 ± 2.79	0 ± 0

(*continued on next page*)

Table 4 (*continued*)

Order	Family, Genus, Species (if identifiable)	Control	Antibiotic Only	Stress Only	Antibiotic + Stress
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	239.5 ± 55.96	1278.17 ± 176.23	460 ± 81.71	998.27 ± 124.22
	Rikenellaceae, Alistipes,	3.43 ± 1.37	0 ± 0	3.19 ± 1.06	0 ± 0
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	85 ± 29.48	0 ± 0	99.31 ± 49.01	3.07 ± 2.2
	Muribaculaceae, Muribaculaceae, uncultured_bacterium	8.57 ± 5.4	0 ± 0	1.38 ± 0.63	0 ± 0
	Prevotellaceae, Prevotellaceae_UCG-003, uncultured_bacterium	43.64 ± 11.39	8.17 ± 4.78	34.63 ± 7.58	0 ± 0
	Prevotellaceae,	156.5 ± 88.8	0 ± 0	265.81 ± 73.18	0 ± 0
	Rikenellaceae, Alistipes, uncultured_bacterium	3.5 ± 1.36	0 ± 0	4.13 ± 1.62	0 ± 0
	Prevotellaceae, Prevotellaceae_UCG-001, uncultured_Bacteroidales	29.86 ± 13.42	384.67 ± 137.6	62.56 ± 39.21	0 ± 0
	Rikenellaceae, Rikenellaceae_RC9_gut_group,	60.14 ± 25.21	0 ± 0	149.19 ± 114.7	0 ± 0
Burkholderiales	Oxalobacteraceae, Oxalobacter, uncultured_bacterium	25.64 ± 8.69	4.83 ± 2.99	28.56 ± 10.32	0 ± 0
	Sutterellaceae, Parasutterella, uncultured_bacterium	57.43 ± 33.82	0 ± 0	4 ± 2.37	0 ± 0
Campylobacterales	Helicobacteraceae, Helicobacter,	219.71 ± 139.93	0 ± 0	296.63 ± 104.66	7.13 ± 3.83
	Helicobacteraceae, Helicobacter, Helicobacter_sp.	143.71 ± 62.43	0.28 ± 0.28	112.63 ± 49.42	0 ± 0
	Helicobacteraceae, Helicobacter, Helicobacter_bilis	759.29 ± 245.78	0.72 ± 0.72	458.94 ± 195.05	0 ± 0
Clostridia_vadinBB60_group	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, unidentified	7.07 ± 2.66	0 ± 0	5.88 ± 1.26	0 ± 0
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_bacterium	7.79 ± 4.35	0 ± 0	18.5 ± 5.3	0 ± 0
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, unidentified	2.14 ± 1.21	0 ± 0	9.5 ± 3.47	0 ± 0
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_Clostridia	5.29 ± 2.27	0 ± 0	14.56 ± 9.52	0 ± 0
	Clostridia_vadinBB60_group, Clostridia_vadinBB60_group, uncultured_bacterium	7.43 ± 3.25	0 ± 0	5.56 ± 2.3	0 ± 0
Coriobacteriales	Coriobacteriales_Incertae_Sedis, uncultured,	3.64 ± 0.76	0 ± 0	2.81 ± 0.73	1.8 ± 0.76
Deferribacterales	Deferribacteraceae, Mucispirillum,	2.93 ± 1.26	0 ± 0	6.75 ± 2.96	113.47 ± 86.14
Desulfovibrionales	Desulfovibrionaceae, Bilophila, uncultured_bacterium	12.29 ± 4.03	0 ± 0	17.63 ± 6.36	0 ± 0
	Desulfovibrionaceae, uncultured, uncultured_bacterium	0 ± 0	10.17 ± 3.29	0 ± 0	9.33 ± 3.07
	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	12.86 ± 3.34	0 ± 0	31.5 ± 12.35	0 ± 0
	Gastranaerophilales, Gastranaerophilales,	5.29 ± 1.6	0 ± 0	14.38 ± 7.25	0 ± 0
	Gastranaerophilales, Gastranaerophilales, uncultured_rumen	7.14 ± 2.06	0 ± 0	9.81 ± 2.4	0 ± 0
	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	45.29 ± 7.31	0 ± 0	51.69 ± 11.65	0 ± 0
	Gastranaerophilales, Gastranaerophilales,	21.5 ± 4.21	0 ± 0	38.44 ± 10.58	0 ± 0
	Gastranaerophilales, Gastranaerophilales,	4.64 ± 2.72	0 ± 0	9.88 ± 2.5	0 ± 0
	Gastranaerophilales, Gastranaerophilales, uncultured_bacterium	21.79 ± 10.66	0 ± 0	44.88 ± 19.03	0 ± 0
	Gastranaerophilales, Gastranaerophilales,	9.14 ± 7.61	0 ± 0	23.19 ± 10.66	0 ± 0
Lachnospirales	Lachnospiraceae, [Eubacterium]_ventriosum_group, uncultured_rumen	11.14 ± 5.61	46.33 ± 12.56	0 ± 0	86.2 ± 29.11
	Lachnospiraceae, Acetatifactor,	0 ± 0	9.17 ± 3.5	0 ± 0	10.27 ± 3.44
Mycoplasmatales	Mycoplasmataceae, Mycoplasma, uncultured_rumen	18.71 ± 5.41	1.39 ± 1.39	32.06 ± 13.7	0 ± 0
	Mycoplasmataceae, Mycoplasma, Malacoplasma_penetrans	40.07 ± 9.56	0.22 ± 0.22	39.94 ± 8.93	11.27 ± 6.38
Oscillospirales	UCG-010, UCG-010, uncultured_bacterium	24.43 ± 6.84	0 ± 0	14.25 ± 5.46	0 ± 0
	Oscillospiraceae, uncultured, Clostridium_sp.	22.71 ± 4.32	58.28 ± 8.93	16.81 ± 2.37	33.8 ± 3.9
Paracaedibacterales	Paracaedibacteraceae, uncultured, uncultured_Alphaproteobacteria	21.5 ± 10.83	0.11 ± 0.11	13.88 ± 8.16	0 ± 0
Peptococcales	Peptococcaceae, Peptococcus, uncultured_bacterium	5.21 ± 2.17	0 ± 0	5.13 ± 1.38	9.6 ± 4.2
Rhodospirillales	uncultured, uncultured, gut_metagenome	3.14 ± 1.1	0 ± 0	5.5 ± 0.83	0 ± 0
Alphaproteobacteria_Order Unknown	Unclassified ASV	18.07 ± 10.43	0 ± 0	12.81 ± 7.07	0 ± 0

compared to male and female offspring from Stress Only mothers, although this interaction was not significant ($p = 0.08$, Table 7). Female offspring from Antibiotic Only mothers (log SI CORT 5.05 ± 0.03) had lower SI-CORT concentrations compared to female offspring from Stress Only mothers (log SI-CORT 5.13 ± 0.05), whereas male offspring from Antibiotic Only mothers (log SI-CORT 5.04 ± 0.03) had slightly higher SI-CORT concentrations compared to male offspring from Stress Only mothers (4.99 ± 0.04). Offspring weight, the main effects of offspring sex and maternal treatment, and the remaining interaction terms did not have an effect on offspring SI-CORT concentrations (Table 7).

4. Discussion

An individual's experiences during the prenatal period, including exposure to maternal stress (Seckl and Meaney, 2004; Duckworth et al., 2015) and the maternal microbiome (Dominguez-Bello et al., 2010) can have profound, long-term effects on offspring development, the foundation and development of offspring's microbiome, and offspring behavior. The HPA axis and the gut microbiome display bidirectional communication such that alterations in one system may affect the function of the other (Cryan and O'Mahony, 2011; Cryan et al., 2019; Cusick et al., 2021b). In this study, we investigated the interactive effects of maternal stress and manipulations of the maternal microbiome on offspring growth, gut microbiome composition and diversity, stress response, and social behavior. Manipulations of the maternal gut microbiome affected the diversity and composition of their offspring's gut microbial communities 40 days after birth. Maternal environment also had sex-specific effects on offspring stress response and aggressive behavior, but did not affect offspring escape behavior.

Table 5
P values for non-parametric pairwise comparisons of the abundance of significant ASVs belonging to 16 Orders detected in offspring fecal samples for which the effect of maternal treatment was significant.

Order	Control vs. Antibiotic Only	Control vs. Stress Only	Control vs. Antibiotic + Stress	Antibiotic Only vs. Stress Only	Antibiotic Only vs. Antibiotic + Stress	Stress Only vs. Antibiotic + Stress
Acholeplasmatales	**<0.001**	0.92	**<0.001**	**<0.001**	N/A	**<0.001**
Bacteroidales	0.34	0.50	**<0.001**	*0.06*	*0.06*	**<0.0001**
Burkholderiales	**<0.001**	0.28	**<0.0001**	**<0.001**	0.13	**<0.001**
Campylobacterales	**<0.0001**	0.50	**<0.0001**	**<0.0001**	0.48	**<0.0001**
Clostridia_vadinBB60_group	**<0.0001**	0.32	**<0.0001**	**<0.0001**	N/A	**<0.0001**
Coriobacteriales	**<0.0001**	0.48	*0.07*	**<0.001**	**<0.01**	0.27
Deferribacterales	**<0.01**	0.81	0.84	**<0.01**	**<0.01**	1.00
Desulfovibrionales*	0.95	0.95	0.95	0.95	0.95	0.95
Gastranaerophilales	**<0.00001**	0.26	**<0.0001**	**<0.00001**	N/A	**<0.00001**
Lachnospirales	**<0.01**	**<0.01**	**<0.01**	**<0.001**	0.57	**<0.001**
Mycoplasmatales	**<0.0001**	0.66	**<0.001**	**<0.0001**	0.48	**<0.001**
Oscillospirales**	0.49	0.18	0.20	0.12	0.20	0.49
Paracaedibacterales	**<0.01**	0.40	**<0.01**	**<0.01**	0.40	**<0.01**
Peptococcales	**<0.01**	0.89	0.94	**<0.001**	**<0.01**	0.89
Rhodospirillales	**<0.01**	*0.08*	**<0.01**	**<0.001**	N/A	**<0.001**
Alphaproteobacteria_Order Unidentified	**<0.01**	0.54	**<0.01**	**<0.01**	N/A	**<0.05**

Pairwise comparisons were calculated using Wilcoxon rank sum test with continuity correction with corrected *p*-values (BH method, Benjamini and Hochberg, 1995). Values reported in table are *p*-values. Bold indicates significant parameters ($p \leq 0.05$) and italicized indicates non-significant ($0.05 > p < 0.1$) parameters trending towards significance. NA indicates comparisons that could not be calculated because ASVs belonging to this Order were not detected in offspring from these treatments.

* Two unique ASVs in the Order *Desulfovibrionales* were identified as being impacted by maternal treatment. "*Desulfovibrionaceae, Bilophila, uncultured_bacterium"* was not detected in offspring produce from mothers that received antibiotics (i.e., Antibiotic Only or Antibiotic + Stress). Meanwhile, "*Desulfovibrionaceae, uncultured, uncultured_*bacterium" was detected in offspring produced by mothers exposed to antibiotics but was not detected in Stress Only or Control offspring (Table 4).

** Two unique ASVs in the Order *Oscillospirales* were identified as being impacted by maternal treatment. "*UCG-010, UCG-010, uncultured_bacterium"* was not detected in offspring produced by Antibiotic Only and Antibiotic + Stress mothers. "*Oscillospiraceae, uncultured, Clostridium_sp."* was detected in greater abundances in offspring produced by Antibiotic Only and Antibiotic + Stress mothers (Table 4).

4.1. Maternal treatment affects offspring gut microbiome

Maternal manipulations impacted the diversity and abundances of ASVs belonging to 16 Orders in the offspring gut microbiome. Mothers exposed to Antibiotics Only and the combined treatment (i.e., Antibiotics + Stress) produced male and female offspring whose gut microbiome was less diverse (i.e., lower Shannon Index). The microbiome of these offspring also differed in the abundance of certain ASVs when compared to offspring produced by control mothers and stressed mothers. For example, *Cyanobacteria* is typically found in the normal gut flora of mammals (Sukenik et al., 2015). Multiple ASVs belonging to the Order *Gastranaerophilales* (Phylum: *Cyanobacteria)* were not observed in offspring produced by mothers that had received antibiotics (i.e., Antibiotic Only or Antibiotic+Stress treatments). Similarly, some ASVs belonging to the Order *Acholeplasmatales* and *Burkholderiales* were also completely absent or significantly reduced in offspring produced by mothers that had received antibiotics as part of their treatment. Many ASVs belonging to the Order *Bacteroidales* (e.g., Family: *Tannerellaceae*) also completely disappeared in offspring produced by mothers that received antibiotics, while other unique ASVs belonging to this Order were observed in higher abundances in these offspring. Similarly, ASVs belonging to the Order *Desulfovibrionales* were also detected in higher abundances in offspring produced by Antibiotic Only or Antibiotic + Stress mothers. Our results also suggest that the maternal microbiome and maternal stress response interact in ways that impact which microbes were detected in their offspring's microbiome. For example, ASVs belonging to the Order *Coriobacteriales* (Phylum: *Actinobacteriota*), and *Deferribacteraceae* (Phylum: *Deferribacterota)* were not detected in the gut microbiome of male and female offspring produced by mothers exposed to only antibiotics (detected in ≤1 individual), but were present in offspring produced by mothers exposed to the combined treatment, as well as offspring from control and stressed mothers.

Mounting evidence indicates there is bidirectional communication between the HPA axis and the gut microbiome. Experiencing stress or manipulations of glucocorticoids can impact the diversity and composition of the microbiome (e.g., Stothart et al., 2016; Noguera et al., 2018), but different types of stressors or hormonal manipulations can alter the gut microbiome in different ways (Williams et al., 2020) such that some types of stressors may have less impact on the microbiome. In the current study, the microbiome diversity and abundances of ASVs detected in offspring produced by stressed mothers were similar to that detected in offspring produced by control mothers. Previous work in our lab also did not detect an effect of stressors on the diversity or composition of the gut microbiome in juvenile Siberian hamsters (Sylvia et al., 2018). There is evidence that prenatal stress can affect the offspring microbiome (Golubeva et al., 2015; Zheng et al., 2020). For example, prenatal stress has been shown to alter the abundance of *Lactobacillus (*Order*: Lactobacillales)*, *Bacteroides (*Order: *Bacteroidales), Oscillibacter,* (Order: *Oscillospirales*) *Anaerotruncus* (Order: *Oscillospirales*), and *Peptococcu (*Order: *Peptococcales)* in offspring, which are bacterial genera from the phyla *Firmicutes* and *Bacteroidota* (formally *Bacteroidetes*). We did observe a significant effect of maternal treatment on the abundance of ASVs belonging to the Genus *Bacteroides* (Family: *Bacteroidaceae*) and Order *Oscillospirales,* however not between offspring produced by Stress Only and Control mothers, but instead in offspring produced by mothers exposed to antibiotics (i.e., Antibiotic Only or Antibiotic + Stress). Changes in glucocorticoid concentrations are associated with differences in parental care behavior (e.g., Dantzer et al., 2017), indicating that changes in maternal behavior due to stress have the potential to mediate the effects of that stress on the offspring microbiome. Further, the long-term effects of maternal treatments on offspring gut microbiome may also differ depending on the treatment. Fecal samples were collected at PND40, 20 days after weaning. It possible that the microbiome of offspring from stressed mothers "recovered" (i.e., became more like offspring of controls) whereas the effects of maternal microbiome manipulations last longer. Although previous work in our lab has confirmed that antibiotics do alter the gut microbiome community of adults (Sylvia et al., 2017), it is possible that antibiotic and stress manipulations also impact other maternal microbe communities (e.g., vaginal microbiome) that can influence offspring in different ways, including the foundation of the offspring's microbiome (e.g., Jasarevic et al., 2018; Jasarevic and Bale, 2019). Our data do support that manipulations of the maternal

Table 6
GLMM coefficients assessing interaction of maternal treatment and offspring sex on offspring aggression scores.

Parameters	Estimate	Std. Error	df	t value	*p* value
Offspring Sex Male vs. Female	**0.48**	**0.20**	**50.00**	**2.36**	**0.02**
Offspring Weight (g)	0.02	0.01	50.00	1.45	0.15
Offspring SI-CORT Concentration	0.06	0.05	50.00	1.08	0.29
Maternal treatment					
Antibiotic + Stress vs. Stress Only	**−0.43**	**0.21**	**50.00**	**−2.04**	**0.05**
Antibiotic Only vs. Stress Only	−0.29	0.19	50.00	−1.47	0.15
Control vs. Stress Only	**−0.49**	**0.20**	**50.00**	**−2.50**	**0.02**
Antibiotic + Stress vs. Control	0.06	0.20	50.00	0.32	0.75
Antibiotic Only vs. Control	0.21	0.18	50.00	1.13	0.26
Antibiotic Only vs. Antibiotic + Stress	0.14	0.20	50.00	0.73	0.47
Offspring Sex * Maternal Treatment Interaction					
Male vs. Female: Antibiotic + Stress vs. Stress Only	**0.58**	**0.28**	**50.00**	**2.05**	**0.05**
Male vs. Female: Antibiotic Only vs. Stress Only	0.42	0.27	50.00	1.57	0.12
Male vs. Female: Control vs. Stress Only	**0.62**	**0.28**	**50.00**	**2.21**	**0.03**
Male vs. Female: Antibiotic + Stress vs. Control	−0.04	0.28	50.00	−0.13	0.90
Male vs. Female: Antibiotic Only vs. Control	−0.20	0.27	50.00	−0.74	0.47
Male vs. Female: Antibiotic Only vs. Antibiotic + Stress	−0.16	0.27	50.00	−0.60	0.55

GLMM model with identity link function was used to assess the interactive effects of maternal treatment and offspring sex, offspring weight, and offspring SI-CORT concentrations (scaled) on offspring aggression scores (PC2, n = 60 individuals). Intruder identity was included as a random effect. Aggression scores were positively associated attack and chase, Table 1). Marginal $R^2_{GLMM} = 0.33$ and Conditional $R^2_{GLMM} = 0.33$. Bold indicates significant parameters ($p \leq 0.05$).

microbiome using antibiotics can impact the abundance of ASVs detected in offspring, including those from Orders that are also typically impacted by maternal stress.

We did not detect sex-specific differences in gut microbiome diversity (as measured by Shannon Index). We did detect a significant interaction between maternal treatment and offspring sex on one unique ASV classified as uncultured_bacterium belonging to the Order *Bacteroidales* and Family *Muribaculaceae*. This ASV was present in male and female offspring produced by Control mothers and Stress Only mothers and was also not detected in male and female offspring produced by Antibiotic Only mothers. This ASV was not detect in male offspring produced by Antibiotic + Stress mothers, however, this ASV was detected in female offspring produced by Antibiotic + Stress mothers, albeit at lower abundances when compared to female offspring produced by Control and Stress only mothers. Previous studies manipulating the microbiome of adult Siberian hamsters did not detect sex-specific differences in the gut microbiome after antibiotic treatment, despite observing sex-specific differences in behavior (Sylvia et al., 2017). This suggests that in general, microbiome manipulations may not have sex-specific effects on the gut microbiome composition itself, but may instead have sex-specific effects on the gut-brain axis due to sex differences in how these systems interact. Sex hormones can affect regulation of the gastrointestinal tract, which could impact normal functioning of the gut microbiome (Mulak et al., 2014; Sylvia and Demas, 2018). Some of these sexually dimorphic differences observed could be related to sex differences in HPA response (Handa et al., 1994; Sylvia and Demas, 2018). For example, female rats tend to have greater endocrine response to various stressors (Viau et al., 2005) and gonadal steroid hormones may play a role in regulating HPA negative feedback (Handa et al., 1994). Microbes may also use sex steroid hormones to manipulate sex steroid receptor signaling (Vom Steeg and Klein, 2017). Another potential reason we did not detect sex-specific effects of maternal treatment on offspring gut microbiome is because gut microbiome sex differences may be more likely to emerge after puberty (Markle et al., 2013; Steegenga et al., 2014). In our study we assessed offspring gut microbiome (40 PND) and offspring behavior (50–55 PND) during the late adolescent stage before individuals complete the pubertal transition (~60 PND). Considering the role of sex is critical for understanding the role of the gut microbiome in development, health and immune system function, and behavior.

A variety of factors (e.g., diet, environment) can alter the maternal microbiome and influence the establishment of the microbial community in offspring (e.g., Reddivari et al., 2017; Hebert et al., 2021). Although current research often focuses on groups that are present in high abundance in the microbiome (Ley et al., 2006), there is growing evidence that those found in lower relative abundance can also be impacted by manipulations or may play an important foundational role. Rare microbes have been shown to play a disproportionate role in organism physiology, reproduction and survival (e.g., Sylvia et al., 2017; Antwis et al., 2019; Robinson et al., 2019) as well as ecosystem processes (e.g., Shade et al., 2014; Jousset et al., 2017), suggesting they play a role in biologically meaningful ways. In our study, there were cases where ASVs not detected in offspring produced by Control mothers appeared in offspring produced by treatment mothers (e.g., ASVs belonging to *Bacteroidales*). In other cases, ASVs detected in low abundances or ASVs belonging to less abundant Orders (e.g., *Acholeplasmatales)* were detected in offspring produced by Control mothers but completely absent in offspring produced by mothers exposed to antibiotics. Further investigation into how the appearance or disappearance of these more rare microbes influence physiology and behavior (e.g. Antwis et al., 2019) or even the foundation and community structure of the gut microbiome (Carlstrom et al., 2019) are important next steps.

4.2. Maternal treatment affects offspring social behavior and SI-CORT concentrations

Maternal treatment had sex-specific effects on offspring aggressive scores, but not escape scores. Male offspring produced by stressed mothers did not differ in their aggressive scores compared to male offspring from other maternal treatment groups. In contrast, female offspring produced by stressed mothers had higher aggressive scores relative to female offspring from control mothers. These results indicate that female offspring were more susceptible to maternal stress than male offspring, consistent with previous work in our lab demonstrating that adult female Siberian hamsters are more affected by stress (e.g., Sylvia et al., 2017; Sylvia et al., 2018). Across many vertebrate species, sex-specific effects of maternal stress on offspring have been documented (e.g., Schmidt et al., 2018; Gu et al., 2018; Thayer et al., 2018; Iturra-Mena et al., 2018) and our results are consistent with well-documented sex differences in behavior due to prenatal stress - often female offspring are more affected by prenatal stress than male offspring (Frye and Wawrzycki, 2003; Schulz et al., 2011; Zagron and Weinstock, 2006). Furthermore, in the current study female offspring from stressed mothers displayed aggression scores similar to control males. This is consistent with other studies demonstrating that prenatal stress can affect female offspring behavior such that they display behavior similar to males (e.g., Sachser and Kaiser, 1996) and more energetically-demanding behavior (Sangenstedt et al., 2018).

Maternal treatment also had sex-specific effects on offspring SI-CORT concentrations. In our study, female offspring from stressed mothers tended to have higher SI-CORT concentrations compared to female offspring from other maternal treatment groups. In contrast, SI-CORT concentrations of male offspring from stressed mothers were similar to

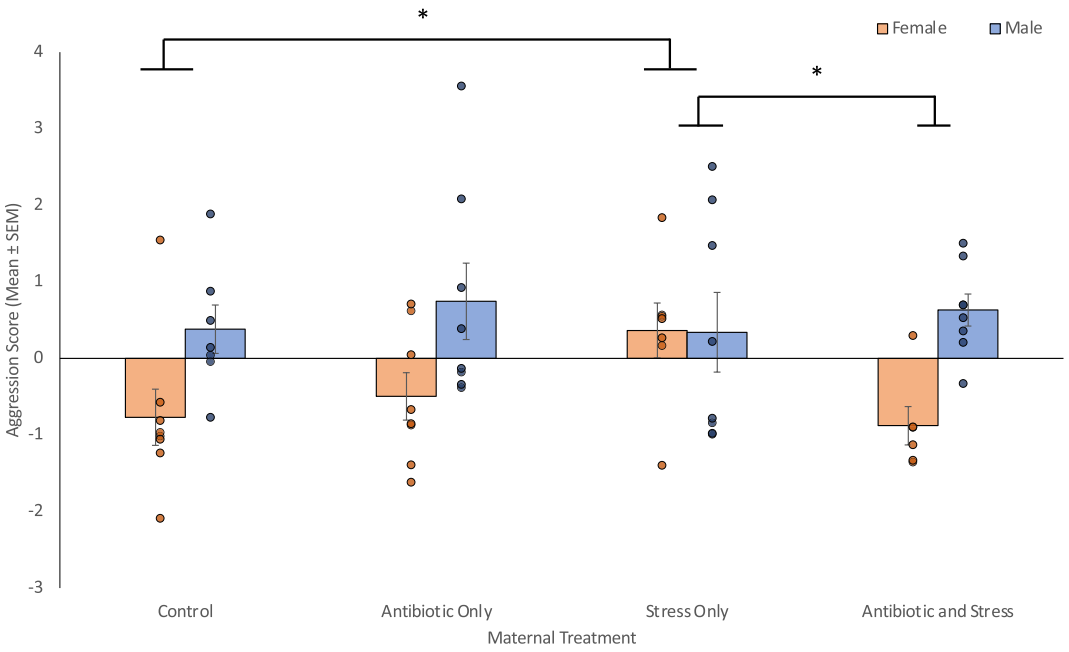

Fig. 4. Interactive effect of maternal treatment and sex on female (orange) and male (blue) offspring aggression scores. We detected a significant interaction between maternal treatment and offspring sex (indicated by "*"). Aggression scores range from positive values (more aggressive) to negative values (less aggressive). Aggression scores of male and female offspring produced by Stress Only mothers differed significantly from male and female offspring produced by Antibiotic + Stress mothers (GLMM: Male vs. Female: Antibiotic + Stress vs. Stress Only: 0.58 ± 0.28, df = 50, $t = 2.05$, $p = 0.05$). The aggression scores of male and female offspring from Control mothers also differed significantly from male and female offspring produced by Stress Only mothers (GLMM Male vs. Female: Control vs. Stress Only: 0.62 ± 0.28, df = 50, $t = 2.21$, $p = 0.03$). Aggression scores were positively associated with attack and chase behaviors (Table 1). Points represent individual datapoints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

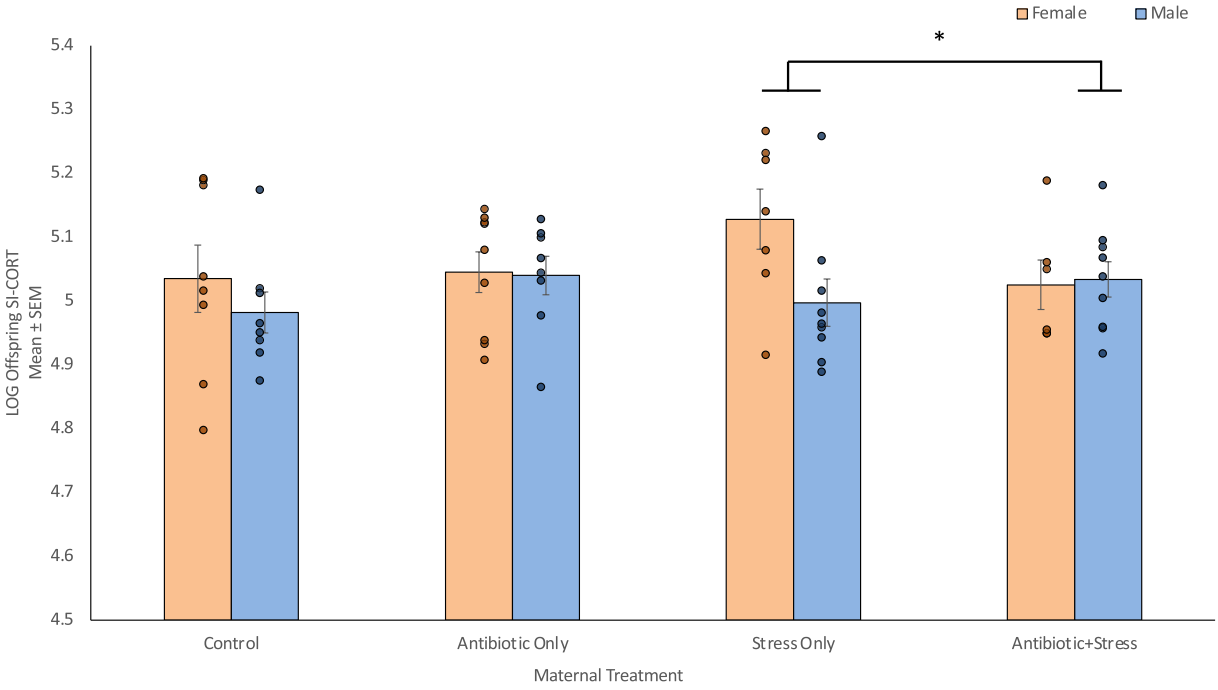

Fig. 5. Interactive effect of maternal treatment and sex on female (orange) and male (blue) offspring stress-induced cortisol (SI-CORT) concentrations. We detected a significant interaction between maternal treatment and offspring sex (indicated by "*"). LOG SI-CORT concentrations of male and female offspring from Stress Only mothers differed significantly from the LOG SI-CORT concentrations of male and female offspring produced by Antibiotic + Stress mothers (GLMM: Male vs. Female: Stress Only vs. Antibiotic + Stress: -0.34 ± 0.18, df = 54.67, $t = -1.93$, p = 0.05). SI-CORT concentrations reflect the cortisol concentration of individuals 30 min after the start of the resident-intruder trial. Points represent individual datapoints. Raw SI-CORT values are displayed in Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 7
GLMM coefficients assessing interaction of maternal treatment and offspring sex on offspring SI-CORT concentrations.

Parameters	Estimate	Std. Error	df	t value	*p* value
Offspring Sex Male vs. Female	−0.02	0.13	39.38	−0.18	0.86
Offspring Weight (g)	0.01	0.01	27.30	1.16	0.25
Maternal Treatment					
Antibiotic Only vs. Antibiotic + Stress	0.05	0.13	33.89	0.39	0.70
Control vs. Antibiotic + Stress	0.01	0.13	28.35	0.08	0.94
Stress Only vs. Antibiotic + Stress	*0.25*	*0.14*	*37.77*	*1.82*	*0.08*
Antibiotic Only vs. Control	0.04	0.12	34.82	0.33	0.74
Stress Only vs. Control	*0.24*	*0.13*	*39.95*	*1.86*	*0.07*
Antibiotic Only vs. Stress Only	−0.20	0.12	46.57	−1.62	0.11
Offspring Sex*Maternal Treatment Interaction					
Male vs. Female: Antibiotic Only vs. Antibiotic + Stress	−0.05	0.18	55.00	−0.28	0.78
Male vs. Female: Control vs. Antibiotic + Stress	−0.12	0.18	46.99	−0.65	0.52
Male vs. Female: Stress Only vs. Antibiotic + Stress	**−0.34**	**0.18**	**54.67**	**−1.93**	**0.05**
Male vs Female: Antibiotic Only vs. Control	0.07	0.17	53.84	0.38	0.70
Male vs. Female: Stress Only vs. Control	−0.23	0.18	52.33	−1.29	0.20
Male vs. Female: Antibiotic Only vs. Stress Only	*0.30*	*0.17*	*37.86*	*1.75*	*0.08*

GLMM model with identity link function was used to assess the interactive effects of maternal treatment and offspring sex, and offspring weight on the log of offspring SI-CORT concentrations (n = 64 individuals). Litter identity was included as a random effect. Offspring SI-CORT concentrations reflect the cortisol concentration of individuals 30 min after the start of the resident-intruder trial. Marginal $R^2_{GLMM} = 0.14$ and Conditional $R^2_{GLMM} = 0.16$. Bold indicates significant parameters ($p \leq 0.05$) and italicized indicates non-significant ($0.05 > p < 0.1$) parameters trending towards significance.

SI-CORT concentrations of male offspring produced by control mothers. During adolescence, the HPA axis and glucocorticoid-sensitive regions in the brain are developing, suggesting that this is a sensitive period for programming (McCormick et al., 2010). Prenatal experiences can have organizational effects on the brain that affect the development of the HPA axis, the effects of which can be observed throughout development and into adulthood (Thayer et al., 2018; Bosch et al., 2007) and are especially strong in females. For example, exposure to stress affects stress-induced glucocorticoid levels in adult females more so than males (e.g., Grippo et al., 2007) and prenatal stress can affect adult female HPA axis activity (Bosch et al., 2007). Prenatal stress may also affect other aspects of male and female offspring stress response (Thayer et al., 2018). For example, glucocorticoid recovery following stress, rather than peak response, may be impacted by prenatal stress (Thayer et al., 2018). We did not quantify baseline CORT concentrations in our study. Thus, the differences in SI-CORT concentrations observed could represent elevations in SI-CORT concentrations above baseline concentrations in response to stress or general elevations in CORT concentrations overall. It is possible that maternal stress impacted measures of HPA axis activity in male and female offspring differently, which may be why we did not detect an effect of maternal stress on male SI-CORT concentrations. Offspring SI-CORT concentrations were also not related to offspring aggression scores in both male and female offspring. Previous work also found that aggression was unrelated to CORT concentrations and experimental elevation of CORT did not alter aggressive behavior in adult male Siberian hamsters (Scotti et al., 2015). However, changes in post-stress glucocorticoids during adolescence has been shown to coincide with transitions from play fighting to adult aggression (Wommack and Delville, 2007). These results suggest that maternal stress can impact both offspring behavior and stress response and suggests maternal environment may impact developmental transitions in social behavior, but not the relationship between SI-CORT and social behavior.

The effects of the maternal microbiome manipulation and combined treatment also differed for male and female offspring. Male offspring produced by mothers exposed to antibiotics and the combined treatment were both slightly more aggressive than male offspring from the other treatment groups. In contrast, female offspring produced by mothers exposed to antibiotics displayed low levels of aggression, similar to that of female offspring from control mothers. Our results are consistent with previous findings from our lab, which revealed sex-specific effects of microbiome manipulations on aggressive behavior in adult Siberian hamsters (Sylvia et al., 2017). Adult females were more susceptible to antibiotic treatment, displayed decreased aggression after a week of treatment, and did not recover behaviorally after treatment ended. Males appeared to be less susceptible and were more likely to behaviorally recover (Sylvia et al., 2017). In this study, we also observed that female offspring produced by mothers exposed to the combined treatment displayed levels of aggression similar to female offspring produced by Control mothers and displayed significantly less aggression than female offspring produced by Stress Only mothers. Similarly, female offspring produced by mothers exposed to the combined treatment displayed SI-CORT concentrations and growth rates more similar to Control female offspring and significantly different from female offspring produced by mothers exposed to stress. Collectively, these data suggest that for female offspring, prenatal alteration of the maternal microbiome may have lessened the effects of the simultaneous exposure to prenatal stress. Previous work has identified how the gut microbiome can mediate the effects of stress in juveniles and adults (e.g., Marin et al., 2017) and that the maternal microbiome may mediate the effects of prenatal stress on male offspring development in some species (Jasarevic et al., 2018). Our results suggest that the maternal microbiome may also mediate the effects of prenatal stress for female offspring. We demonstrate that maternal systems involving the gut microbiome influence other physiological systems and affect offspring development and behavior in sex-specific ways that last well after offspring are weaned and living independently from mothers.

We did not observe an effect of maternal treatment on offspring escape behavior. Previous studies have identified effects of maternal environment on certain aspects of offspring social avoidance or anxiety-like behaviors; in some cases these effects were long lasting and in others they were not. For example, manipulations of the maternal microbiome resulted in offspring that exhibited lower activity and exploration of familiar and novel environments compared to control offspring at postnatal week four, but this difference disappeared at postnatal weeks 7–8 and could be "recovered" by "normal" maternal care (i.e., mothers that were not exposed to antibiotics; Tochitani et al., 2016). Additional studies have shown that prenatal stress can negatively affect adult social-approach behavior towards a conspecific when given the choice between interacting with a conspecific and a novel object (Gur et al., 2019). There is evidence that certain measures of social avoidance, including those that are more similar to the behaviors associated with individuals' escape scores measured in the current study, may not be impacted by the prenatal environment (Brachetta et al., 2018). In the subterranean rodent, *Ctenomys talarum*, prenatal stress did not affect the time offspring spent at the wall during both an open field test and a predator cue test. We measured a similar behavior in our study (i.e., jump), which correlated positively with individuals' escape score and was unaffected by maternal treatment. There is also increasing evidence that although individuals may behave similarly, they may achieve these outcomes using different strategies mediated by physiological systems that are differentially affected by the prenatal environment (Davidson et al., 2018).

5. Conclusion

Experiences during prenatal development, like maternal stress or manipulations of the maternal microbiome, can alter the development of offspring and have long-lasting effects on offspring behavior. These maternal physiological systems do not function in isolation. Consideration of how the maternal microbiome interacts with other maternal systems and their long-term, sex-specific effects on offspring development and behavior is still needed and may provide important insight into the complex role of the gut microbiome in mediating development and behavior. Here, we show that manipulations of the maternal microbiome have lasting effects on offspring's gut microbiome diversity and composition. Further, we demonstrate that maternal stress can interact with the maternal microbiome, producing long-lasting, sex-specific effects on offspring development and social behavior. Understanding how maternal systems interact to affect offspring phenotypes, identifying the mechanisms that mediate CNS-microbiome cross-talk (e.g., the immune system, HPA axis, microbial by-products), and investigating additional factors that may reduce or enhance these effects (e.g., maternal body weight, maternal body condition, or parental care behavior) can help elucidate the complex physiological processes that create individual behavioral phenotypes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105146.

Data availability

The data from this study are available upon request.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by National Institutes of Health (NIH) training grant (T32HD049336, awarded to JAC) and by National Science Foundation (NSF) grant (IOS-1656414, awarded to GED and CLW). We thank all members of the Demas Lab, including Kat Munley, Beth Morrison, Jessica Deyoe, Kate Adaniya, Luke Gohmann, and all the undergraduate volunteers that assisted with the study. Thank you to the Indiana University Veterinary and LAR staff, including Alicia Meranda, Calan Quate, and Dr. Randalyn Shepherd for animal care. We thank Indiana University's Center for Genomics and Bioinformatics, including Dr. Sumitha Nallu, Dr. Chunlin Yang, and Christopher Hemmerich for microbiome sample processing and bioinformatics, and Dr. Irene Newton, Audrey Parish, Delaney Miller for training in sampling, extraction, amplification protocols. We thank Dr. Margaret Vogel for discussions and training in sampling, extraction, amplification protocols, and bioinformatics.

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